# Studies into the Isolation and Cryopreservation of Human and Porcine Hepatocytes and their suitability for use in Bioartificial Liver Development.

by

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# Contents

# Preface

It is widely accepted that the treatment of hepatic failure from any cause, (parenchymal dysfunction, mechanical obstruction due to tumour or as a temporary lack of functional capacity after a surgical procedure) is one of the most challenging problems in medicine today. Despite many years of extensive research and clinical experience of tens of thousands of patients, care is still restricted to supportive measures in the vast majority of patients. An alternative in a small number of clinically eligible patients with end stage disease is a liver transplant but there remains a shortage of donor organs worldwide, which is unlikely to change in the foreseeable future despite attempts to engineer a source of xenografts.

This unacceptable clinical situation with the added problem of a steady increase in the incidence of liver disease has over the last 40 years been the stimulus for research aimed at developing methods of replicating the livers endogenous functions. These studies have produced steady progress but major improvements have not occurred as anticipated and the *Holy Grail* of an artificial liver analogous to a dialysis machine remains elusive. It is now widely believed that for this artificial liver to be clinically applicable, a biological component (hepatocytes) will be required to augment the role of the residual diseased liver. Controversy surrounds the exact specifications needed to construct such a device and the source of the hepatocytes. Human hepatocytes are believed to be an ideal source but are in very short supply and lack the necessary extensive evaluation. An effective programme to acquire human or porcine tissue and then produce and store the hepatocytes until they are required is currently regarded as the most important single rate-limiting step preventing the introduction of a treatment based on a bioartificial liver (BAL). For a clinically successful BAL programme to be

effective, the hepatocytes need to be readily available for use at any time and at short notice. Cryopreservation of hepatocytes is believed to be the only solution likely to facilitate the storage of hepatocytes in a viable condition with sufficient retention of endogenous function to be clinically useful. Unfortunately published studies have reported that cryopreserved hepatocytes prepared by current methods function poorly after thawing and there is in addition a viability loss of 70-80%, which reduces the number of cells available.

As part of the bioartificial liver development programme being undertaken in the Department of Surgery at the Leicester General hospital, this thesis examines the effects that different methods of cryopreservation, acquisition and hepatocyte isolation techniques have on the function of human and porcine hepatocytes. These studies have allowed a thorough and detailed evaluation of these cryopreservation effects together with the impact of hepatocyte isolation on the functional viability of recovered cells. As a result it is now possible to assess their potential for use in the construction of a bioartificial liver, predict useful areas to research in the future and hopefully develop treatment programmes in a clinical setting.

# Abstract

*Introduction.* **Clinical bioartificial liver development for the treatment of liver failure has been actively researched for many years. The development of an easily accessible cell source that also provides an adequate range of functions has proved a major block to its development. The aim of these studies was to evaluate and develop methods for the successful cryopreservation of human and porcine hepatocytes for potential use within a bioartificial liver.**

*Methods.* **Both porcine and human hepatocytes were studied. Following informed patient consent, human liver tissue was extracted from discarded specimens removed during liver resection. Porcine tissue was sourced from an abattoir. The hepatocytes were then isolated from the tissue, and investigations into cryopreservation parameters performed. Measurements of cellular return, LDH leakage, attachment, bilirubin conjugation and lignocaine metabolism were performed on the thawed hepatocyte cultures and compared to untreated hepatocyte controls. Patient, operative and isolation parameters were reviewed to determine their influence on pre- and post- hepatocyte function and quality.**

*Results.* **The processing of surgically resected liver tissue provided consistently viable and functionally high quality hepatocytes. Large tissue samples and methods of cannulation influenced the level of cell viability following isolation. No other pre-operative factors were identified as being of any influence. The temperature reduction method had no significant affect on the function of the hepatocytes contrary to reports in the literature. The concentration of hepatocytes, and the pre-incubation of hepatocytes did not significantly affect the quality of the hepatocytes following post cryopreservation culture. Hepatocyte functional data proved similar for hepatocytes cultured following isolation and post- cryopreservation.**

*Conclusions.* **Human and porcine hepatocytes can be successfully cryopreserved using a gradual temperature reduction at an optimal concentration of 5 x $10^6$. There are minimal differences within the functional parameters between fresh and post-cryopreservation with human or porcine hepatocytes. A reduction of approximately 70% of viable hepatocytes was found following cryopreservation. Further method optimisation will be required to reduce this loss.**

# Acknowledgements

The work presented in this thesis was undertaken whilst a clinical research fellow in the department of surgery at the Leicester General Hospital. I am indebted to Mr Ashley Dennison for supervising and allowing me the opportunity to carry out this work. The study was sponsored by the UK human tissue bank under the directorship of Samantha Orr. I am grateful for her supervision, constructive criticism, financial support and laboratory access.

In the early days of the project I was grateful to receive help and guidance from Dr Jo Davies and Dr Heather Clayton who also contributed in setting up this work. Their laboratory experience and expertise in hepatocyte isolations and functional assays was invaluable in guiding me at the start of this project.

I am also grateful to the technical staff in the UK human tissue bank, Rakhee Patel, Gerard Crees, Seema Chavda and Shaun Kingston who assisted me with the tissue collection and the many hepatocyte isolations at all hours of the day.

Statistical analysis and guidance was provided by Joy Wilkinson and Dr Sanjoy Paul, and I thank them for their patience in analysing the data. Many of the diagrams were provided courtesy of Mr Andrew Featherstone and I am grateful for the hours he has spent doing this for me.

Through the latter part of the study close links with the Fondation Transplantique in Strasbourg developed under the leadership of Professor Lysiane Richert and Dr Eliane Alexandre. Their helpful comments, expertise and hospitality were invaluable in completing this thesis.

Finally I am indebted to my family. My father, for his constructive criticism and patient proof reading, while writing this thesis. Also to my wife and daughter for their support, encouragement and acceptance of my frequent absence during the 2 years, without which I probably would not have finished this work.

# Thesis Outline

## Purpose of the work

The work described in this thesis was designed to evaluate the isolation of hepatocytes from human livers and develop a protocol for the cryopreservation of human and porcine hepatocytes. It was anticipated that a store of functioning hepatocytes would allow the future evaluation of a clinical bioartificial liver program.

## Presentation of the thesis

The thesis consists of 11 chapters, which broadly fit into three sections. Chapter 1-3 provides the background to the thesis. Chapter 1 examines the current literature and discusses unresolved issues surrounding bioartificial liver development. Chapter 2 deals with liver anatomy and function *in vivo* to illustrate the number and type of processes that an artificial device needs to be able to replicate. Chapter 3 critically reviews the literature on hepatocyte cryopreservation particularly the various methods employed and there perceived success.

The second section, chapters 4 – 8, concerns the experimental methods (chapter 4) and the results. Chapter 5 presents data evaluating patient data, the operative and isolation factors and their effect on the quality and suitability of hepatocytes isolated from human livers. Chapter 6 presents the effect of the rate at which the temperature is reduced on both human and porcine hepatocytes and their subsequent function. Chapter 7 examines the effect on function of differing concentrations, again on both human and porcine hepatocytes, within the cryopreservation solution during freezing. Chapter 8 investigates the approach of pre-incubating the hepatocytes following

isolation and prior to cryopreservation. Each chapter has a discussion of the results and also a summary conclusion at the end.

Finally the last two chapters provide a conclusion regarding the methods and results of the experiments and discuss their application to future developments. Other areas that have potential in furthering the development of a clinical bioartificial liver are also reviewed.

The work arising from this thesis that has been presented is listed, followed by a bibliography.

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# Abbreviations

| | |
|---|---|
| **ALF** | Acute Liver failure |
| **ALT** | Alanine Transaminase |
| **BAL** | Bioartificial liver |
| **BMI** | Body mass index |
| **CMO** | Chief medical officer |
| **CEA** | Carcinoembryonic antigen |
| **CNS** | Central nervous system |
| **CT** | Computer Tomography |
| **CYP** | Cytochrome P450 |
| **Da** | Dalton (atomic mass unit) |
| **DMEM** | Dulbeccos modified eagles medium |
| **DMSO** | Dimethyl sulphoxide |
| **DNA** | Deoxyribonucleic acid |
| **ECVAM** | European Centre for the Validation of Alternative Methods |
| **ELAD** | Extracorporeal liver assist device |
| **FBS** | Foetal bovine Serum |
| **GSH** | Reduced glutathione |
| **GST** | Glutathione S-transferase |
| **GX** | Glycinexylidide |
| **HBSS** | Hanks balanced salt solution |
| **HCL** | Hydrochloric acid |
| **HEPA** | High efficiency particulate air |
| **HGF** | Hepatocyte Growth Factor |
| **HIV** | Human immunodeficiency virus |
| **HLA** | Human leukocyte antigens |
| **HPLC** | High-performance liquid chromatography |
| **IVC** | Inferior Vena Cava |
| **Il** | Interleukin |
| **LDH** | Lactate Dehydrogenase |
| **LDM** | Liver Digest Medium |
| **LREC** | Leicestershire Research ethics committee |
| **MARS** | Molecular Adsorbent Recirculating System |
| **MEGX** | Monoethylglycinexylidide |
| **MELS** | Modular Extracorporeal Liver Support |
| **MEM** | Modified Eagles Medium |
| **MOD** | Multi Organ Donor |
| **MRI** | Magnetic resonance imaging |
| **MRSA** | Methicillin Resistant Staphylococcus aureus |
| **MSC** | Microbiological Safety Cabinet |
| **MSO** | Methyl Sulphoxide |
| **$NADP^+$** | Oxidised nicotinamide adenine dinucleotide phosphate |

| | |
|---|---|
| **NADPH** | Reduced nicotinamide adenine dinucleotide phosphate |
| **NaOH** | Sodium Hydroxide |
| **NHS** | National Health Service |
| **PERV** | Porcine endogenous retrovirus |
| **UDP** | Uridine 5'-diphosphate |
| **UK** | United Kingdom |
| **UK HTB** | United Kingdom Human Tissue Bank |
| **UMIST** | University of Manchester Institute of Science and Technology |
| **ST** | Phenol sulfotransferase |
| **TGF** | Tumour Growth Factor |

# Thesis hypothesis

This study aims to examine the effect of isolation and cryopreservation techniques on porcine and human hepatocyte viability and function, in order to establish whether they can be considered as a reproducible source of cells for a clinically applicable bioartificial liver programme.

# Chapter 1

# The Bioartificial liver

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## 1.1 Introduction

The treatment of hepatic failure has been a clinical challenge for over 40 years. The livers complex and varied functions (outlined in chapter 2) have proved difficult to duplicate and have made realising the concept of artificial hepatic support difficult. Davies and Hodgon (1995) summarised this difficulty by comparing organs in terms of their primary functions, "...hearts are pumps, kidneys are filters, lungs are membranes.... However, the liver is described as a multifunctional unit that includes being, ...a chemical factory, a detoxification plant, and a nutrient processor with a sophisticated command and control function as a chemical servo-mechanism".

Initially, research concentrated on attempting to detoxify the blood (Kiley *et al.*, 1951; Kiley *et al.*, 1956). With advances in knowledge of cell biology and *in-vitro* cell culture methods, the concept of the bioartificial liver using functional hepatocytes (the biological component) emerged and became an attractive strategy for the future (Dunn *et al.*, 1991; Dixit, 1994; Kasai *et al.*, 1994). Under defined conditions, and in sufficient numbers, hepatocytes have the potential to maintain normal liver function for many weeks (Ferrini *et al.*, 1997; Chen *et al.*, 1998; Zeilinger *et al.*, 2002).

The need to develop such an artificial support system was first proposed by Sorrentino in 1956 (Sorrentino, 1956). Nose developed a BAL in 1959 using liver slices and granules, but the first clinical device utilising isolated hepatocytes is credited to Matsumara in 1987 (Matsumara *et al.*, 1987; Nose, 2001). The developments since these early examples have been towards optimising the treatment of patients suffering from fulminant hepatic failure.

## 1.2 Historical review

The main cause of death from hepatic failure is coma, and was initially believed to be due to the accumulation of toxins of less than 5 kDa. Based on this theory, much of the early research was aimed at developing systems to remove these toxins. Hui *et al* called this approach "non biological", while the term "biological systems" referred to those hepatocytes emulating the functions of the natural liver and are currently the main area of research (Hui *et al.*). Most developing systems use a combination of both types and are referred to as hybrid. The following sections outline some of the developments in the two different devices.

### 1.2.1 Nonbiological liver support systems

#### 1.2.1.1 Haemodialysis

Kiley *et al.* in the 1950's reported the successful use of a kidney haemodialyser for the treatment of animals and later patients with ammonia intoxication (Platt, 2000; Kiley *et al.*, 1956). It was postulated that these results could have implications for the treatment of hepatic coma by the removal of toxins. In 1976, Opolon *et al.* used a polyacrylonitrile membrane to treat 24 patients suffering from acute fulminant viral hepatitis (Opolon *et al.*, 1976). The membrane allowed the passage of particles up to a molecular weight of 15000 Daltons, which includes many of the substances associated with metabolic encephalopathy. An improvement in the patients' coma and condition had been achieved by these studies, but no long-term survival benefit was demonstrated compared with historical controls.

### 1.2.1.2 Charcoal

Charcoal haemoperfusion has been extensively investigated as a possible option for non-biological treatment in acute liver failure (Gimson *et al,*. 1982; Chang, 1972; Van Berlo *et al.*, 1985; Matsubara, 1994). It utilises the properties of charcoal to bind water-soluble molecules that contribute to metabolic encephalopathy (Table 1). Chang found an improvement in biochemical parameters and consciousness in a 50 year old woman with a history of alcohol abuse, following treatment with an albumin encapsulated charcoal (Chang, 1972). A larger study was conducted at Kings College London two years later, on patients with fulminant hepatic failure and grade 4 encephalopathy. Of the 22 treated, 11 regained consciousness and 10 were discharged home, demonstrating significant survival advantages (Gazzard *et al.,* 1974). An extension of this trial revealed problems with hypotension linked to platelet activation and aggregation as they passed through the column (Silk and Williams, 1978). Although this was successfully overcome using prostacyclin infusions, the documented improvement in mental status and biochemical correction seen in the earlier work has not lead to any proven benefit in long term survival in rats or humans in larger trials (Gimson *et al.*, 1982; O'Grady *et al.*, 1988)

**Molecules absorbed during activated charcoal haemoperfusion:**

- γ -Aminobutyric acid
- Theophylline
- Acetaminophen
- Methaqualone
- Phenobarbital
- Bile salts
- Aromatic amino acids
- Mercaptans
- Salicylate
- Phencyclidine
- Secobarbital

**Table 1. Molecules adsorbed during activated charcoal haemoperfusion.**

### 1.2.1.3 Blood and plasma exchange

This technique, involving the removal of patients' plasma and its replacement with donor plasma, was investigated as a means of extracting all metabolites possibly responsible for causing the various manifestations associated with fulminant hepatic failure. Although trial results have demonstrated improved responses in hepatic coma and some biochemical changes, the system did not improve patients survival (Lepore *et al.*, 1972; Freeman and Matthewsson, 1986). There were also considerable side effects associated with this approach that included chemical toxicity, viral infections, and deaths from pulmonary and cerebral complications (Brunner and Losgen, 1987).

Despite these limitations, the use of plasma exchange still remains the most frequently used treatment for acute liver failure in Japan (Matsubara, 1994). Matsubara calculated that following one plasma volume exchange, bilirubin and bile acids levels decreased by 40% and 25% with endotoxins reducing by 30% (Matsubara, 1994).

### 1.2.1.4 Molecular Recirculating Adsorbent System (MARS)

The MARS system is based upon albumin dialysis (Figure 1). The system was designed to remove water soluble toxins by utilising a semi-permeable membrane that allowed passage of albumin bound toxins, mimicking the hepatocyte cell membrane. The membrane itself permits the release of the albumin-ligand complex present in the blood due to its physiochemical ability to interact with lipophillic binding domains. It is also impermeable to proteins including hormones, clotting factors, antithrombin III and albumin itself. The albumin is recycled and other properties of the system allow the maintenance of electrolytes, acid/base balance, fluid balance and glucose levels. (Stange *et al.*, 2001; Di Campli *et al.*, 2003). A recent review by Di Campli has concluded that the system was safe and has improved survival outcome in hepatorenal

syndrome post surgical resection and hyperacute liver failure and a survival advantage in patients with liver failure (Di Campli *et al.*, 2003; Schmidt *et al.*, 2003; Mitzner *et al.*, 2000, Kellersmann *et al.*, 2002).

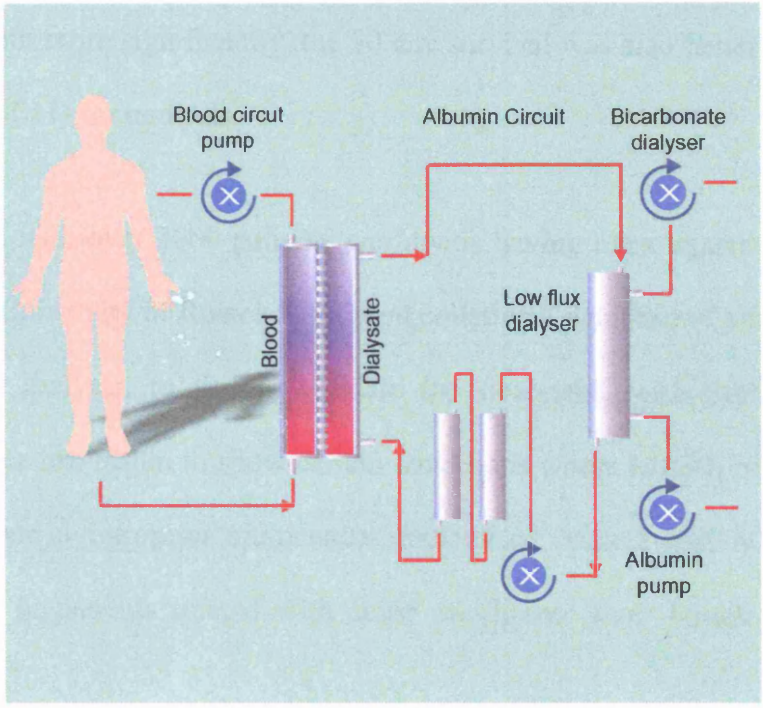


**Figure 1. Diagram illustrating the Molecular recirculating adsorbent system (MARS).**

However, the true benefits and indications for the MARS system have not been fully evaluated. No large-scale randomised trial has yet been completed so opinion is based upon an ever increasing number of small trials and case reports. One of the difficulties experienced by centres attempting to establish a trial is a standard definition of when to start treatment and also to ensure there are sufficient numbers of patients, with similar comparable pathologies (Jalan *et al.*, 2004). The largest randomised control trial was undertaken in Rostock and Essen,

### Indications

- Acute on chronic liver failure
- Severe acute alcoholic hepatitis
- Severe pruritus due to cholestasis
- Intoxication from protein bound substances

### Relative contraindications

- Progressive coagulopathy indicative of DIC
- Uncontrolled sepsis
- Uncontrolled bleeding

### Monitoring during therapy

- Electrolytes including phosphates, magnesium and calcium
- Coagulation
- Drug levels (protein bound drugs may be removed)

**Table 2. Indications and contraindications of MARS therapy (from Jalen *et al.*, 2004)**

and consisted of 24 patients (Heemann *et al.*, 2002). Biochemical improvements was found in the MARS group, but more significantly, the 30 day survival was also better (11 out of 12) versus (6 out of 11) for controls.

Safety appears to be assured with over 3000 patients worldwide having been treated with MARS. In Europe, the University of Rostok have been collating a database of all patients undergoing MARS dialysis, to further validate the treatment, with 500 patients to date. The evidence has begun to show certain conditions where MARS is not appropriate (Table 2), and a European multicentre randomised control trial is ongoing to assess mortality in patients treated with acute on chronic liver failure (Jalen *et al.* 2004).

MARS appears to hold great promise for the future and much time and resources are being devoted to its evaluation. Its true benefits will not be completely known until randomised controlled trials are completed.

### 1.2.2 Biological methods

The failure and perceived disadvantage of these early treatment methods has been attributed to the fact that workers concentrated solely upon detoxification. Minimal consideration had been placed on replacing the synthetic functions of the liver. Many factors that could potentially be helpful in the regeneration of a damaged liver are not retained by these methods due their non-selective design. This was demonstrated by Seldon and co workers, who found the plasma of patients with fulminant hepatic failure contains stimulants such as hepatocyte growth factor necessary for hepatic repair (Seldon *et al.*, 1986). Therefore, the incorporation of hepatocytes able to

reproduce these functions provides the theoretical basis for developing bioartifical systems.

### 1.2.2.1 Extracorporeal liver perfusion

Variable results have been observed using livers derived from monkey, rat and pig sources, as extracorporeal units. Studies undertaken consisted of using these livers *ex-vivo* and perfusing them with the patients' blood (Figure 2). In 1970, Abouna *et al.* reported the use of 16 livers from 5 animal species used over a 10-week period on a single patient (Abouna *et al.*, 1970). Although improvements were found in the patient's neurological status, no long-term improvement in expected survival was reported.

The use of xenogenic organs in this way is controversial due to the potential immunological problems that could be encountered. The animal organs may be the source of immunogens causing a variety of detrimental immunological responses. However, Abouna and colleagues have recently reintroduced experiments using *ex vivo* whole organ liver perfusions for hepatic failure as an alternative to transplantation (Abouna *et al.*, 2001). Five out of eight dogs with surgically induced hepatic ischaemia successfully bridged to complete recovery, using

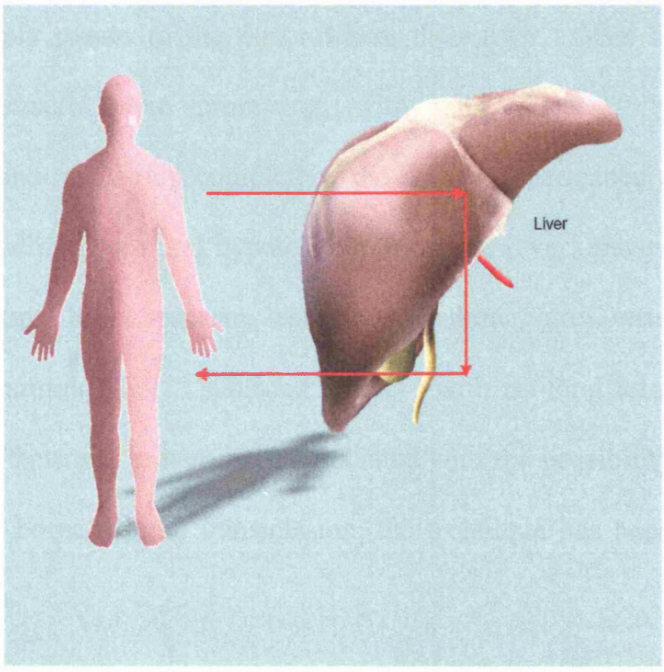


**Figure 2. Extracorporeal perfusion.**

a calf liver. The authors claim that the liver showed only early signs of xenograft rejection and effectively allowed the dogs' liver to regenerate sufficiently while correcting abnormal blood parameters. They argue that this relatively simple procedure, in comparison to the costly and more complex liver transplantation, provides an efficient method to allow recovery of patients with hepatic failure. Even if human organs were considered for this use, the undoubted success of liver transplantation would raise the ethical question of using these limited resources on an alternative clinically unproven treatment (Ting and Demetriou, 2000).

### 1.2.2.2 Cross-circulation

Four patients, three with hepatic coma following halothane anaesthesia and one with post-partum hepatic coma, were treated by Burnell *et al.* with cross-circulation (Burnell *et al.*, 1967). This technique involved direct linkage of a patient to a healthy donor. The patient's blood supply passes to the donor where their liver assists in detoxification. Although the results were promising, with three successfully recovering from the coma and one recovering completely, the donors experienced a range of complications. These included postural hypotension, urticaria, fever, nausea and anorexia, low platelet count, bronchospasm, cardiac arrhythmia, and were described as "... temporarily alarming" but "..subsided rapidly" with no long term sequelae. Together with these effects and proven risks associated with the possibility of increased incidence of blood-borne disease transmission, the technique has been abandoned.

## 1.3 Bioartificial Livers

In 1969, Berry and Friend developed a method for isolating fresh hepatocytes that enabled the consideration and study of isolated hepatocytes in an extra corporeal bioreactor (Berry and Friend, 1969). Modifications to this method, by Seglen in 1976 led to the successful production of a new generation of artificial livers being applied to animal models, and more recently human trials (Seglen, 1976; Fremond *et al.*, 1993; Sheil *et al.*, 1996; Patzer *et al.*, 1999; Nyberg *et al.*, 1993; Detry *et al.*, 1999; Watanabe *et al.*, 1997).

### 1.3.1 Design of the bioreactor

The construction of a clinically applicable BAL has raised many unresolved issues. All BAL designs postulated over recent years have attempted to incorporate a number of parameters that ensure successful application. These include a requirement for:

1. A large cell mass being made available for exposure to patients' plasma/blood.
2. Mass transfer considerations
3. Cells to receive sufficient nutrition and oxygenation.
4. Consistent cell configuration.
5. A suitable fresh cell source.
6. Simple "scale up" to the clinical setting.

One of the earliest and simplest models was proposed by Olumide *et al.* (Olumide *et al.*, 1977). They described a BAL using hepatocytes circulated in a suspension utilising a renal dialysis unit (Figure 3). They studied its value on an-hepatic pigs and observed coma improvement after its introduction 24 hours after surgery. This has not been evaluated in a clinical setting.

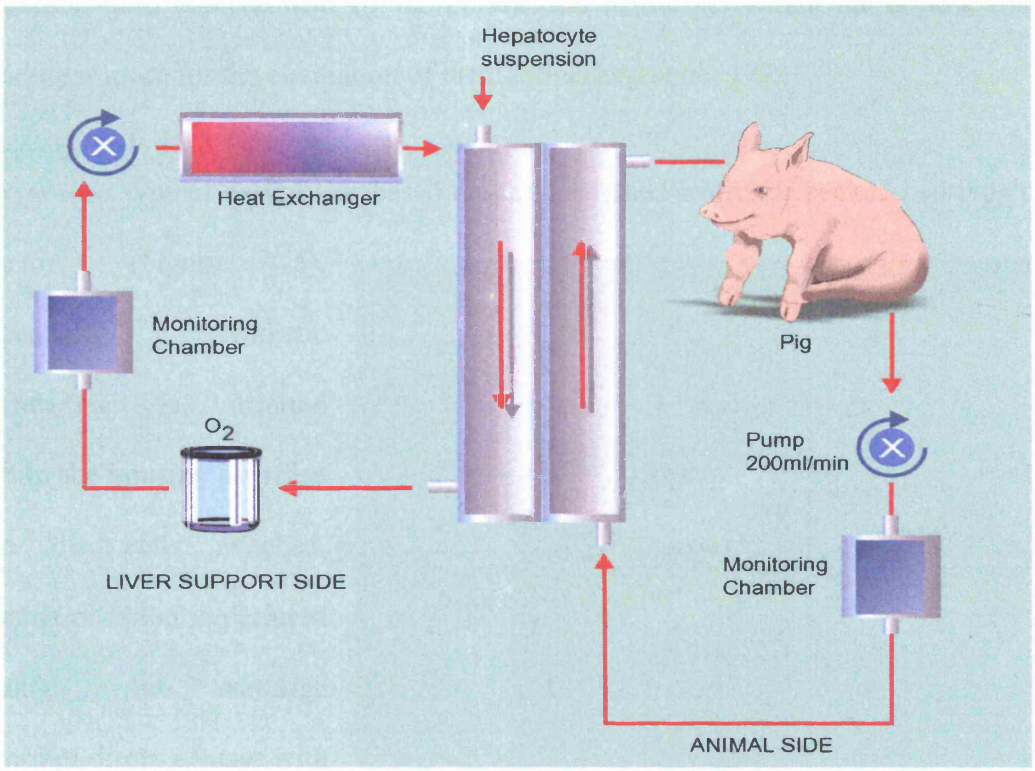


**Figure 3. BAL with hepatocyte suspensions (Olumide *et al.*, 1977).**

The hollow fibre type reactor has been the design most fully evaluated of all bioartificial liver designs. Essentially, the bioreactor consists of a cartridge containing hollow fibres in which the hepatocytes, entrapped in a gel, are inoculated into the fibres (Figure 4).

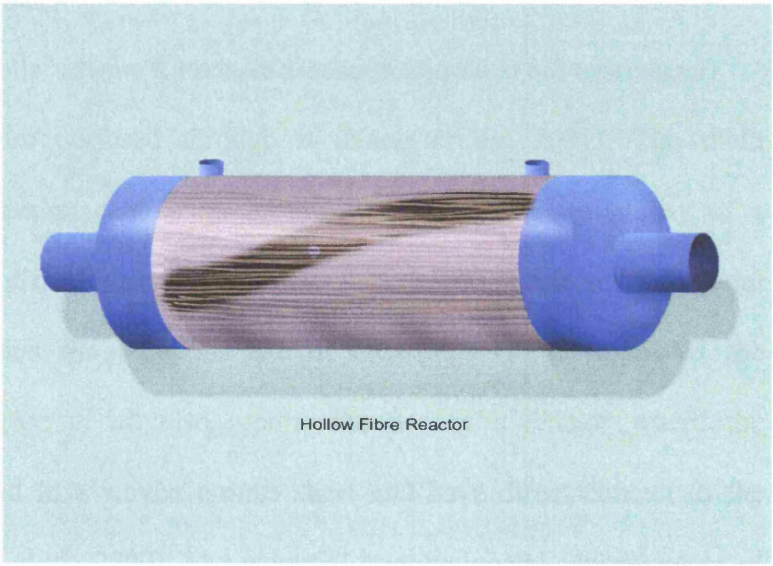


**Figure 4. Hollow fibre reactor.**

The plasma or blood is then circulated through the "extracapillary space" allowing diffusion of toxic substances across the interface. Various adaptations to this design have been made to increase efficiency and cell survival. For example, Shatford *et al.*

created a third compartment by the contraction of the gel within the hollow fibre creating a space for the circulation of media (Shatford *et al.*, 1992).

The second type of design was based on perfused beds/scaffolds/ packed bed type of reactor (Figure 5). Essentially a synthetic framework is inserted within the housing cartridge and the cells attached. Plasma or blood is perfused through the cartridge ensuring direct contact with the hepatocytes. Yang *et al.* utilised a 3-D porous material into which the cells are inoculated and serum perfused through it (Yang *et al.*, 2001). The main disadvantage with this design centres on its dependence on the availability of a sufficient concentration of freshly acquired hepatocytes able to attach to the material used. They suggested that the judicious use of centrifugal force improved the attachment process. Naruse *et al.* also reports the use of a reactor where the hepatocytes are entrapped in a woven porous sheet and have direct contact to the plasma or blood (Naruse *et al.*, 2000). The potential immunological consequences of this method were overcome by incorporating an immunosorbent column. On evaluating the system using porcine hepatocytes on a dog with hepatic failure, they demonstrated that the system reversed hepatic encephalopathy, increased survival and resulted in positive biochemical advantages over a range of parameters. It also

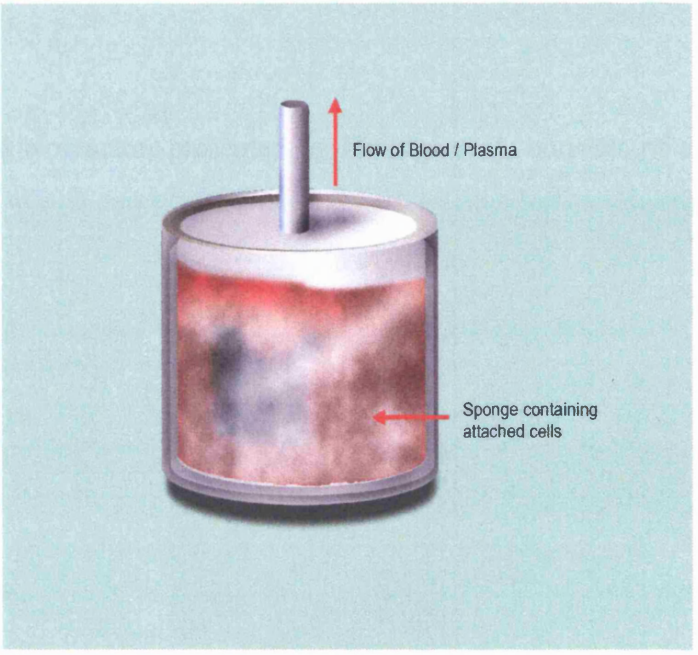


**Figure 5. Perfused beds/scaffolds/packed bed type reactor.**

demonstrated that the immunosorbent column worked sufficiently well to allow for xenogeneic perfusion. Sosef *et al.* devised a spirally wound, nonwoven polyester matrix within a cartridge BAL (Sosef *et al.*, 2002). Early experimental studies on porcine anhepatic models have demonstrated increased survival rates amongst treated groups.

The third type, a fluidised-bed bioreactor, presented by Hwang *et al.*, consists of a column of encapsulated hepatocytes (Figure 6) (Hwang *et al.*, 2000). The blood was introduced at a rate of 20ml/min and the bed became "fluidised". The alginate used for the encapsulation allowed the diffusion of important elements including essential nutrients and oxygen but prevented immunoglobulin. An acute liver failure model induced in pigs was evaluated and demonstrated an improvement in ammonia levels and control of intra-cranial pressure.

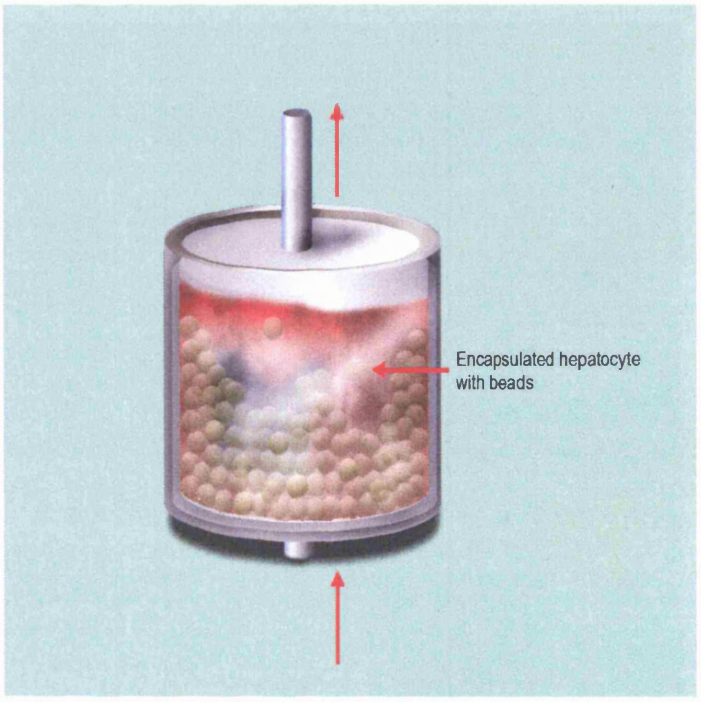


**Figure 6. Fluidised bed reactor.**

Currently, work continues to optimise the materials and parameters to improve their function and safety (McClelland and Coger, 2000; Iwata *et al.*, 2000; Ambrosino *et al.*, 2002; Beresford, 2001; Chen *et al.*, 2001; Jasmund *et al.*, 2002; De Bartolo and Bader, 2001).

### 1.3.2 Clinical Trials

In 1987, Matsumara presented results of the first clinical trial involving a bioartificial liver. A patient with liver failure due to an inoperable cholangiocarcinoma was dialysed using cryopreserved rabbit hepatocytes. Biochemical improvement was noted; although there was no evidence that it improved the clinical course of the patients' condition. This single patient study did however, demonstrate the potential for further development of a BAL.

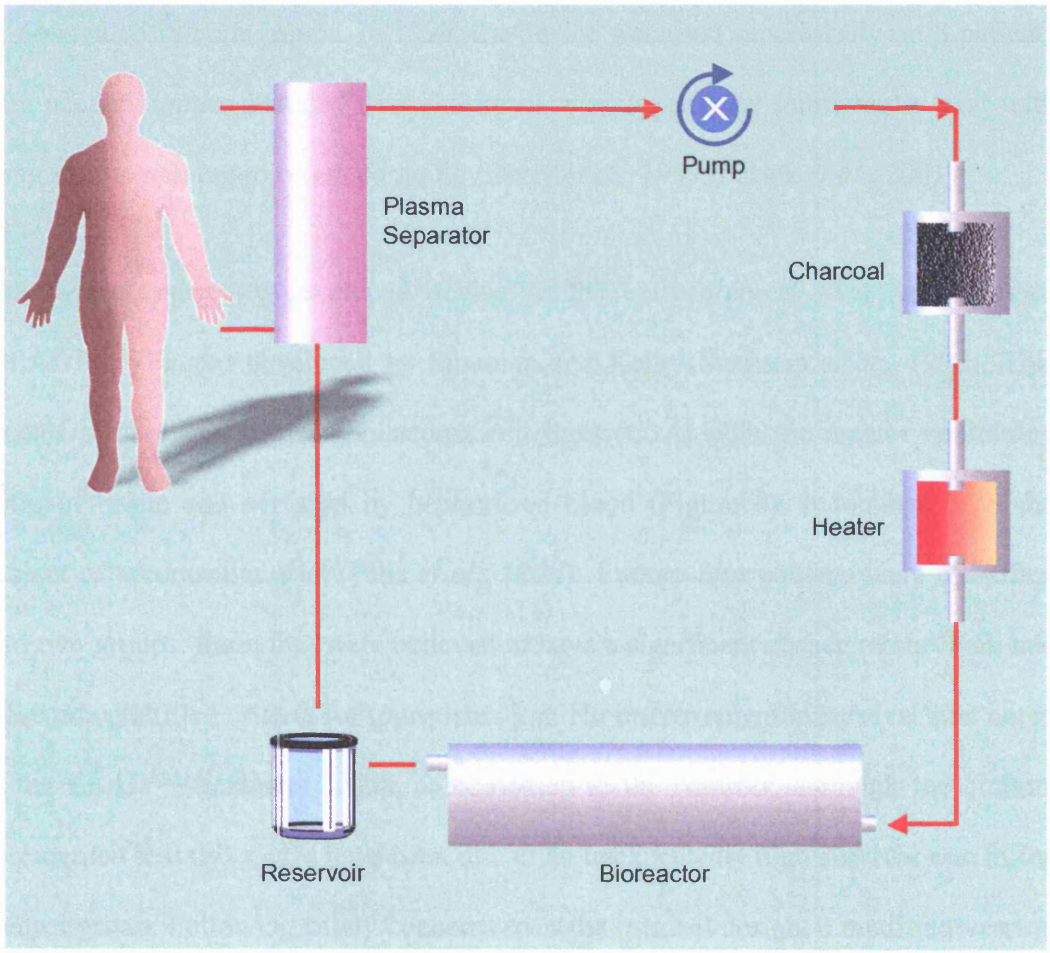


**Figure 7. The HepAssist bioartificial liver.**

The HepatAssist BAL (Circe Biomedical) developed by Demetriou *et al.* is the most evaluated system (Figure 7). It consists of a plasma separator and a high flow plasma recirculation system, primed with 5-7x$10^9$ cryopreserved porcine cells attached to microcarriers within the bioreactor. In addition, the inclusion of a charcoal column

protects hepatocytes from hepatic failure plasma. This system was initially trialled on a number of animal models and humans (Demetriou *et al.*, 1995; LePlage *et al.*, 2001; Rozga *et al.*, 2001; Rakela *et al.*, 1985; Detry *et al.*, 1999). A phase I clinical trial has now been completed (Watanabe *et al.*, 1997). They successfully bridged 16 out of 18 patients with fulminant hepatic failure to transplant. In a second group of 3 patients, with non-function of their transplanted liver, successful bridging to a further transplant was achieved. Improved neurological and biochemical parameters were demonstrated in both groups. In 1999, the device was used successfully on 8 patients with acetaminophen induced liver failure. It is now part of a multi-centre trial with early results proving to be encouraging (Detry *et al.*, 1999; Susick *et al.*, 2001)

The second, currently developed device, is the extracorporeal liver assist device (ELAD™, Vitagen) developed by Sussman and Kelly (Sussman *et al.*, 1992). This reactor utilises human hepatoblastoma cell lines (C3A) with the reactor containing 200g of tissue and perfused by heparinised blood (Figure 8). It too has been the subject of a controlled trial (Ellis *et al.*, 1996). Twenty-four patients were classified into two groups: those that were believed to have a significant chance of survival, and those who fulfilled criteria for transplantation. No improvement in survival was noted in the ELAD™ treatment group, as compared to the controls, although the authors commented that this might have been due to an unexpectedly high survival rate in the control group. Following safety concerns over the original design, a modified version of the original ELAD™ was trialled in five patients with fulminant hepatic failure awaiting transplantation (Millis *et al.*, 2002). All five survived to transplantation and no clinical problems were observed while using the device. The authors

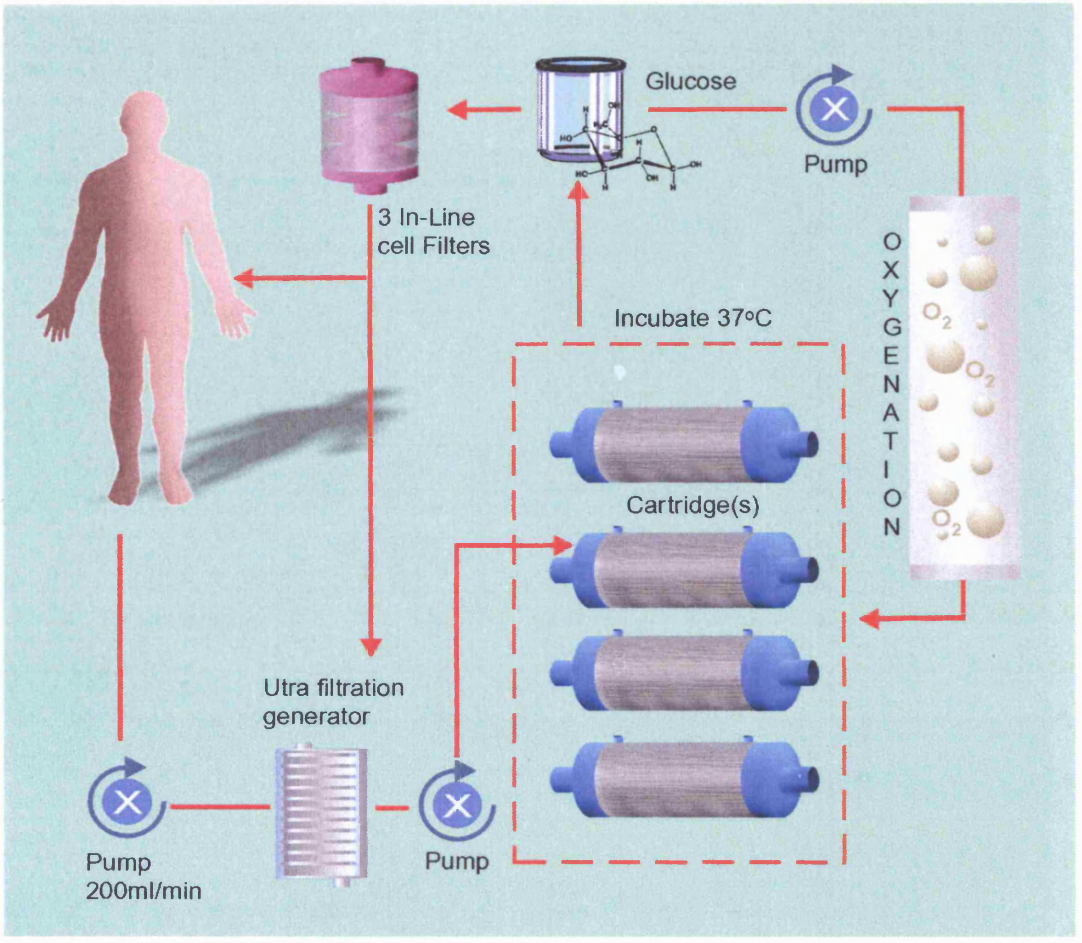


**Figure 8. The ELAD bioartificial liver.**

also commented that the cells within the bioreactor were metabolically active throughout, and that these results would allow further multicentre studies.

The Excorp Medical Bioartificial Liver Support System (BLSS) is currently undergoing phase I clinical trials (Figure 9). This device consists of a hollow fibre design utilising greater than 70g of primary porcine hepatocytes, infused into the extra-capillary space of the reactor (Patzer *et al.*, 1999; Mazariegos *et al.*, 2002; Patzer *et al.*, 2002). It is also unique in being the only device to be perfused by whole blood rather than plasma. The initial pre-clinical work on canines established the BLSS to be safe and to provide adequate hepatic support for canines with D-galactosamine induced hepatic failure (Patzer *et al.*, 2002).

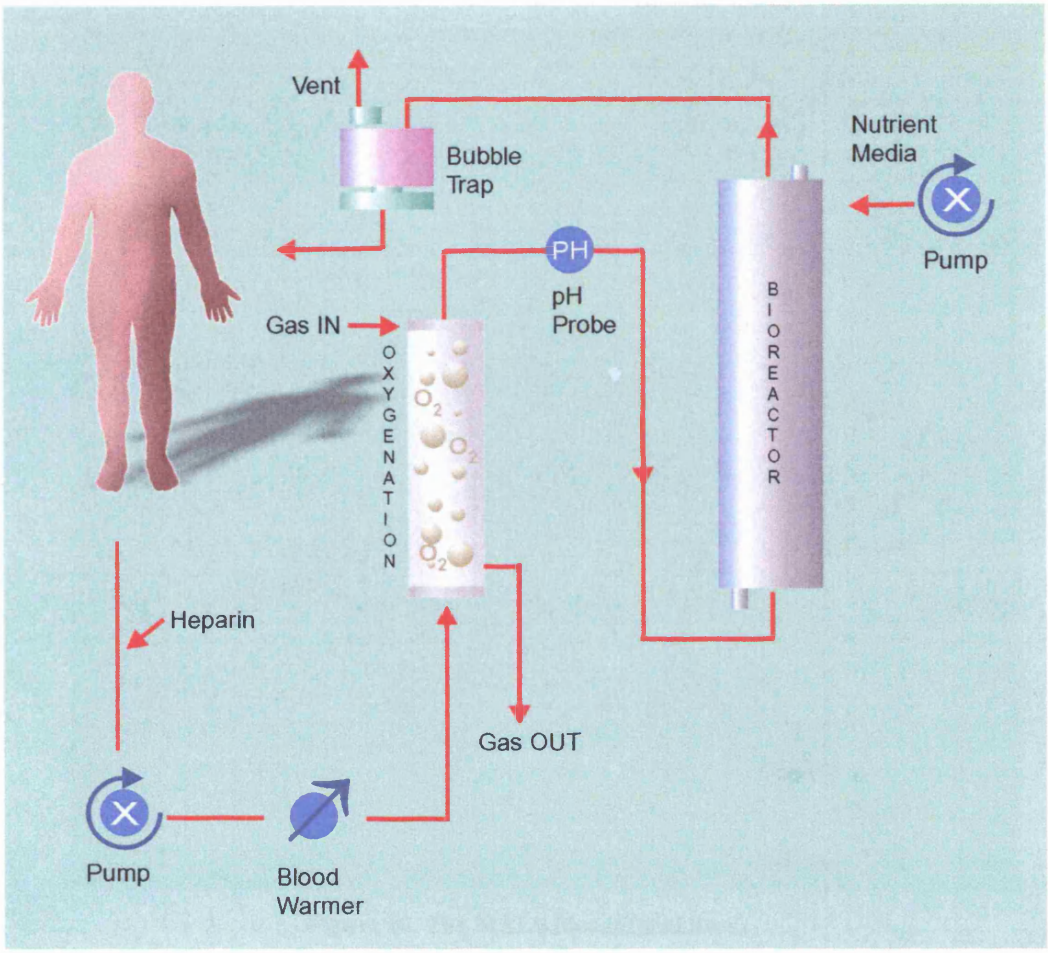


**Figure 9. BLSS bioartificial liver.**

The group has also reported the successful treatment of one patient, a 41 year old with fulminant hepatic failure, although they concluded that a full trial is needed for appropriate evaluation (Mazariegos *et al.*, 2002).

The Modular extracorporeal liver support (MELS) device is the fourth undergoing a current phase I trial (Figure 10). This is a multi-compartment hollow-fibre design that utilises the extra-compartment space of a tripartite arrangement of hollow fibres. The group recently presented early results of this phase I trial at the British Transplantation Society meeting (April 2003). For this trial they have used human hepatocytes from livers rejected for transplantation.

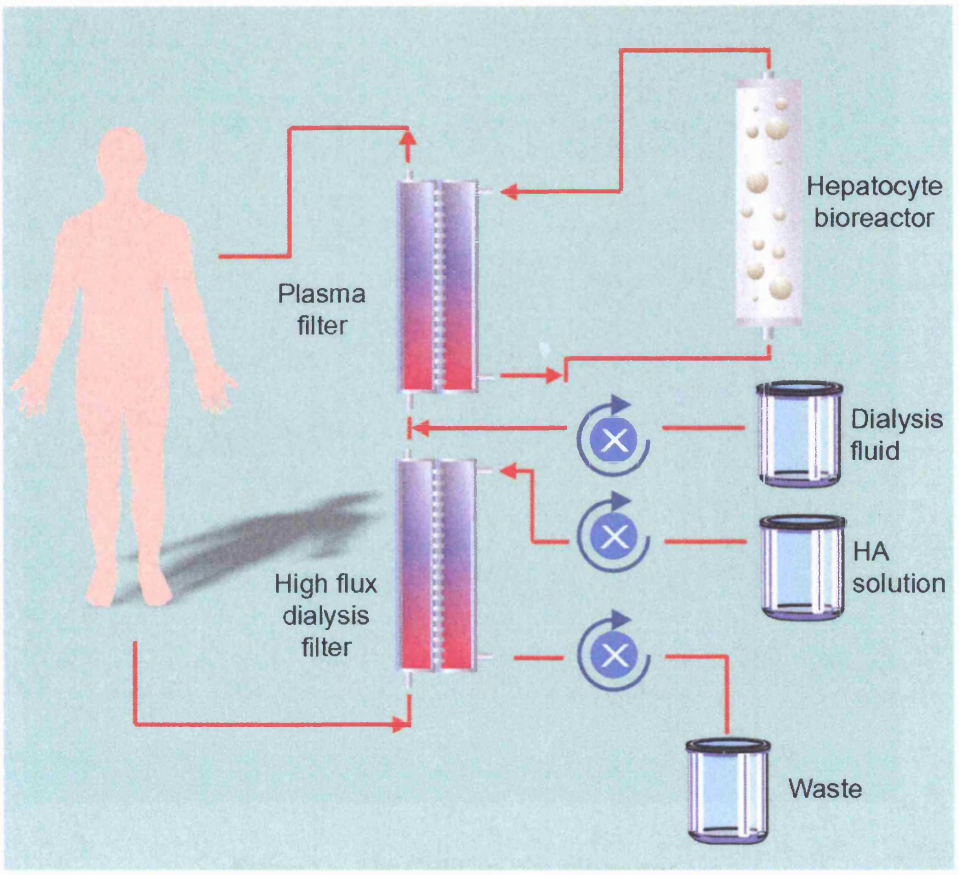


**Figure 10. The MELS bioartificial liver.**

They treated 9 patients, successfully bridging 7 of them to transplantation: 2 with acute liver failure, 2 with acute-on-chronic failure, 3 with primary non-function of their liver graft. A further 2 were not eligible for transplantation but were supported during acute deterioration. They state that all the patients tolerated the treatment well and no adverse effects were demonstrated, and that further trials are now advocated (Sauer *et al.*, 2003).

Shiel *et al.*, reported a novel bioreactor (CelliGen Plus®) evaluated on a porcine model (Figure 11). The design consisted of attaching the hepatocytes in culture to a number of polyester disks (Fibra-Cel®) and bathing them in porcine serum (Sheil *et al.*, 2000). The serum was then pumped through a separate dialysis cartridge and dialysed against the patients' blood. Immunological complexes and possible viral

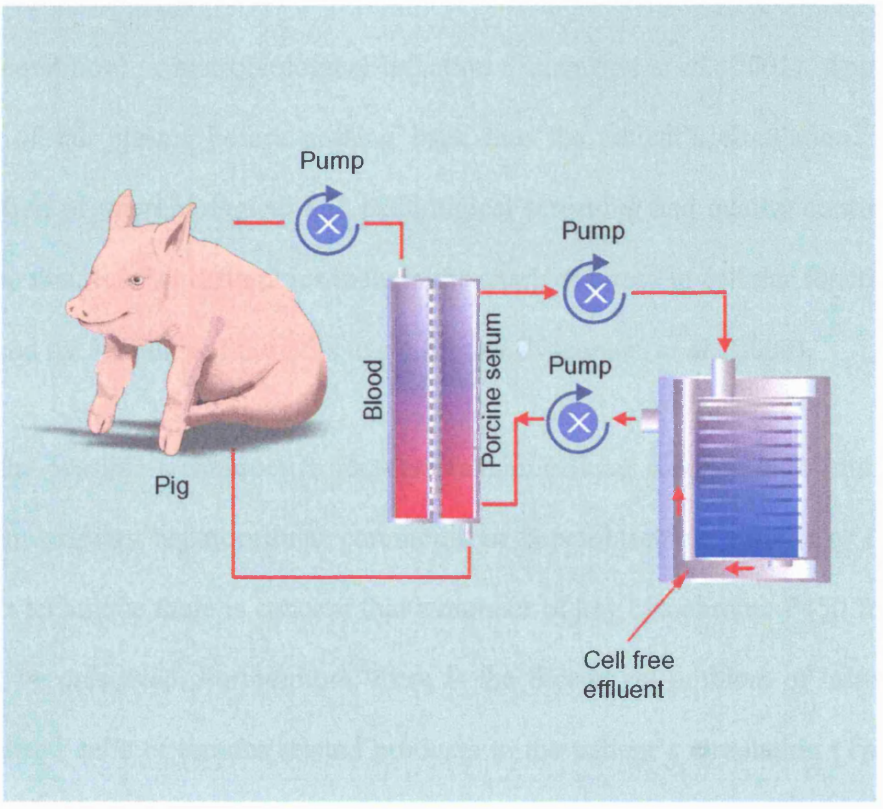


**Figure 11. The CelliGen Plus bioreactor.**

transmission were reduced by the inclusion of biological filtration for removal. The filter construction allowed small molecules, including electrolytes, enzymes, ammonia and lactate to pass back into the circulation. They achieved improvement in biochemical parameters in comparison to control group.

### 1.3.3 Cell types

#### 1.3.3.1 Human

Although human cells are seen as the ideal tissue source for a human BAL their use is restricted by low availability (Morsiani *et al.*, 2002). The current sources are restricted to surgically resected material derived from patients undergoing liver resections, or from livers rejected for transplant. The use of this tissue is subject to complex and poorly defined legal conditions, which vary in different countries (Orr *et al.*, 2002).

There is also concern over the possible transmission of carcinoma (from a resection for this condition) or microbiological infection (Tsiaoussis *et al.*, 2001). Appropriate filtering of the plasma before passing back into the patient's circulation, and the introduction of microbiological and pathological screening and quality control could overcome this. Further difficulties include the sharp decrease in cellular function after culture and the inability of the cells to proliferate (Nagamori *et al.*, 2000).

One of the possible techniques to preserve human cellular function is to immortalise cells from primary hepatocellular carcinoma or hepatoblastoma cells (e.g. HepG2). With this technique there is concern that a number of key cytochrome P450 functions may not be preserved. Furthermore, there is the theoretical problem of transmitting immortalised cells or tumour related products to the patient's circulation (Tsiaoussis *et al.*, 2001). The advantage is that the cell line is potentially unlimited. Kobayashi *et al.* presented the use of immortalised foetal hepatocytes by introducing a plasmid, SV3neo that expresses a simian virus 40 large T-antigen (SV40Tag) gene (Kobayashi *et al.*, 2001). They state that it provided metabolic support in an experimental acute liver failure model in rats. A recent paper by Kono *et al.* presented the immortalised human cell line HHY41 by culturing the hepatocytes in a sandwich layer (Kono *et al.*, 1995). After 4 weeks the cells were seen to be dividing and continued subcultures were possible.

Nagaki *et al.* reported the use of HepG2 cells and primary porcine cells in a BAL. They found that the primary cells performed significantly better than the HepG2 in the porcine model developed (Nagaki *et al.*, 2001). As described above, Sussman *et al.* used hepatocytes from the hepatoma cell line (C3A) within their BAL (Sussman *et al.*, 1992). The potential disadvantage of these cells relates to the reduced range of

functional capacity in comparison to fresh cells and their theoretical tumorigenic potential (Nyberg *et al.*, 1994). Despite these concerns and limitations early trial data have indicated that they perform adequately and that concerns may be unfounded (Mazariegos *et al.*, 2002).

### 1.3.3.2 Xenogenic hepatocytes

The use of hepatocytes derived from other species is an attractive option (Table 2). Rats are easily obtainable and their hepatocytes are simple to isolate. They have the advantage of being metabolically very active in comparison to larger animal cells. Unfortunately, a large number of animals would be required to provide enough cells to service a clinical BAL (Tsiaoussis *et al.*, 2001).

Porcine cells are seen as the superior available option and have been used extensively as the main cell of choice for the current trials of clinical BALs. A single liver can produce sufficient quantities of cells that have similar characteristics to human hepatocytes. The problem with using xenogenic hepatocytes are summarised by Tsiaoussis *et al.* into three categories (Tsiaoussis *et al.*, 2001):

*Immunogenicity*: Immunogenicity is considered a major problem as it has been estimated that 7% of the population have pre-formed IgM against porcine hepatocytes, more specifically GAL- alpha GAL membrane epitope (Takahashi *et al.*, 1993). These antibodies have been found to play a role in the hyperacute rejection process of xenografts. During perfusion these antibodies clear from the circulation and deposit in the donor organ aiding rejection (Parker *et al.*, 1994). No adverse effects have been discovered to date, although Tsiaoussis concludes that this could be due to the immunosuppression of the patients suffering from acute liver failure (ALF),

although few studies have examined this phenomenon. Bacquerizo *et al.* demonstrated a two-to three-fold increase in anti-pig antibodies in patients receiving 2 or more treatments with a porcine bioartificial liver (Baquerizo *et al.*, 1997). The risk of hypersensitivity reactions and humoral sensitisation with the production of antibodies that would cross-react with human antigens (i.e. HLA) is considered a high priority (Dowling and Mutimer, 1999). Currently, this has not been shown experimentally (Cotterell *et al.*, 1995). The potential effects of adverse immunological reactions to both patient and reactor are being studied where repeated use of porcine bioartifical livers is being undertaken.

*Zoonoses*: There is the potential that animal cells may pass on infections to their human hosts. The recent discovery of the porcine endogenous retrovirus (PERV) has raised questions about the suitability of using porcine tissue for human utilisation (Quarto and Santi, 2000). This virus, which is found within porcine cells, has been introduced to human cells in vitro and successfully replicated (Patience *et al.*, 1997; Le Tissier *et al.*, 1997; Martin *et al.*, 1998,). Le Tissier *et al.* concluded that to breed virus free pig "would if at all possible, ... represent a complex task." (Le Tissier *et al.*, 1997). Despite these concerns it has not yet been demonstrated that patients exposed to porcine BAL's and xenografts have developed any signs or symptoms of infection (Kuddus *et al.*, 2002; Patzer, 2001; Falasca *et al.*, 2001; Pitkin *et al.*, 1999; Heneine *et al.*, 1998; Patience *et al.*, 1998). Therefore, Platt argues that this is probably not a substantial risk, especially if tissue sources and organs are suitably screened (Platt, 2000).

Bornemann *et al.* have cautioned interpretation of many studies looking at PERV infection following xenotransplant or exposure of patients to bioartificial perfusions.

The details of the study need to be looked at carefully to see if the perfusion was in direct contact with the patient or was a membrane placed to filter out PERV (Bornemann *et al.* 1996). Further hypothesis have been put forward regarding the differences in the demonstration of infection between *in vitro* and *in vivo* studies. Fuijita and colleagues demonstrated that human serum might have an inhibitory effect on PERV and prevent its infectivity (Fuijita *et al.*, 2003). They demonstrated that the classical complement pathway is activated by the binding of natural antibodies to the α1-3Gal epitopes, and possibly others yet to be defined. It remains to be demonstrated whether appropriate filtering membranes and the types of patients treated with bioartificial livers, will provide the safety to prevent PERV establishing within the human population.

*Biocompatability*: Although porcine cells are very similar in their function to human cells, there is still concern that products from a different species may not exert an appropriate or even sufficient biochemical response in the human circulation

### 1.3.3.3 Progenitor or Stem cells

Recently, it has been accepted that the liver contains cells with stem-like properties which, when activated, could proliferate and mature into all types of hepatic cells. These cells can now be separated by flow cytometry and are in the early stages of evaluation (Susick *et al.,* 2001). The perceived advantages over mature cells include their ability to replicate and differentiate into specific cell types and have superior cryopreservation tolerance characteristics with minimal immunogenicity (Samuel *et al.*, 2002; Ilan, 2002; Shafritz, 2000).

| Cell Source | Pros | Cons |
|---|---|---|
| Primary human hepatocytes | Allo-compatible<br>Functionally active | Scarce availability<br>Difficult to culture<br>Potential cancer transmission |
| Human liver cell lines | Highly allo-compatible<br>Largely available | Limited function<br>Ethical considerations |
| Hepatoma derived cell lines | Largely available | Potentially tumourgenic |
| Transfected human cell lines | Highly allo-compatible<br>Largely available | Limited function<br>Potentially tumourgenic<br>Potential viral transmission |
| Primary porcine hepatocytes | Largely available<br>Functionally active and similar to human | Xenoreactions<br>Zoonosis<br>Immunogenicity |
| Small animal i.e. rat | Largely available<br>Functionally active | Xenoreactions<br>Zoonoses<br>Immunogenicity<br>Large numbers of animals required |

**Table 3. Summary of potential cells in bioartificial liver construction (adapted from Morsiani *et al.*, 2002).**

It is too early to fully appreciate what role this cell type will have on hepatic support therapies, and it should be remembered that there is no guarantee that the cells will dedifferentiate to the correct characteristics (Kobayashi *et al.* 2003). The future for this new development has considerable theoretical potential.

## 1.3.4 Number of cells required

The optimum number of cells required to support a patient with liver failure is currently uncertain. It has been hypothesised from liver resection data that the body requires 10-30% of the normal liver mass to support life, although this figure is considered by some to be an underestimate (Riordan and Williams, 1999; Nagasue *et al.*, 1987). This equates to approximately 100-400g of cells being required by a bioreactor, assuming there is no endogenous liver function (Rosenthal, 2000). Hui *et*

*al.* states that there is functioning liver in most patients with liver failure, so providing clinical management support rather than complete replacement should be the preferred option (Hui *et al.*, 2001).

In Leicester, the bioartificial liver under development for large volume liver resections, may only require relatively small cell numbers. Patients undergoing this type of surgery generally do not have abnormal liver function markers (unpublished data). On the whole, tumours do not affect the function of the liver unless they directly occlude bile drainage or a large vessel. Therefore, the patient is starting their treatment at a potentially "normal" level, rather than the artificial liver being required to not only maintain function but to correct abnormalities, as in the "bridging" to transplant system. They also have a small volume of remaining liver tissue, which should, with adequate physiological support, regenerate.

Currently, in the clinically developed BAL to date, wide variations in cell numbers have been required. The HepatAssist device requires approximately $6x10^9$ porcine cells which corresponds to 2% of normal cell mass (Watanabe *et al.*, 1997). The ELAD device uses $4x10^{10}$ C3A cells, equivalent to 15% of normal hepatocyte mass, while the MELs device uses $1x10^{11}$ porcine hepatocytes or human cells (equivalent to 400-600g of tissue), 33% of the hepatocyte mass. By comparison the BLSS utilises 70g of porcine hepatocytes (Sussman *et al.*, 1992; Sauer and Gerlach, 2002; Gerlach *et al.*, 1994; Patzer *et al.*, 1999; Mazariegos *et al.*, 2002).

Sauer *et al.* commented that it has proven difficult to equate the required cell mass with an equivalent function as most cells within the BAL will have reduced functional ability (Sauer *et al.*, 2001). There is still much controversy as to whether such a small number of cells can provide adequate support (Iwata *et al.*, 1999). Future

developments that improve our understanding of cellular behaviour in BAL designs and determine the optimum criteria for patient selection, will ultimately dictate the cell number that should be used.

### 1.3.5 Treatment regime

The treatment regime for BAL's to become established in a clinical setting has yet to be ascertained (Dixit, 1994). It is uncertain whether patients will need continuous or intermittent treatment similar to the regimes undertaken for patients requiring renal dialysis. This may also depend on patient and pathological factors that will require individually tailored programmes. In the human trials already undertaken, a number of different protocols have been evaluated using different devices. Demetriou *et al.* (HepAssist) used 6-7 hours for their studies with beneficial effects being maintained for up to 18 hours following cessation of the treatment (Demetriou *et al.*, 1995; LePlage *et al.*, 2001; Watanabe *et al.*, 1997). The ELAD and BELS favour continuous perfusion, while the preclinical work of the BLSS group suggest that 12 hour perfusion periods are adequate (Mazariegos *et al.*, 2002; Patzer *et al.*, 2002).

Supporters of continuous support site the situation *in vivo* whereby the liver is continuously perfused. However, published work by Stefanovitch *et al.* has demonstrated the effect of plasma on rat hepatocytes following plasma perfusion (Stefanovich *et al.*, 1996). They found that the cells produced urea to normal levels after 20 hours of continuous perfusion. Following, this period of treatment there was a significant reduction in hepatocyte function. When the perfusion time was divided into 6 hour time periods, the total time before loss of function was 96 hours. This strategy indicated that an intermittent regime provides longer periods of effective hepatocyte function.

## 1.4 The Leicester model

The Leicester model (Figure 12), as already outlined above, has different potential treatment uses compared with alternative artificial livers being developed. In collaboration with the University of Manchester Institute of Science and Technology (UMIST) a design has been proposed that will be developed over the coming years.

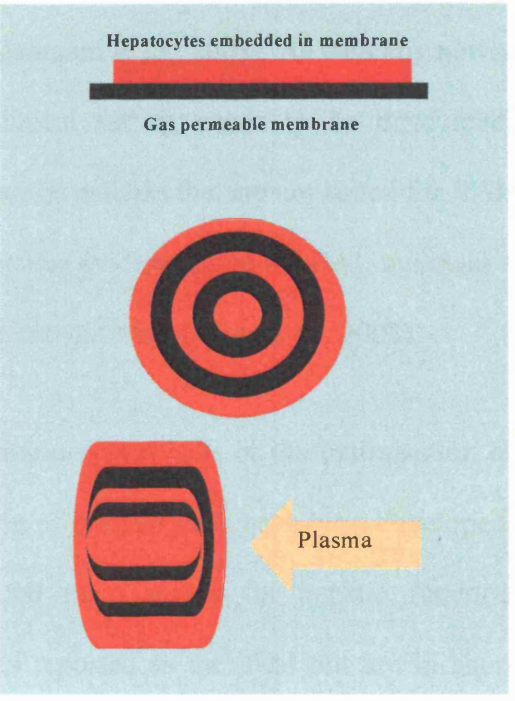


**Figure 12. Leicester BAL design plan**

It is based on attaching hepatocytes to a porous sheet, that will then be rolled and plasma perfused through it. Initial tests will be performed on a porcine model being developed in Adelaide. It is anticipated that cryopreserved cells will also be used either wholly or partly as the tissue source. Due to the relatively small number of patients that are likely to be treated and for the reasons discussed above, it is anticipated that a sufficient number of isolated and cryopreserved human cells from liver resections will provide the cell source.

## 1.5 Summary

Significant progress has been made into the development of a bioartificial liver, yet the parameters for a successful BAL model are far from clear. The various designs, tissue types, cell numbers required and range of disease that may potentially be treated have complicated the development process. Animal and human trials to date have yielded encouraging results. A Cochrane review of artificial and bioartificial

human clinical trials did not conclude specifically the bioartificial liver position, but as a whole, both artificial and bioartificial treatments show some benefit in the treatment of acute-on-chronic liver failure (Kjaergard *et al.*, 2003). As with any newly developed system, its place within the clinical setting needs to be developed, established and clinically evaluated. There may be patients that are not suited for BAL treatment, and further ethical debates may become evident: could the BAL maintain a patient's consciousness and when should it be discontinued (Nitta *et al.*, 2002).

Until a tissue type such as stem cells, safe immortalised cells or the optimisation of conditions that allow the successful replication of primary cells have been developed, primary cells need to be considered as the main source for hepatic function replacement therapy. Human cells have been reported as the ideal but are in short supply. In Leicester, in association with the UK Human Tissue Bank, a large number of surgically resected liver specimens provide the basis for cellular isolation. For a clinically successful BAL project identification of hepatocyte sources need to be identified for use at any time. This thesis examines the problems surrounding cryopreservation and hepatocyte isolations from human and porcine livers and uses this knowledge to evaluate and optimise hepatocyte isolation, cryopreservation and determine the functional status of hepatocytes for potential use within bioartificial liver systems.

# Chapter 2

# Basic liver anatomy and function

2.1 Clinical Background

2.2 Structure and function of the liver

- 2.2.1 Macroscopic structure
- 2.2.2 Microscopic structure
- 2.2.3 The functional unit
- 2.2.4 Hepatocyte structure and function
- 2.2.5 Kupffer cells

2.3 Liver drug metabolism and waste handling

- 2.3.1 Phase I
- 2.3.2 Phase II
- 2.3.2 Coculture

2.4 Hepatic regeneration

## 2.1 Clinical Background

The treatment of liver disease and hepatic failure, irrespective of the cause has been a significant challenge to the medical profession. According to Department of Health statistics in 2000, 17.8 people per 100,000 died of chronic liver disease and cirrhosis in the UK. The National Statistics Office reported that 9000 patients with alcoholic liver disease and 3000 cases of cirrhosis were admitted to hospital in the UK in the previous 12 months. The predominant cause was alcohol abuse, the remainder being due to viral infections (hepatitis A-E; cytomegalovirus), drug abuse, neoplasms, vascular abnormalities and occasionally, pregnancy. The rise in the incidence of cirrhosis has been linked to changing attitudes within society towards alcohol. Since 1970, there has been a steady rise in mortality from cirrhosis that correlates with a 'binge' drinking culture and an acceptance of this social behaviour in the younger age groups. Over the last 30 years this has resulted in an 8-fold increase in cirrhosis in the 35-44 age group in men and 7 fold in women. The conclusion drawn in the Chief Medical Officers report (Donaldson, 2001) anticipated that the incidence of alcoholic liver disease would continue to rise unless public health measures are implemented to educate the population about the dangers of excessive alcohol consumption.

In the past, the prognosis of patients with hepatic failure was considered universally poor as they would invariably develop cerebral oedema, gross coagulopathies, renal failure and septicaemia, which lead to a 80-94% mortality from multiple organ failure (Rakela *et al.*, 1985; Typstrup *et al.*, 1981). With developments in liver transplantation techniques over the last 20 years, patient prognosis has improved with survival now being 65-75%

(Chapman *et al.*, 1990). However, as with all transplant programmes, a chronic shortage of donors has proved a significant limiting factor, preventing further improvements in the survival statistics. Many either die before a transplant can be undertaken, or deteriorate to such an extent that they are no longer suitable candidates (Dowling and Mutimer, 1999; Riordan and Williams, 2000).

Although consideration of hepatic malignancies is beyond the scope of this study there is a potential need for hepatic support in the management of such patients. Primary hepatocellular carcinoma remains unusual in the United Kingdom yet it is one of the most common solid organ tumours world-wide, primarily caused by Hepatitis virus strains B and C, especially in the Far East, where it is endemic. In the United Kingdom the most common liver malignancy is secondary spread from colonic and rectal primaries, although it is also found in other malignancies such as renal and breast (McCarter *et al.*, 2000). This can either be recognised at the time of surgery for the colonic primary or years later during routine follow up. The 5-year survival of patients with established, untreated, liver metastasis was found to be less than 5%, and the median survival time is approximately 6 months (Fong *et al.*, 1999). Despite this poor prognosis, hepatic resection, in the absence of extrahepatic disease, has been associated with a 5-year survival rate of 20-65%, with an operative mortality of less than 5% (Nordlinger *et al.*, 1996; Ueno *et al.*, 2000; Fong *et al.*, 1999; Nagakura *et al.*, 2002).

Hepatic resection is currently the only proven curative option for patients with colorectal metastatic deposits. Over recent years surgical and anaesthetic techniques have advanced

with liver surgery becoming almost routine. Various groups have studied a wide range of prognostic indicators (Table 4) in an attempt to predict postoperative survival (Nordlinger *et al.*, 1996; Ueno *et al.*, 2000; Fong *et al.*, 1999). Despite these studies the biology of the disease is such that there remains a 60% recurrence risk after hepatic resection although recent studies have shown that repeated local resections of these recurrences can improve the overall predicted survival by as much as 11% (Nagakura *et al.*, 2002). Unfortunately, the removal of approximately 80% and greater of the functioning liver, will compromise the patient and without adequate liver support lead to death. Below this level the liver rapidly regenerates over the first 2 weeks and by 1 year 80% of the original liver is present (Nagino *et al.*, 2001). In many situations the degree of tissue removal has to be moderated to ensure that the patients remaining liver is able to cope with the metabolic demands placed on it post operatively, whilst simultaneously being able to regenerate. Such patients are the elderly, those with cirrhosis or gross steatosis in those where there is a small left lobe (if a right hepatectomy is contemplated), and patients with extensive metastatic disease.

| |
|---|
| • 20 years of age |
| • Largest lesion being >5 cm |
| • Serosal extension by the primary cancer |
| • Lymphatic spread of the primary cancer |
| • Preoperative CEA of > 200ng/ml |
| • Bilobar disease |
| • >4 lymph nodes at primary resection, margin <1 cm |
| • Diagnosis of metastasis at time of primary surgery or within 1 year |

**Table 4. Poor prognostic indicators for liver resection.**

Recently further strategies to reduce this possible liver failure in patients undergoing liver resection have been evaluated. In patients with large tumours, who would be left with an unacceptably small remnant can undergo portal vein embolization in an attempt to increase the size of the remnant. Approximately 2 months before surgery is contemplated, the affected lobe is embolized via the portal vein, to induce atrophy. This leads to compensatory hypertrophy of the future remnant. This technique is largely experimental and only routine in a few centres, but appears to be well tolerated by patients (Azoulay *et al.*, (2000).

Despite bilobar disease being quoted as a poor prognostic factor (Table 4), some patients are now being offered two-stage surgery with curative intent. One side is removed during the first operation followed by the second 1-2 months later following adequate regeneration of the remnant (Elias *et al.*, 1995). This approach is often combined with chemotherapy, although the optimal order and drugs used have not been fully defined (personal communication with own centre). Large numbers are not reported in the literature although a 3-year survival rate of 35% and median survival of 31 months have been reported (Adam *et al.* 2000).

The resultant liver failure, whether as a result of the disease process or surgery, represents an extremely complex metabolic situation and a difficult clinical entity to manage. Currently, supportive management is the only widespread treatment that can be offered. With this the native liver will either begin to regenerate or will fail, necessitating a liver transplant (assuming eligibility). There is an acute need for a treatment option that replicates liver function to either 'bridge' patients to a transplantation or create a suitable

environment to allow time for regeneration of the native liver (Riordan and Williams, 1999; Schulte arn Esch *et al.*, 2002).

It was realised that hepatocytes were crucial to achieve this and their isolation from the liver has been successfully achieved over the last 30 years. These cells have formed the basis of developmental bioartificial liver systems or alternatively used directly in hepatocyte transplantation to improve patient management and clinical outcome.

## 2.2 Structure and function of the liver

The liver is one of the largest (1200-1600g) and most complex organs in the body. It accounts for 20-30% of the total oxygen consumption and requires 20-25% of the cardiac output. It has many functions summarised in Table 5.

| | |
|---|---|
| 1. | Uptake nutrients delivered from the digestive tract via the portal vein. |
| 2. | Synthesise, store, interconvert and degrade metabolites. |
| 3. | Regulate supply of energy rich intermediates and building blocks for biosynthetic reactions. |
| 4. | Detoxify harmful compounds by biotransformation. |
| 5. | Excrete substances with the bile as well as synthesise and degrade many blood plasma constituents. |
| 6. | Metabolise carbohydrates, lipids, amino acids and proteins, maintaining them at sufficient plasma levels. |
| 7. | Heat production. |
| 8. | Activate own immune system particularly by antigen recognition. |

**Table 5. Roles of the liver**

### 2.2.1 Macroscopic structure

When viewed from the front they are separated by the falciform fissure. On the inferior aspect the right lobe is seen to consist of two further lobes, the quadrate and caudate (Spigelian) lobes (Figure 13).

The functional anatomy is very different from this simple left and right, being based upon the division of the liver into eight segments following the distribution of the portal pedicle and the location of the hepatic veins, as described by Couinaud (Figure 14). The four sectors individualised by the portal veins are called portal sectors as each is supplied by independent portal pedicles. This separates the liver into left and right functional divisions (Figure 15).

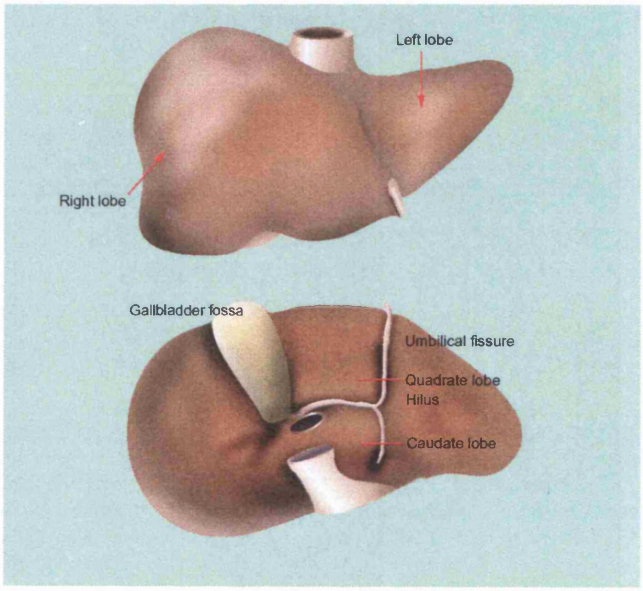


**Figure 13. Morphology of the liver**

The right liver is divided into two sectors by the right portal scissura. Each of these sectors is further divided into the anterior sector segments, V inferiorly and segment VIII superiorly; and the posterior sector, segment VI inferiorly and segment VII superiorly (Figures 14 and 15).

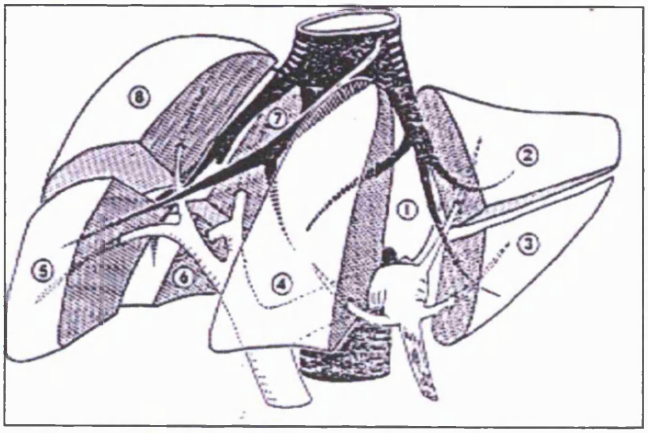


**Figure 14. Couinauds segments (McIntyre *et al* 1991).**

The left liver is divided into two sectors by the left portal scissura where the left hepatic vein runs. The anterior sector is divided by the umbilical fissure, medially segment IV the anterior part of which is the quadrate lobe and laterally segmentIII which is the anterior part of the left lobe. The posterior part only has one segment, segment II, which is the posterior part of the left lobe (Figure 2 and 3). The caudate lobe (segment I) must be considered, from the functional point of view, as an autonomous segment, or "mini-liver", for its vascular supply is independent of the portal divisions.

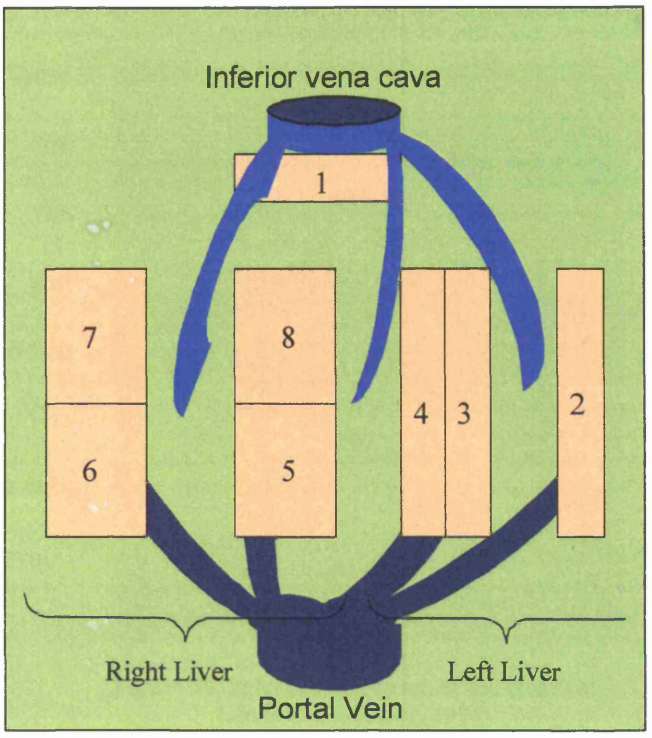


**Figure 15. Vascular supply to the segments (adapted from McIntyre *et al.* 1991).**

## 2.2.2 Microscopic structure

The liver is a large organ that is highly vascular and surrounded by a very thin capsule (Glissons capsule). This capsule is composed of collagen fibres and scattered fibroblasts. Eighty percent of the blood entering the liver enters through the portal vein. Accompanying the portal vein at the hilum are the hepatic artery (20% of the blood supply) and the exiting bile duct. Within the liver, blood passes from the portal tract through a number of small channels called sinusoids. These are lined by endothelial and

Kupffer cells which lie one cell deep with hepatocytes below. From the sinusoids blood passes to the centrilobular vein (Figure 16). During the flow through the sinusoids, blood is exposed to the hepatocytes which detoxify and biotransform the contents of the blood to enable their subsequent excretion. The centrilobular vein drains eventually into the hepatic veins and inferior vena cava.

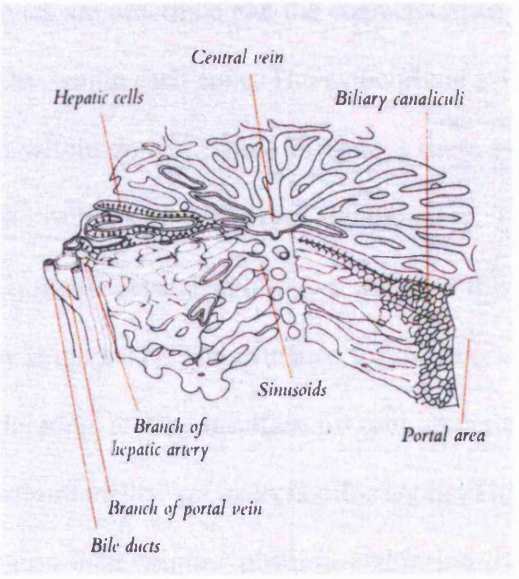


**Figure 16. Schematic diagram of the classical lobule (from Leevy, 1974)**

## 2.2.3 The functional unit

The classical lobule is illustrated in figure 16 and consists of a central vein with a hexagonal area surrounding it. At each corner is found a portal tract, and the sinusoids are located between it and the central vein. Functionally, it is the description by Rappaport (Rappaport's acinus) that is generally believed to reflect the situation most accurately (Figure 17). Rappaport's acinus consists of an axis, which is a small radicle of the portal triad containing a terminal portal vein, hepatic arteriole, nerves, lymph vessels and bile ducts. Blood flows from this axis to the centrilobular veins. This

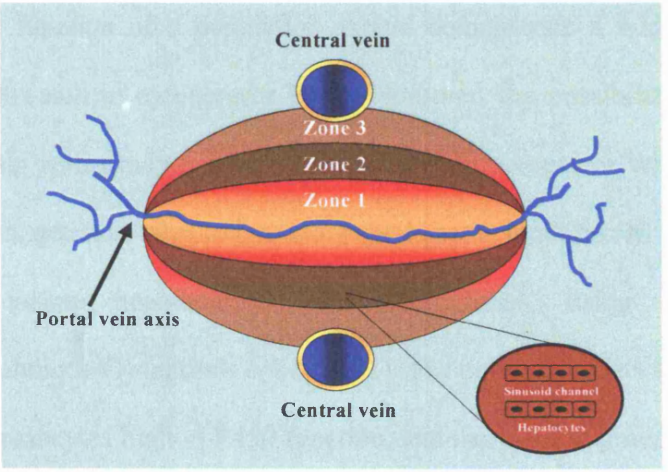


**Figure 17. Rapport's ascinus and zonal distribution**

concept is particularly useful in understanding the gradients of metabolic activity that occur in zones surrounding the axis. Three zones are described and the characteristics of hepatocytes in each differ, conferring specificity within each zone. The cytochrome P450 enzymes are found at a higher concentration within zone 1, while in zone 3 there is a higher concentration of enzymes such as urea synthesis enzymes and glutaminases. The hepatocytes in zone 3 are more important for bile-salt dependent bile formation, as this is the area in closest contact with the blood flow (Figure 17). The evidence for this work is vast. Many studies have been undertaken looking at the specifics of each function. Hepatocytes have been cultured in the same culture media, yet express different activities of glycolysis and gluconeogenesis, depending on their original position within the liver (Probst *et al.,* 1982). This work has been extensively reviewed by Jungermen (Jungerman *et al.* 1989). The zonations of liver functions have importance for bioartificial liver development. Ideally the cellular function of a population should demonstrate a wide range, but particularly contain cells rich in cytochrome P450. However, the possibility exists that hepatocytes from certain zones may not survive the isolation process or will not culture, thus leading to a homogeneous population and poor hepatic function. It is now possible to selectively isolate hepatocytes from these zones, using a digitonin/collagenase perfusion technique (Tsiaoussis *et al.,* 2001). The potential exists to enrich a bioartificial liver with hepatocytes high in P450 function, and therefore augment the biotransformation and detoxification functions.

Other cells are found accompanying the hepatocytes along the sinusoids: Kupffer cells (2% of the total cellular mass), endothelial cells (3%) and lipocytes (2%).

## 2.2.4 Hepatocyte structure and function

Hepatocytes are large cells, 10-30μm in diameter and polyhedral in shape. They are arranged in plates dispersed between vascular channels and the sinusoids. Between them lie tiny biliary spaces or bile cannaliculi, where bile is excreted. As the distribution of nutrients, biologically active molecules, and oxygen is different depending on the situation of the hepatocytes, the individual hepatocyte cell structure and function differs depending upon its position in the liver (Table 6).

The unique characteristic function of the hepatocyte is its ability to detoxify many different molecules and adapt to a huge array of chemical substances. These may be endogenous substances such as bilirubin, or exogenous substances particularly drugs and foodstuffs. Almost any harmful substance can be converted to one that may be excreted in the bile or blood utilising innumerable chemical reactions. Overall this process is referred to as biotransformation and it is so complex that it cannot be duplicated artificially. These problems underpin many of the design considerations of biological hepatic support systems.

- Protein synthesis and secretion.
- Bile formation and secretion.
- Metabolism and detoxification of lipid soluble drugs.
- Metabolism of steroids and cholesterol synthesis.
- Lipoprotein synthesis and secretion.
- Carbohydrate metabolism.
- Urea formation.

**Table 6. Summary of Hepatocyte functions.**

## 2.2.5 Kupffer cells.

These cells are phagocytic and are derived from monocytes. They are responsible for destroying many potentially pathological substances, including bacteria, immune

complexes, and particles. With appropriate stimulation these cells release mediators and cytotoxic agents to aid destruction. Their microscopic structure is typical of cells performing this function, and consists of a large volume of cytoplasm with complex and extensive organelles (Ross and Rommel, 1990).

## 2.3 Liver drug metabolism and waste handling

Hepatocyte metabolism of drugs and endogenous products is multi-faceted, but can be subdivided into two interlinked systems: phase I (function reactions) and phase II (conjugation reactions) (Figure 18). Phase I reactions as a general rule act to prepare the substrate for phase II. During this phase apolar compounds have new functional groups added to them (-OH, -$NH_2$,-SH, -COOH etc:) to increase the polarity and decrease the biological activity or toxicity. However, it should be noted that some drugs only become biologically active once

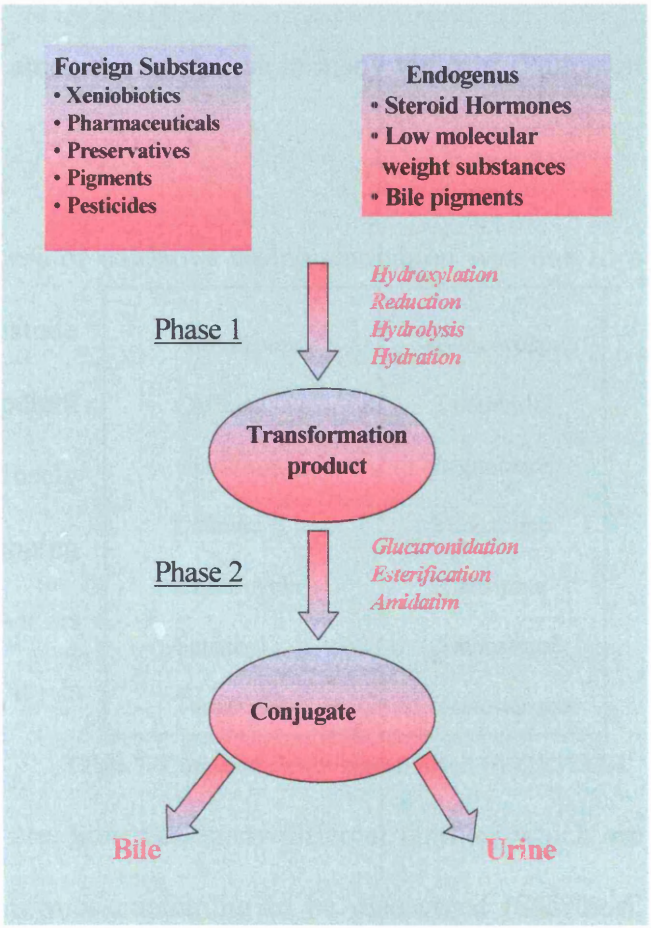


**Figure 18. Biotransformation by phase I and phase II (adapted from Koolman *et al* ).**

they have been subjected to phase I reactions. Oxidation is the most important reaction and is performed by the highly diverse mixed-functional oxidase system called Cytochrome P450.

P450 isoenzymes perform oxidative biotransformation of endogenous substrates as well as drugs and carcinogens. Each individual organism possesses a large number of different P450 isoenzymes, many of which are selectively inducible. They are located in the membranes of the endoplasmic reticulum of hepatocytes and are also found in the kidney, skin, lungs and CNS (in much smaller amounts) as well as in many tumours (Patterson and Murray, 2002).

Originally it was thought that the process of oxidative biotransformation was due to a single haemoprotein with broad substrate specificity. However, it has become apparent that there was a large number of closely related isoenzymes with some overlapping function. Cytochrome 3A (CYP3A) is clinically the most important as it is responsible for the biotransformation of many common drugs (Table 7). There are, however, many different families which are numerous and diverse with new isoenzymes continuing to be discovered (Saarikosi, 2003).

| | |
|---|---|
| Amlodipine | Hydrcortisone |
| Caffeine | Laoperidol |
| Clarithromycin | Lignocaine |
| Codeine | Midazolam |
| Erythroycin | Nifedipine |
| Fentanyl | Salbutamol |
| Tamoxifen | Testosterone |

**Table 7. Common drugs metabolised by CYP 3A4**

The characteristics of the P450 isoenzymes are very important for the development of new drugs and for studying the possible polypharmacological interactions directly on

patients. The P450 enzymes are selectively induced by drugs and, therefore, enable drug metabolism to take place i.e. rifampicin induces and is metabolised by CYP3A4. The administration of other drugs (e.g., an oral contraceptive) to patients will also induce CYP3A4 and will lead to enhanced metabolic clearance and a sub-therapeutic plasma/tissue drug level. Conversely, some drugs are potent inhibitors of selective isoenzymes i.e. Itraconazole, Ketoconazole and Troleandomycin are inhibitors of CYP3A4 (Li, 1998). The addition of a drug that induces this enzyme would lead to the accumulation of the compound, as inhibitors prevent it from being metabolised. These studies using hepatocytes provides a simple basis for the evaluation and isoenzyme identification of any new drug therapies. Freshly isolated human hepatocytes represent the best model for these studies particularly for the pharmaceutical industry (Hengstler *et al.*, 2000; Li *et al.*, 1997).

### 2.3.1 Phase I

The overall purpose of this oxidative reaction is to attach a hydroxyl group to the substrate (S).

$$SH + O_2 + NADPH + H^+ \rightarrow SOH + H_2O + NADP^+$$

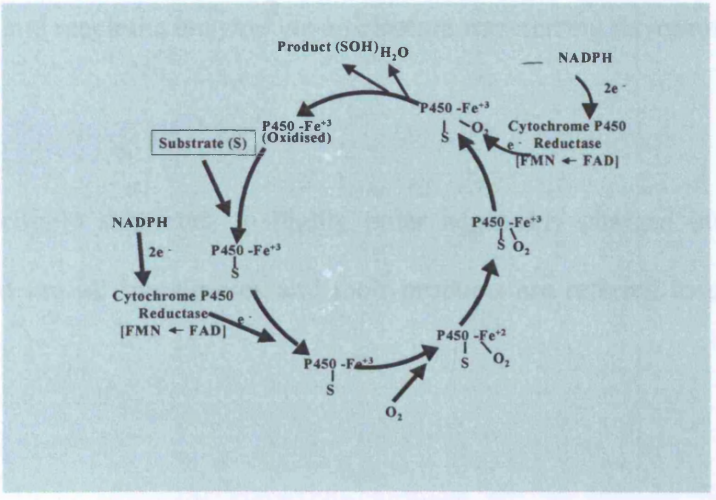




**Figure 19. The cytochrome P450 cycle (Oxford textbook of Hepatology 2nd edition).**

Depending on whether the substance is endogenous (metabolism of steroid hormones, thyroid hormones, fatty acids, prostaglandins) or exogenous (drugs), different reactions

| Reaction | Examples of substrates metabolised |
|---|---|
| Oxidation involving cytochrome P450<br>• Aromatic hydroxylation<br>• Aliphatic hydroxylation<br>• Epoxidation<br>• *N*-Dealkylation<br>• *O*-Dealkylation<br>• *S*-Dealkylation<br>• Oxidative deamination<br>• *N*-Oxidation<br>• Ethanol | Lignocaine<br>Pentobarbitone<br>Benzo[*a*]pyrene<br>Diazepam<br>Codeine<br>6-Methylthiopurine<br>Amphetamine<br>3-Methylpyridine<br>Alcohol |
| Reduction | Azo compounds<br>Nitro compounds |
| Hydrolysis | Ester compounds<br>Amides |
| Hydration | Epoxides |

**Table 8. Summary of phase I reactions (adapted from Gibson and Skett, 1996)**

occur leading to hydroxylation, the most important being cytochrome P450 oxidation (Figure 19 and Table 7). The cytochrome P450 enzymes catalyse the reductive cleavage of molecular oxygen. One of the two oxygen molecules is incorporated into the substrate while the other is released as water. The necessary reducing equivalents are derived from NADPH $^{+}$and $H^{+}$ and reach the enzyme via an electron transferring flavoprotein.

## 2.3.2. Phase II

These reactions couple substrates to highly polar negatively charged molecules. The enzymes involved are all transferases and their products are referred to as conjugates.

Phase II reactions convert the products of phase I to a highly water-soluble product that can be excreted in bile or urine.

The major route of conjugation is glucuronidation (conjugation with a D-glucuronic acid) and is important not only in endogenous substances (bilirubin and steroid hormones), but also exogenous metabolism i.e. drugs. The reaction is catalysed by glucuronyl transferase and allows bilirubin to be excreted within the bile (Figure 20). Other phase II reactions involving endogenous compounds are summarised in Table 9.

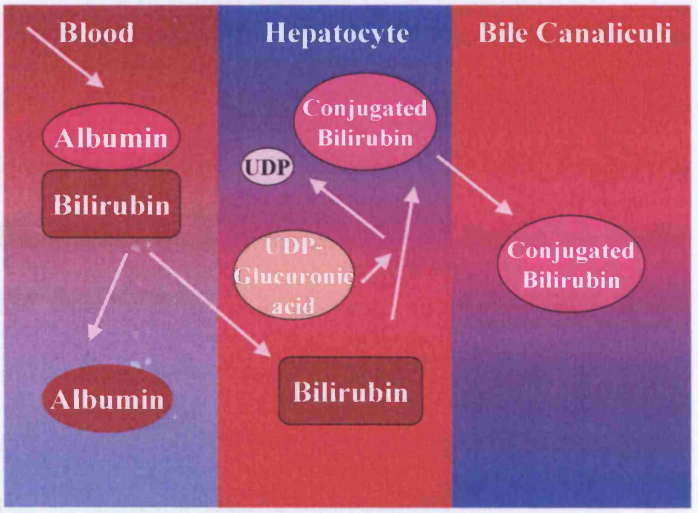


**Figure 20. Bilirubin conjugation.**

| Reaction | Substrates metabolised |
|---|---|
| Glucuronidation | Steroids, Thyroxine, Bilirubin, Catecholamines |
| Sulfation | Steroids, Carbohydrates |
| Methylation | Biogenic amines |
| Acetylation | Serotonin |
| Amino acid conjugation | Bile acids |
| Glutathione conjugation | Arachidonic acid metabolites (leukotrienes) |

**Table 9. Phase II metabolism of endogenous compounds.**

### 2.3.3 Coculture

Hepatocytes in culture lose their differential function after only a few days. Much work has been undertaken to enhance and maintain differential function including the culturing of other cells with the hepatocytes to synergistically improve function and thus reduce the number of required hepatocytes. Cells such as bone marrow stem cells and fibroblasts have been used successfully, and can induce a 3-15 fold increase in albumin secretion, and maintain liver specific function for up to 3 weeks (Murakami *et al.*, 2004, Gregory *et al.*, 2001; Bhatia *et al.*, 1999). The mechanism is believed to be the expression, by the coculture cells, of substances that can improve liver specific functions (HGF, EGF, cytokines etc). The optimisation of the cell type and culturing conditions to induce maximum efficiency and function from hepatocytes is ongoing, but has the potentially to significantly improve the function of isolated hepatocytes within a bioartificial liver (Murakami *et al.*, 2004).

## 2.4 Hepatic regeneration

The phenomenon of liver regeneration was not formally described until 1889 by Ponfick. It has been found in most species and following the removal of a portion of the liver the remnant gains increasing DNA content (hypertrophy) and liver cell number (hyperplasia), until the original mass is restored. This growth potential is retained after second or subsequent liver resections (MacSween *et al.*, 2002). The subject of liver regeneration has been extensively investigated over many years but the exact trigger and control mechanisms are not fully understood.

Regeneration occurs in the physiologically normal individual without damage or surgical insult to the liver. As a person ages, increases in body length, surface area and weight correspond to increases in liver mass. It is the ability of the liver to recover from damage that has intrigued hepatologists, but unfortunately, laboratory experiments have often been difficult to relate to the *in vivo* situation. Potential growth promoters have been identified including prostaglandins, platelet-derived growth factor, epidermal growth factor, hepatocyte growth factor (HGF) and epinephrine. The most important growth inhibitor discovered to date has been tumour growth factor-beta (TGF-β1). Rozga believes that after massive tissue loss or damage the normal proliferative mechanisms do not occur (Rozga, 2002). It appears that in this situation imbalance exists between the growth stimulators (e.g. HGF) and inhibitors (e.g. TGF-β1) as well as other abnormal blood cytokines (IL-6), which interfere with the normal process. He also concluded that addition of HGF as a stimulating therapy would therefore be unsuccessful. Therapies that will be able to induce hepatic regeneration directly remain elusive.

# Chapter 3

# Cryopreservation of hepatocytes

## 3.1 Introduction

One of the rate-limiting factors of BAL development, and pharmacological research using human hepatocytes, has been their source and availability. Methods for isolating and maintaining hepatocytes in culture are well documented and have been successfully isolated from human livers and various animal (rat, porcine, rabbit) species (Loretz *et al.*, 1989; Wang *et al.*, 2000; Morsiani *et al.*, 1994; Takahashi *et al.*, 1993; Donini *et al.*, 1997). Extracting fresh human hepatocytes for research and clinical application relies on the availability of liver tissue from liver resections or from non-transplantable livers from multi-organ donors (MOD). This supply is erratic and often geographically inconvenient, especially when cells have limited functional time once isolated (Smrzova *et al.*, 2001). Many cells are also wasted, as even moderate tissue yields, in excess of $10^9$ cells, are more than most large-scale experiments can use at once. Such large yields would be suitable if a BAL was constructed immediately after isolation. A reproducible protocol for the long-term storage of functioning hepatocytes is therefore required both for the construction of a clinical BAL or maximise their use in pharmotoxicological research, whilst minimising wastage (Strain, 1994).

Cryopreservation is the storage of cells in liquid nitrogen (-196°C) or in its gas phase (-150°C). It has been for many years viewed, as the system to use for storage. Many studies have been performed to try and establish a technique that allows hepatocytes to function normally on thawing, especially preserving the important phase I and II metabolic and enzymatic pathways responsible for detoxification.

Ideally any clinically applicable device would need an "off-the-shelf" supply of cells. Despite the last 20 years research into the cryopreservation of hepatocytes, no universally acceptable protocol has been proven effective. Comparative studies are difficult to assess as the variability of methods and results lack consistency. Published procedures describe numerous hepatocyte isolation procedures and cryopreservation protocols that have been applied to many different animal species. An international panel of experts in 1999 recognised the importance of continued development and advised that "...research should continue to improve the cryopreservation procedures..." (Li *et al.*, 1999).

The major factors that have been determined for a successful protocol are: rate of freeze, concentration of hepatocytes, type of cryoprotectant and final cooling temperature which must be "precisely controlled and specified in each particular case" (Kasai and Mito, 1993). More recently approaches other than freezing in suspension have been used with increasing success, although a decrease in viability on thawing is beginning to appear inevitable. The universally agreed steps are illustrated in Table 10. (Li *et al.*, 1999).

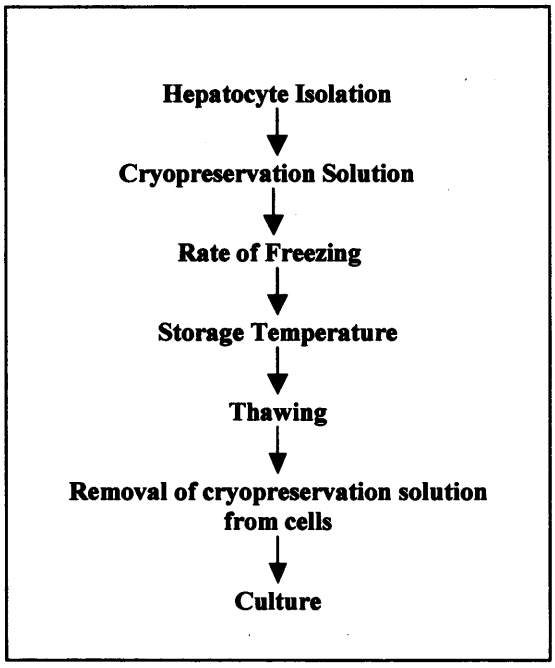


**Table 10. The stages of cryopreservation.**

## 3.2 Pre-freeze processing

### 3.2.1 Cell density and volume of storage

Storage of cells at high cell density would be considered cost effective through the saving of storage space. However, large volume cell storage would negate the time consuming step of filling small cryovials, when cryopreserving in solution, and thus ensure a quick and easy thawing and access process. Published studies have investigated variable cell densities for cryopreserving, but few comparative studies have been made that determine optimum levels. A review by Madan *et al.* concluded that the optimal concentration of storage ranged between $10^6$-$10^7$ cells/ml in solution (Madan *et al.*, 1999). While Wu *et al.* recently showed that at concentrations of $4x10^7$cells/ml cryopreserved human hepatocytes, using stepwise freezing protocols, showed improved post-thaw viability (as measured by trypan blue exclusion) when stored in 50ml bags rather than in 100ml bags (Wu *et al.*, 2000).

De Loecker *et al.* studied the effect of rat hepatocyte cell densities and the effect of freezing the cells (De Loecker *et al.*, 1998). Rat hepatocytes were suspended at the same concentration ($5x10^6$ cells/ml) and exposed to 1.8M Dimethyl Sulphoxide (DMSO) thus ensuring that the cells had a constant exposure. The hepatocytes were diluted and frozen at different concentrations expressed as a percentage packing of the original sample. As the density of cells decreased, so the viability, as measured by trypan blue, increased. In addition, as the density increased, the attachment measured by radioactive uptake reduced following 1 hour of culture. Kasai *et al.* and Diener *et al.* found in both dogs and rats, respectively, that viability measured by trypan blue exclusion, was effective at

concentrations of less than $10^7$cells/ml compared with higher densities (Diener *et al.*, 1993; Kasai and Mito, 1993). These findings seem to further validate the hypothesis put forward by De Loecker *et al* (De Loecker *et al.*, 1998). It states that although there were a number of scenarios to explain cellular injury caused during the freezing process, membrane-membrane contact between hepatocytes was considered the most plausible scientific explanation that leads to serious physiological cell damage. Therefore, based on this hypothesis it was suggested that lower densities should provide improved hepatocyte viability and functional yield.

### 3.2.2 Culture prior to cryopreservation (pre-incubation)

Prior to cryopreservation, the culturing of hepatocytes in suspension is thought to improve the cells ability to recover from isolation before being exposed to the biophysical rigors of the freeze process. Darr and Hubel cultured porcine hepatocytes in Williams E cryoprotectant medium (with additives), in a spinner cultures for between 4 and 48 hours at 90 rpm following isolation, before the cells were seeded for culture (Darr and Hubel, 2001). They measured the hepatocytes ability to secrete albumin as an indicator of hepatocyte functional viability. Growth in fresh culture progressively increased albumin secretion as pre-incubation period increased (0.25μg/ml/hr at time 0 raising to 1.40μg/ml/hr at 48 hours). Further detailed studies continued to examine the possible advantages of pre-incubation on cellular activity after subsequent cryopreservation and thawing. Pre-incubation periods up to 24 hours conveyed significant advantages on albumin production on post-cryopreservation thawing. Comparative studies with fresh cells found that pre-incubation periods greater than 24 hours induced a marked reduction in albumin secretion. They concluded that aggregates

of cells form during growth in suspension cultures, which do not survive the freezing process as efficiently as homogeneous cell suspensions and therefore do not survive in post-thaw cultures.

Koebe *et al.* performed a series of experiments involving the cryopreservation of hepatocytes in monolayer culture, using both rat and porcine cells (Koebe *et al.*, 1996a; Koebe *et al.*, 1996b). Prior to cryopreservation hepatocytes were cultured on a monolayer in Dulbeccos Modified Eagles Medium (DMEM with additives). It was found that culturing for 3 days before cryopreserving improved cell yield and functionality. Additionally, culturing for 7 days prior to freezing in a sandwich configuration conferred survival benefits after thawing (Koebe *et al.*, 1990).

## 3.3 Freezing protocol

### 3.3.1 Cryopreservation Solution

#### 3.3.1.1 Classic cryoprotectants

The most significant influence on cell survival is thought to be dependent upon the formulation of the type and concentration of the cryoprotectant. Studies by Loretz *et al.* compared glycerol, polyvinylpyrrolidine, dextran and DMSO for the cryopreservation of human and rat hepatocytes (Loretz *et al.*, 1989). They found that DMSO preserved the attachment and viability characteristics of hepatocytes at concentration of between 10-20% v/v more effectively than other cryoprotectants. Further work on rat, human and other species found that the optimum DMSO concentration of between 10-12% proved the ideal concentration. Kasai *et al.* found no statistical difference on the viability of dog

hepatocytes, as measured by trypan blue exclusion, when compared with either 10% Glycerol or DMSO (Kasai and Mito, 1993). However, DMSO was found to be superior in preservation of cell attachment capabilities. Chesne *et al.* cryopreserved a number of different species and found that each had a different concentration of DMSO (of the total volume) as the ideal (Chesne *et al.*, 1993). Human cells retained their attachment and viability characteristics at concentrations between 10–12%, 16% for rat, while mice, rabbit, hamsters and monkeys were optimal at 14%. Dog hepatocytes where found to be sensitive to minor (±1%) fluctuations of the optimum concentration of 16%. Possibly due to other factors and varying experimental protocols the literature reviewed was found to vary in a number of similar studies (Chesne *et al.*, 1993). Diener *et al.* found that a 10% concentration of the final volume was the most effective in maintaining post thaw viability for rat hepatocytes. Lawrence *et al.* found a 10% concentration superior to a 20% concentration when studying attachment and post thaw viability (Lawrence and Benford, 1991; Diener *et al.*, 1993).

These results demonstrate that DMSO is widely considered the cryoprotectant of choice due to its superior properties when compared with all other solutions examined to date. The alternative use of Glycerol has been replaced as the cryoprotectant of choice, following the observations that post-thaw attachment efficiency of cells deceased markedly (Loretz *et al.*, 1989; Lawrence and Benford, 1991; Kasai and Mito, 1993).

### 3.3.1.2 Culture configurations

### i) Alginate bead entrapment

By entrapping the cells in beads it has been proposed that cellular pelleting and aggregation formation is avoided, so protecting the cells from damage. The alginate structure has the potential to protect hepatocytes from the biophysical rigors of the freezing process. Guyomard *et al.* undertook a series of studies to entrap rat and human hepatocytes in calcium-alginate gel, prior to cryopreservation (Guyomard *et al.*, 1996; Rialland *et al.*, 2000). It was found that both species of hepatocytes were protected from the rigors of the freeze thaw process, with no significant difference of cellular viability between fresh and post-cyopreserved thawing of cells being observed. The hepatocytes, once released from the beads, were found to have attachment properties comparable to fresh monolayer cultures. Subsequent culture and ability to respond to inducers of various cytochrome P450 pathways e.g. CYP1A, 1A2, 2C9, 2C19, 2D6 and 3A4, demonstrated preservation of cellular function slightly reduced to the levels normally observed with fresh monolayer cultures.

Dixit *et al.* performed a similar technique by micro-encapsulating rat hepatocytes with ultra-thin sodium alginate (Dixit *et al.*, 1993; Dixit, 1994). Ureagenesis and protein production were measured, showing that post-cryopreservation production was at comparable levels with fresh cultures. Following implantation of these cells into the peritoneum of Gunn rats, the induced hyperbilirubinaemia was reduced indicating successful function of the implanted cells. Both these studies, although differing in

methodology, demonstrated that hepatocytes could be cryopreserved with minimal damage and preservation of cellular function post-thawing.

### ii) Storing in monolayer culture

For many years, the standard method for cryopreserving hepatocytes was to suspend them in solution. Koebe *et al.* demonstrated that by freezing porcine hepatocytes at -80°C, in an immobilising gel culture as a monolayer, albumin secretion and cytochrome P450 function could be improved on subsequent thawing (Koebe *et al.*, 1996a). Watts *et al.* obtained similar results on rat hepatocytes frozen on a monolayer culture frozen at -70°C (Watts and Grant, 1996). They observed that cellular viability, immediately following thawing was approximately 10% but microscope examination showed significant cellular damage. Culturing the hepatocytes for an additional 24 hours demonstrated cellular repair with viability increasing too greater than 80%. Cytochrome P450 function was maintained following thawing and both ureagenesis and reduced gluthione (GSH) activity was found to be less than 25%, when compared to fresh cell cultures. Recent studies by the same group evaluated this observation on rat hepatocyte monolayers after one month storage at –70°C. (McKay *et al.*, 2002). They also compared the level of cytochrome P450 with freshly cultured cells and those that had been frozen and subsequently thawed. They found no significant difference between equivalent time points, indicating good preservation and reproducibility of cellular repair after thawing.

### iii) Sandwich culture and cryopreservation

There has been reported success with the in-vitro culture of hepatocytes within a collagen sandwich configuration. This has been adapted as the main method for many laboratories

undertaking pharmacological research. According to Darr and Hubel, the disadvantage of this cryopreservant method is that only low cell densities can be stored (Darr and Hubel, 2001). Koebe *et al.* published in 1990 the successful cryopreservation using this culturing method with rat hepatocytes (Koebe *et al.*, 1990). They were initially cultured on collagen plates, and then frozen to -70°C following the addition of a collagen layer over a monolayer of hepatocytes. Culturing the cells for upwards of 1 week before cryopreservation maintained good post thaw albumin production. In a later publication it was reported that cells cultured for 3 days before freezing and subsequent culture initially expressed CYP1A1 activity of 45.6±8.7% of the unfrozen control group (Koebe *et al.*, 1996b). However, the level rose to 93±14.5% after 1 week of culture. Only 30% of the cells survived the procedure, but this was considered an improvement on frozen cell suspensions. This has now become a successfully reproducible technique (Koebe *et al.*, 1999). Borel-Rinkes *et al.* went on to characterise a freeze thaw program for sandwich cultured cells. They initially cultured the cells in the sandwich formation for between 7 to 10 days, based on the research of Dunn *et al.* (Borel-Rinkes *et al.*, 1992; Dunn *et al.*, 1991; Dunn *et al.*, 1992). This allowed sufficient time for the hepatocytes to reach a steady state. Before the introduction of this improvement they appeared to be more susceptible to the cryoprotectant (Koebe *et al.*, 1990). The cells were cooled at various rates and thawed rapidly at >400°C/min. Albumin secretion was found to recover to normal levels after 2 days of culture at a freezing rate of -5°C/min and maintained comparable levels using fresh hepatocytes cultured for up to 20 days. Borel-Rinkes *et al.* also assessed the affects of DMSO on hepatocytes in the collagen sandwich configuration (Borel-Rinkes *et al.*, 1992). They concluded that the gel allowed a gradual exposure of

cells to the DMSO. This also stabilised hepatocytes within the sandwich formation, as this was thought to make them less vulnerable to the toxic affects of DMSO. They concluded that when heaptocytes were placed in a sandwich configuration and exposed to DMSO for 60 to 120 minutes at 22°C, cellular morphology and cytoskeletal organisation proved to be unaffected.

### 3.3.1.3 Foetal Bovine Serum

Foetal bovine serum (FBS) is routinely used when cryopreserving with DMSO. Studies have been performed to assess different concentrations of FBS within the cryopreserving solution. Its increase in concentration was initially thought to improve cell viability on thawing, however experiments have not proved this. Loretz *et al.* found that with concentrations between 90% and 10% (of the final solution volume) there was little effect on post thaw viability and attachment efficiency (Loretz *et al.*, 1989). Other groups have confirmed these findings with a range of alternative freezing protocols (Maganto *et al.*, 1988; Jamal *et al.*, 2000; Hengstler *et al.*, 2000; Diener *et al.*, 1993).

### 3.3.1.4 Suspension medium

The suspension solution seems to have only a minor influence on cellular storage, post-thaw viability and function. A number of different types of cell culture medium, normally used for in-vitro cell culture, has been used, although few direct studies have been undertaken to examine their characteristics. Naik *et al.* found that hepatocytes frozen in Chee's modified Eagles culture media had superior preservation of diazepam metabolism, used as a marker of P450 function (specifically the CYP2C19 isoenzyme), when compared to University of Wisconsin solution (Naik *et al.*, 1997). Chesne *et al.*

compared a number of tissue culture media formulations using intracellular LDH measurements as the comparative assay (Chesne and Guillouzo, 1988). They found that L-15 gave a statistically significant advantage as compared to Euro-Collins, Phosphate Buffered Saline (PBS), Hepes and MEM-199. Diener *et al.* and Smrzova *et al.* in contrast, found that little difference existed when a number of media (modified Krebs-Henseleit, Williams medium E, Foetal calf serum) were examined, and the subsequent cell viability measured by trypan blue (Diener *et al.*, 1993; Smrzova *et al.*, 2001).

### 3.3.1.5 DMSO addition and removal

The rate by which DMSO is added has been regarded as having an effect on subsequent cellular viability and function. A cell suspension containing DMSO has an osmolarity many times higher than the cell suspension. It has been proposed that the properties of this cryoprotectant can cause biochemical damage to the cells through the destabilisation of intracellular proteins when in a non-frozen state (Arakawa *et al.*, 1990). The slow addition and removal of DMSO may allow the cells to adjust to the sudden osmotic and biochemical stress and as a consequence, increase survival. Diener *et al.* showed that there can be a loss of viability (measured by trypan blue exclusion) of 6 to 8% in rat hepatocytes with a one step addition and removal, compared to gradual addition (Hengstler *et al.*, 2000; Diener *et al.*, 1993). Other studies report no such variation on viability with progressive addition and removal (Maganto *et al.*, 1988; Loretz *et al.*, 1989). Naik *et al.* demonstrated the importance of removing the DMSO quickly once the cells have been thawed to avoid further damage (Naik *et al.*, 1997). They showed that by exposing the cells to DMSO for 15 and 30 minutes following thawing that there was a dramatic reduction on the P450 function (CYP 2C19) measured by diazepam metabolism.

### 3.3.2 Rates of freezing and thawing

The freezing rate is considered to be one of the major factors that affect the success of post-thaw viability of hepatocytes. Many studies have compared simple freezing rates (e.g. placing directly into refrigerators) and more complex controlled rates, where the temperature has been reduced in a controlled fashion using sophisticated freezers. The controlled rate methods have been advocated following observations from experiments using rat hepatocytes (Harris *et al.*, 1991). The whole freeze process hinges on the avoidance of intracellular ice formation, especially for cells in suspension. Although DMSO provides some protection by allowing a more controlled cellular dehydration, the rate of freezing is also important. If the rate is too rapid then water cannot exit and intracellular ice forms damaging the cell (Mazur, 1965). If too slow, the cell is exposed to excessive cellular dehydration and the mechanical effects of the external ice can be seen microscopically (Hubel *et al.*, 1991). Controlling and altering the freeze process is required to optimise time and temperature at critical stages of cryopreservation. Some processes also build in a shock-cooling phase (Hengstler *et al.*, 2000). Here the temperature is suddenly reduced, to compensate for the latent heat of fusion. This increase in temperature as the ice forms is possibly harmful to the cell. By quickly reducing the temperature this extra energy is absorbed and a smoother cooling progression occurs.

The simplest method of cryopreservation is to place the hepatocytes directly into liquid nitrogen, although this results in a marked reduction on viability (Donini *et al.*, 2001). Fautrel *et al.* reported a drop of viability from around 80% to 50-60% post thaw using rat

hepatocytes frozen in a two step method that included –20°C for 12 minutes followed by –80°C for 2 hours then directly into liquid nitrogen (Fautrel *et al.*, 1997). A selection of the published work in this area is summarised in Table 11.

Overall conclusions on the correct freeze rates required are difficult to draw as authors use a variety of techniques for different species (Table 11). An overall consensus drawn from a variety of studies suggests that a slower rate of between 1-5°C/min improves preservation of subsequent cellular viability and function. Additionally, the controlled or variable rate programmes are superior when taking account of the latent heat of fusion. The ideal protocol is far from clear, and inconsistencies exist mainly due to hepatocyte preparation and experimental variation (Lawrence and Benford, 1991; Gomez-Lechon *et al.*, 1984). Indeed Guillouzo *et al.* reviewed the present situation in 1999 and concluded that there was no convincing benefit in complex controlled cooling compared to placing in a -20°C, followed by placing into a -80°C freezer (Guillouzo *et al.*, 1999).

A rapid rate of thawing using gentle agitation in a 37°C water bath greatly improves post thaw viability compared to thawing at room temperature. Thus, agitation at 37°C with agitation has been the adopted method in virtually all the studies examined by this review (Chesne and Guillouzo, 1988).

| Cellular condition | Paper | Species | Cryo-protectant | Freeze rate | P.P | Storage Temperature | Conclusion |
|---|---|---|---|---|---|---|---|
| Cells in Suspension | (Diener *et al.*, 1993) | R | DMSO | 1. –38° C/min<br>2. -2° C/min<br>3. Slow variable<br>4. Optimised | Y | Liquid $N_2$ | Program 4 showed the best post thaw viability measured by T.B. |
| | (Utesch *et al.*, 1992) | R | DMSO | Variable rate | N | Liquid $N_2$ | TB viability dropped from 82±7% to 60±7% |
| | (De Loecker *et al.*, 1993) | R | DMSO | -1° C/min to -38° C then into liquid $N_2$ | N | Liquid $N_2$ | Viability measured by TB dropped from 92-95% to 88-84% |
| | (Lawrence and Benford, 1991) | R | DMSO | Cooling rates 0.05-50° C/min | Y | Liquid $N_2$ | Rates of 1-5° C/min gave the best viability/attachment ratios |
| | (Wu *et al.*, 2000) | P | DMSO | 1. Deiner (Diener et al., 1993) optimised<br>2. Modified variable | Y | Liquid $N_2$ | Neither rate significantly altered the viability measured by TB (77-80%) |
| | (Sun *et al.*, 1990) | M | DMSO | Variable rate | Y | Liquid $N_2$ | Measured attachment and concluded that cells were 80% as viable |
| | (Li *et al.*, 1999) | H | DMSO | Variable rate | N | Liquid $N_2$ | TB viability ranged from 55-83% |
| | Coundouris *et al*, 1993 | H | DMSO | -1° C/min | N | -80° C | TB viability 30-405 of fresh, after 72hrs of storage. |
| | (Chesne *et al.*, 1993) | H, R, D, Mi, Ha, M, Ra | DMSO | -20° C for 12 minutes, -70° C for 2 hours | Y | Liquid $N_2$ | TB viability reduced by<10% for, R, D, H, Ha. 15% to25% for Ra, M and Mi. |
| Monolayer cultures | (Watts and Grant, 1996) | R | DMSO | Initial 20hr culture -1° C/min to -70° C | N | -70° C | Initial TB showed 10% viability, after 48hrs >80% of the cells viable |
| Sandwich culture | (Koebe *et al.*, 1990) | R | DMSO | -5° C/min to -20° C, held for 30 minutes then -1° C/min to -70° C | N | Gas phase of $N_2$ | |
| Immobilised gel culture | (Koebe *et al.*, 1996) | P | | 1.- 1° C/min to –4 to –10 in 50s -10° C/min to -80° C<br>2. -1° C/min to -80° C<br>3. -10° C/min to -80° C | N | Liquid $N_2$ | Method 1 showed improved viability TB and morphological changes. |

R – Rat P.P. – Percol Purification
P – Porcine T.B – Trypan Blue
H- Human Variable Rate – Generalised term to describe methods with stepwise temperature
Mi- Mouse reduction. Refer to papers for details
Ra – Rabbit M- Monkey
D – Dog Ha – Hamster

**Table 11. Summary of freeze rate comparison studies.**

The length of storage in liquid nitrogen of cryopreserved cells appear to have little influence on post thaw cellular function (Koebe *et al.*, 1996a; Koebe *et al.*, 1996b; Rialland *et al.*, 2000; Li *et al.*, 1999; Dou *et al.*, 1992)

## 3.4 Post freeze processing

### 3.4.1 Percol purification

The introduction of a Percol density gradient following thawing of the cells is designed to increase the number of viable cells by separating them from the non-viable, using a density gradient (Sun *et al.*, 1990). The action has been found to improve attachment of the remaining hepatocytes, perhaps by removing cells that would otherwise obstruct attachment. Improvements in the hepatocytes cultured following percol have also been found, including P450 function (Lawrence and Benford, 1991; Dou *et al.*, 1992; Utesch *et al.*, 1992; Alexandre *et al.*, 2003). Diener *et al.* found that this step increased the activities of glutathione S-transferase (GST) and phenol sulfotransferase (ST) to the level of cells that had not undergone cryopreservation (Diener *et al.*, 1993). Introducing a Percol gradient has the disadvantage that many healthy cells can be lost during the process thus reducing the overall yield. Disappointingly, Utesch *et al.* found that of the original cells isolated for Percol purification, only 22% of the viable cells that were initially added were finally recovered. However, an improvement of viability as measured by trypan blue exclusion from 69±6% to 86±3% was found (Utesch *et al.*, 1992). This has raised the question of whether the losses of valuable cells and the time involved in performing this procedure are worth the overall gain especially with humans and large animals (Rialland *et al.*, 2000). Wang *et al.* commented that the optimisation of the isolation process might well provide good viability without having to add in the time

and expense of Percol purification (Wang *et al.,* 2000). It is also uncertain as to whether the process will select a different population of hepatocytes, due to their weight. This has the potential to select hepatocytes, with a more specific function.

### 3.4.2 Cellular function following cryopreservation

An universally accepted standard and consistent method of measuring hepatocyte function is lacking between all the studies that had been reviewed. Due to the complexities of the hepatocyte, groups have chosen a variety of ways to assess function, making comparison difficult. The methodology used tends to depend on how the various groups wish to use the isolated hepatocytes (i.e. BAL or pharmacokinetics).

#### 3.4.2.1 Measuring hepatocyte viability

The simplest and most consistently quoted method to measure hepatocyte viability is by trypan blue exclusion. However, following isolation the cells often form three-dimensional clumps consisting of up to 20 cells, limiting the technique to "operator-introduced subjectivity and a reliance on estimation" (Stegemann *et al.*, 2000). This method provides no assessment of the success of the protocol on cellular function. Although the cell membranes may remain intact this does not necessarily correlate with attachment. Caution should therefore be employed in comparing these figures as the calculations can also be performed in different ways. Many researchers do not state the yield of the returned cells that inevitably drops during cryopreservation and Percol purification, so an impressive viability percentage may mask a huge decrease in the yield or actual number of viable cells (Utesch *et al.,* 1992). More accurate methods to obtain a relative viability of the cells have included measuring lactate dehydrogenase release,

erythrosin B exclusion, albumin secretion, carboxyfluorescin diactate staining and confocal laser scanning microscopy (Chesne *et al.*, 1993; Pathernik *et al.*, 1996; Darr and Hubel, 2001; Hengstler *et al.*, 2000; Dou *et al.*, 1992; Naruse *et al.*, 2000). In reviewing papers, the exact method of viability calculation should be carefully sought to allow better comparison of results.

Cryopreservation causes an overall drop in cellular viability, certainly when performed in solution (Dou *et al.*, 1992). The level of this decrease is thought to be about 25-35% of the original number, although exceptions exist (Chen *et al.*, 2001). Following cryopreservation the ability of the hepatocyte to attach to a surface is key for their survival. Hepatocytes will only function when attached, if they do not they die (Smets *et al.*, 2002; Alexandre *et al.*, 2002). Loretz *et al.* calculated that only 50% of hepatocytes cryopreserved in solution retain their ability to attach to plastic (Loretz *et al.*, 1989). De Sousa *et al.* achieved similar figures for dog and monkey hepatocytes (De Sousa *et al.*, 1999). These results show that attachment ability seems to be severely impaired in most cryopreservation protocols.

This poor recovery of cells and their attachment capabilities after cryopreservation in solution is one of the key areas that require further study. This will ensure that cryopreservation becomes a viable option, for the utilisation of large animal and human isolated hepatocytes in the clinical application of BAL construction or hepatocyte transplantation (Sun *et al.*, 1990).

### 3.4.2.2 Donor to donor variation

There is some variation from each liver and each species (Hewitt *et al.*, 2001). This can be as a result of the isolation process as well as the genetic constituents of the species. Small animals kept in the same environment and with minimal genetic variation will demonstrate less variability between livers than humans. Human tissue is obtained from resected specimens or multi-organ donors. A number of factors that could affect the quality of the hepatocytes isolated range from, the transfer time from source to laboratory for processing, the individual genetic make up of the donor, donor medication and the underlying disease process. (Hawksworth, 1994). Using cellular functions as a marker of success for cryopreservation in this setting is difficult and caution is needed. Li *et al.* demonstrated a considerable range of cytochrome P450 levels (including 1A2, 2A6, 2C9, 2C19, 2D6, 2E1 and 3A4 pathways) in 17 human donors following cryopreservation, illustrating this point (Li *et al.*, 1999).

### 3.4.2.3 Cytochrome P450

The ability of cryopreserved cells to metabolise drugs using the major drug-metabolising pathways appears to be unaffected when compared with fresh cells (Li *et al.*, 1999). Cytochrome P450 (phase I and phase II) induction and inhibition are the most important mechanisms in drug-drug interactions (Li *et al.*, 1997). Human hepatocytes, as opposed to other species, have been found to be the most useful model for the prediction of induction of certain isoforms in man. Studies are performed on cultures of primary human hepatocytes to induce pathways to ensure that multiple pharmacology will not ultimately be harmful to humans (Li, 1998). Unfortunately, in cultured cells the

cytochrome P450 function deteriorates rapidly so the window to perform such experiments is narrow. A successful cryopreservation protocol that preserves these important enzymes and functional pathways will allow planned studies that would dramatically increase the scope of research into this field (Li *et al.,* 1997).

Dou *et al.* and Hewitt *et al.* demonstrated good preservation of cytochrome P450 function in cryopreserved human and rat, dog and human cells respectively, almost achieving levels comparable to fresh cells (Dou *et al.,* 1992; Hewitt *et al.*, 2001). It was also found that the function seemed to improve over time implying that thawed cells require a recovery period to regenerate before induction studies should be carried out. Madan *et al.* similarly demonstrated good cytochrome P450 function in thawed cryopreserved rat hepatocytes, again virtually equivalent to fresh cells (Madan *et al.*, 1999). This confirms the findings of others, that the cytochrome P450 function deteriorate over a few days in post thaw cultures (Fautrel *et al.*, 1997).

## 3.5 Conclusion

The aim of hepatocyte cryopreservation has been to provide cells, that on thawing, maintain normal or near normal enzyme activities for drug metabolism studies, BAL construction, cellular transplantation or hepatocyte research to be undertaken. The liver has many complex functions including drug metabolism by phase I and II enzymes, protein synthesis, bilirubin conjugation, production of bile etc. Examination of all cellular biochemical parameters has proved a formidable challenge. Therefore, all studies have selected different end points to measure the success of their methods. In addition, the variability in hepatocyte isolation, culturing methods and viability measurements, has

resulted in inconclusive and inconsistent comparative studies comparisons. The need to standardise this research is apparent (Skett and Bayliss, 1996).

Research has revealed that although there is a rapid deterioration once cultured, cryopreserved hepatocytes do maintain a number of cellular activities. This includes Cytochrome P450 function, which has been claimed to represent a viable model for the study of pharmaceuticals and drug-drug interaction (Hewitt *et al.*, 2001). For BAL development and transplants, cryopreserved hepatocytes do show adequate function; however, the loss of viable cells and reduction in attachment ability, crucial for successful functioning, requires a more complex solution to allow this to be clinically adopted. Therefore, optimisation of the freeze thaw-process for storage and large-scale clinical usage is still required (Ting and Demetriou, 2000). It would be difficult to conclude an ideal protocol from the data currently available. Clearly the use of DMSO, a gradual reduction in temperature and a fast thaw is seen to be an essential baseline for a successful method. The details within this framework will depend on the ultimate use of the hepatocytes and the species being studied. For example, interest in drug development would require methods that may preserve certain cytochrome P450 pathways while BAL and cellular transplants require large numbers of cells with a range of functional abilities. Any future projects would, therefore, require a tailor made cryopreservation programme to take this into account.

Although the utilisation of human cells is not without difficulty, their use in any future BAL development should be advocated (and may well be mandatory), especially with the recent concerns over the use of porcine hepatocytes (Julez *et al.*, 2000). A national and

possibly Europe-wide co-ordinated tissue bank system would go some way in achieving this aim (Anderson *et al.*, 2001; Orr *et al.*, 2002). With economic, legal and transportation barriers being changed across Europe it is hoped to expand such a system to encompass many more centres across Europe (Orr *et al.*, 2002). Even with this promise there is still an acute need for an efficient storage programme to be established. Such a programme is required locally for a developing bioartificial liver project. The method needs to be simple and reproducible and needs to take into account the often unsocial hours of hepatocyte isolation. A number of variables were therefore studied in both human and porcine (to integrate with the creation of a porcine model for BAL testing) hepatocytes, with these criteria in mind.

## 3.6 Hypothesis

This study aims to examine the effect of isolation and cryopreservation techniques on porcine and human hepatocyte viability and function, in order to establish whether they can be considered as a reproducible source of cells for a clinically applicable bioartifical liver programme.

# Chapter 4

# Methods and Materials

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## 4.1 Laboratory Safety

An essential aspect of tissue culture relies on the need to ensure that good quality material is produced in a safe and efficient manner. All experimental work was conducted within a containment level 2 laboratory based on the guidelines produced by the Committee on Dangerous Pathogens (ACDP, 1995). All harvesting and culturing techniques were undertaken within a Class 2 Microbiological Safety Cabinet (MSC II) where protection of the operator from aerosols and cell work from contamination was provided by the use of high efficiency particulate air (HEPA) filters. To reduce the possibility from microbiological contamination the surfaces of the MSC II cabinet were routinely decontaminated with 70% ethanol after each session. All procedures followed were subjected to a risk assessment in accordance with the legal requirement of the Health and Safety at Work Act 1978, UK. The assessments were undertaken prior to starting any activity and consisted of two elements: Identifying and evaluating the risks and defining ways of minimising or avoiding the risk. As the cell cultures forming the basis of the work originated from primary human and porcine sources the risk classification was defined as Medium to High due to the poor characterisation and origins of the cells i.e. no routine virology screening for HIV I and II and Hepatitis B.

All tissue culture waste (culture medium) was inactivated overnight in a Trigene solution prior to disposal to drain with an excess of water. All contaminated pipettes were placed in a "sharps bin" before disposal by incineration. All solid waste such as flasks, centrifuge tubes, contaminated gloves, tissues etc. were placed inside heavy duty sacks for contaminate waste and autoclaved prior to incineration.

## 4.2 Source and Isolation of hepatocytes

### 4.2.1 Human

The Leicestershire Research Ethics committee (LREC) granted approval for the use of human tissue in this study in November 2000. Patients known to be hepatitis B, C or HIV positive and MRSA carriers were excluded from the study. Each patient was interviewed 24 hrs prior to the surgery to gain consent for the use of the resected liver tissue for research. The interview outlined the BAL project and the need for tissue. A diagram was used to illustrate how the tissue was acquired emphasising that no additional tissue would be removed other than would be normal for the type of operation (Figure 21). The role of the UK Human Tissue Bank (UK HTB) in distribution of unused cells to other UK researchers was also outlined. Patients were informed that the UK HTB were sponsors of this project and that a cost recovery fee was charged for cell distribution. Patients were assured of confidentiality and anonymity, as they would expect for any medical treatment. They were informed of the right to withdraw from the study at anytime prior to the surgery without compromising their treatment.

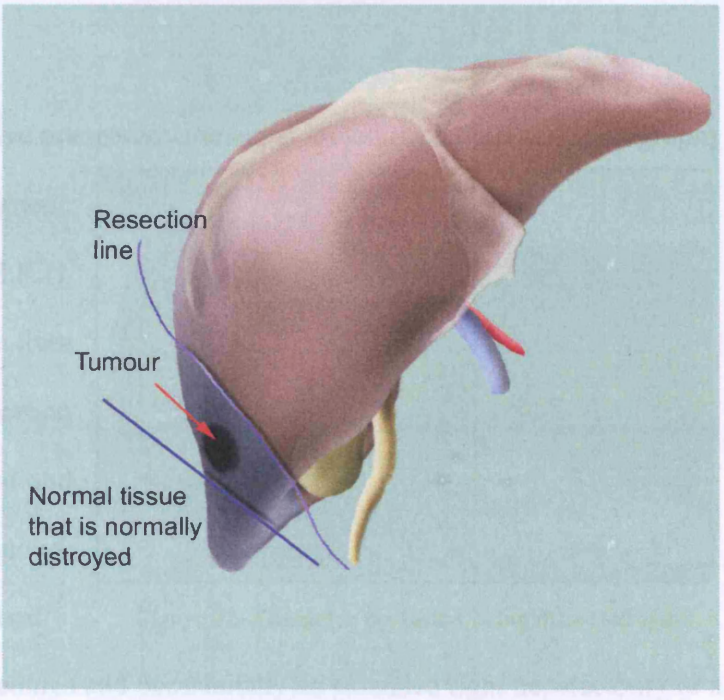



**Figure 21. Diagram used to explain to patients, how tissue is acquired**

An anonymous 'Donor Information Sheet' (Appendix 1) was designed to examine any patient factors that might influence the recovery and cell viability of hepatocytes. Primary areas of interest included demographics, preoperative pathology, medication status, and social behaviour.

All patients underwent extensive preoperative imaging including; Computer Tomography (C.T.), Ultrasound and Magnetic Resonance Imaging (M.R.I). During surgery intraoperative liver ultrasound provided an indication of the procedure to be adopted and extent of tissue resection required. Most patients underwent standard right or left hepatectomy procedures and occasionally an extended right hepatectomy or a modified segmental resection.

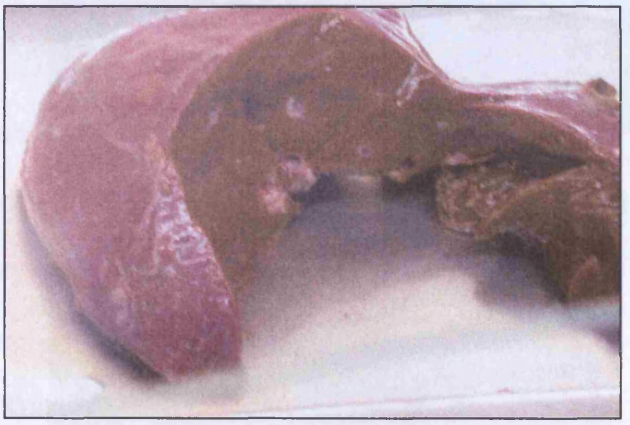


**Figure 22. Tissue for perfusion being dissected clear**

### 4.2.1.1 Postoperative in-vitro liver isolation procedures

Immediately following surgical removal, the tumour was dissected from the normal tissue and sent for histopathological examination (Figure 22). Two methods of cannulation of the normal liver venous system were assessed. Method one used an IV cannula (Optiva*214-20 gauge, vein size dependent) secured into position through suturing into position (Figure 23). A maximum of 4 cannulae were used on the largest vessels exposed at the cut surface. Other minor vessels were sutured to maintain perfusion pressure during

subsequent isolation procedures. Method 2 involved similar identification of the four major blood vessels with 'dog catheters' (Arnolds, Shrewsbury. cat. no. AS89) secured by the use of 'medical adhesive' (Figure 24). The adhesive was also spread over the entire cut surface of the tissue to "reform" Glissens capsule. Each cannula was connected to a tubing set, and the liver immediately perfused with 0.5- 1 litre of ice cold Soltran Kidney perfusion solution (Baxter Healthcare Ltd, Thetford, U.K. cat no. B4708) (Figure 25). Liver immersion in $+4^0$C Soltran aimed to reduce cellular deterioration during transport to the laboratory, which was facilitated in an organ box on melting ice. The hepatocyte performed ateither the UK Human Tissue Bank laboratory or at the Clinical Sciences Laboratory, Leicester General Hospital.

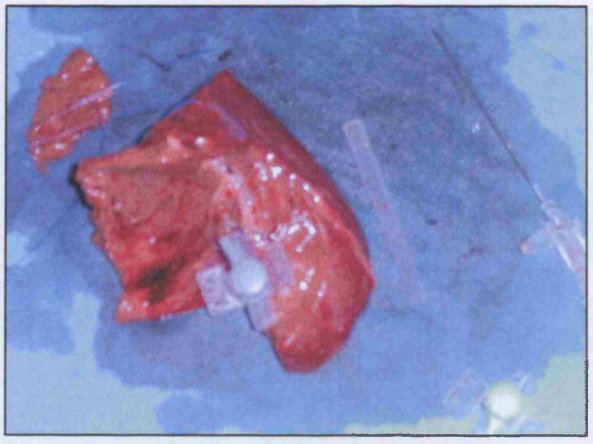


**Figure 23. Cannulae sewn into tissue.**

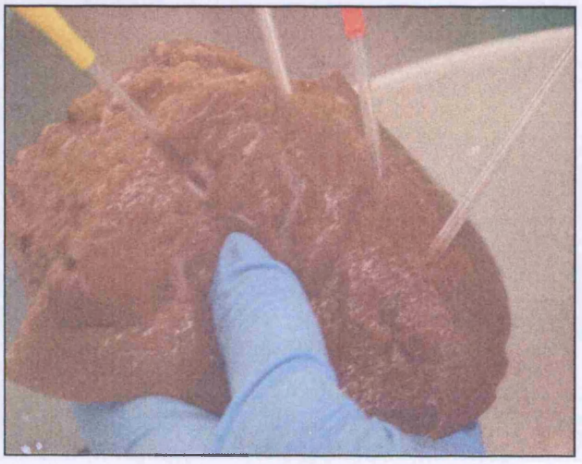


**Figure 24. Dog catheters in place for glue method**

### 4.2.1.2 In-vitro hepatocyte isolation

Each liver was immediately removed from the transportation bag and placed in a stainless steel bowl within a Class II biosafety cabinet (Microflow Biological safety cabinet). Once the perfusion system was connected to the liver tissue via the cannula (Figure 26), 500ml

of oxygenated pre-warmed (37°C) Hepatocyte Liver Perfusion Medium (Gibco, Paisley, Scotland cat no.17701-038) was circulated through the system at a rate of 180mls/min for 20 minutes. On completion, the initial perfusion medium was discarded and replaced with Liver Digest Medium (LGM) (Gibco, Paisley, Scotland cat no.17703-034). The LGM was recirculated at a rate of 180mls/min for between 15-30 minutes, depending on the rapidity of liver digestion, which was estimated by the manual palpation of the liver. Once it had become soft to the touch, the cannulae and tubing were removed and the liver capsule and tissue disrupted with scissors. The resulting cell suspension was passed through a consecutive serious of filters of decreasing pore size (250µm, 100µm and a 75µm). Pre-cooled (+4$^0$C) hepatocyte wash buffer (Appendix 2) was added to the cell suspension and transferred to 200ml conical tubes, centrifuged (+4$^0$C) for 5 minutes at 100g. The cell pellet was washed twice and finally resuspended in 200mls of pre-cooled hepatocyte wash buffer. Aseptic techniques were maintained at all stages of the isolation process. A

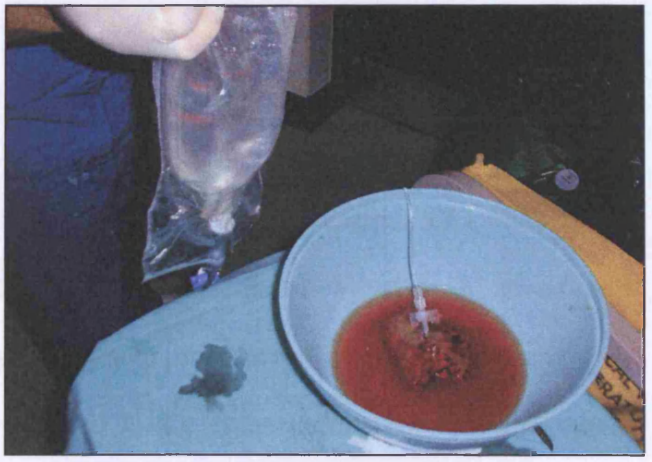


**Figure 25. Soltran perfusion in theatre**

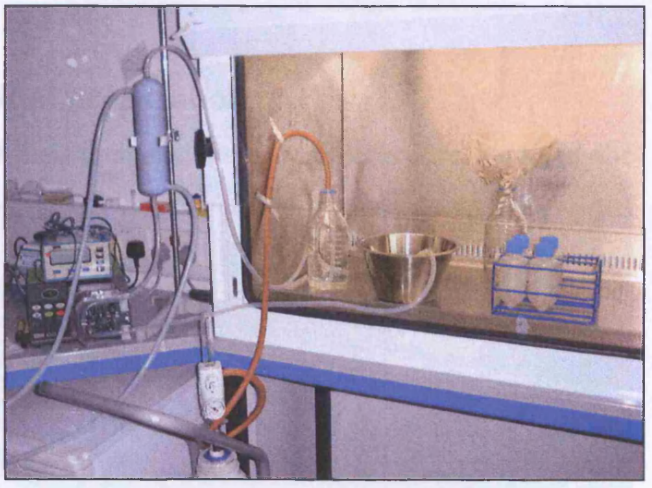


**Figure 26. Isolation equipment**

total viable cell count using trypan blue exclusion was determined using a haemocytometer grid (Appendix 2).

### 4.2.2 Porcine

Porcine livers were obtained from the abattoir located at the campus of University of Nottingham, Sutton Bonnington. The pigs were stunned by electric shock and immediately exsanguinated. All standard abattoir procedures for animal treatment were followed. The pigs weighed between 15 and 20 kg except during the foot and mouth restrictions, when only 40-kg pigs were available.

#### 4.2.2.1 Liver removal and initial preparation

Once the pig had been stunned and exsanguinated the initial procedure involved the removal of the intestines and gall bladder. The liver was removed by dissecting through the diaphragm and inferior vena cava, ensuring a cuff of tissue remained to allow for easy cannulation. The inferior vena cava was cannulated with a large tube and sutured into position (Figure 27). The liver was then perfused with pre-cooled (4°C) Soltran kidney perfusion solution (Baxter Healthcare Ltd, Thetford, U.K. cat. no. B4708), with the addition of 1µl/ml of Gentamicin (Gibco, Paisley, Scotland. cat. no. 15290/026), and introduced using an Arthroscopy set (Medi Plus, High Wycombe, U.K. cat. no.

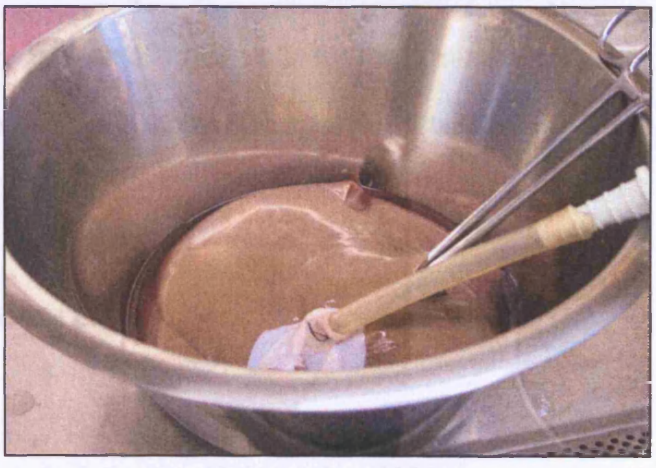


**Figure 27. Cannulated IVC of a pig liver**

MED9700). A clip was placed on the portal vein to prevent uncontrolled leakage and maintain adequate tissue perfusion. The liver was immediately transported to the laboratory on ice.

#### 4.2.2.2 In-vitro hepatocyte isolation

The isolation was performed using the same apparatus as for the human isolation. A modified procedure for porcine hepatocyte isolation, based on the method outlined by Koebe *et al.* was used (Koebe *et al.*, 1995). A series of Oxygenated Porcine Perfusion Buffers I –III (PPB) (Appendix 3) were sequentially perfused through the liver circuit prior to liver digestion and discarded. Liver digestion was achieved by the addition of 800ml of pre-cooled oxygenated Porcine Digestion Medium (Buffer IV), containing 0.5mg of collagenase (Sigma-Aldrich, Gillingham, UK cat. no. C-5138) and perfused through the liver. This was circulated at a rate of 180mls/min for 15-25 minutes and when soft to the touch the liver emulsified manually and cell suspension filtered, washed and counted as described in section 4.1.2.

## 4.3 In-vitro hepatocyte culture

### 4.3.1 Cell culture

Prior to use, twelve well plates (Sarstedt, Leicester, U.K. cat. No. 3225) were pre-coated with rats tail collagen type I (BD Bioscience). The collagen (2mg/ml) was resuspended in 200 ml of sterile water and 2 ml of acetic acid. To each well, 1.4ml of collagen solution was added and the plates incubated at room temperature for 1 hour. After removal of the collagen solution, each plate was washed twice with 1.4 ml of Hanks balanced salt

solution (HBSS) (Gibco, Paisley, Scotland. cat. no. 24020-133) and dried within the hood for an hour, wrapped in cling film and stored at 4°C prior to use.

Immediately following isolation, the hepatocytes were cultured in 12 well plates (Becton Dickinson, Berges, France. cat. no. 353225). Using a concentration of $1x10^6$ hepatocytes/ml, 0.7 ml of cell suspension were added to each well giving a final total cell concentration of $7x10^5$ cells/well.

Human hepatocytes were cultured in Hepatozyme-SFM (Life Technologies, Paisley, Scotland. cat.no.177505-02) with the addition of 1.6 ml of Fungizone (Gibco, Paisley, Scotland. cat. no. 15290/026), and 500µl/litre of Gentamicin (50mg/ml Gibco cat. no.15750-037). Porcine hepatocytes were cultured in tissue culture medium described by Koebe *et al* (Koebe *et al.*, 1995). This consisted of Dulbecco Modified Eagles Medium (DMEM) supplemented with 4.5g/l glucose (Life Technologies, Paisley, Scotland. cat.no.42430-027), 125mu/ml of insulin, 60ng/ml of hydrocortisone, 10ng/ml of glucagon, 10% FCS and 1.6 ml of Fungizone (cat. no. 15290-026, Gibco, Paisley, Scotland). The selection of both human and porcine growth medium was based on selected previous studies undertaken in the by Department of Surgery, Leicester General Hospital (personal communication, Dr J. Davies, Dr H. Clayton).

For every isolation, 12 well plates were set up in triplicate for the assessment of cell function. This allowed each assessment to be performed with six replicates on day 2 and day 5 of each culture. The plates were incubated at 37 °C in 5% $CO_2$. The media was

replaced at 4 hours post inoculation and at 72 hours. Functional assays were undertaken on both fresh and cryopreserved cells after 2 and 5 days of culture.

### 4.3.2 Cell Culture post-cryopreservation

Following thawing, hepatocytes were transferred from the cryovials into 50 ml DMEM (Life Technologies, Paisley, Scotland cat. no.42430-027), supplemented with 4.5g/l glucose (Life technologies, Paisley, Scotland. cat no. 42430-027), centrifuged for 5 minutes at 100g and 4 °C and the supernatant aspirated and discarded. The pellet was washed twice in DMEM, supplemented with 4.5g/l glucose (Life Technologies cat. no. 42430-027), centrifuged and pelleted. The cells were finally resuspended in 10ml of DMEM (Life Technologies, Paisley, Scotland cat. no.42430-027) and total cell count performed. The cells were cultured as described in section 4.4.2.

## 4.4. Functional Assays

### 4.4.1 Bilirubin conjugation assay

The principle of the test is based on the reaction of conjugated and unconjugated bilirubin with diazotized sulfanilic acid in the presence of (DMSO) to give azobilirubin. The cells are exposed to bilirubin mixed isomers and the diazo reaction that occurs in the absence of methyl sulfoxide (MSO), provides a measure of direct (conjugated) bilirubin. The azobilirubin formed from the reaction was measured at a wavelength of 560nm.

The ability of hepatocytes to conjugate bilirubin was determined by the Sigma (Diagnostics) bilirubin assay kit (Sigma-Aldrich, Gillingham, UK. cat. no. 551-A) Briefly, 2.9mg of bilirubin mixed isomers (Sigma-Aldrich, Gillingham, UK. cat. no.

B4126) was dissolved in 1.25ml 50mM NaOH and vortexed until the solution was optically clear. The solution was neutralised by the addition of 3.75ml 100mM Tris hydrochloric acid (HCL), pH 7.8 and used within 5 minutes, before degradation occurred. Twenty-nine µls of this solution was pipetted into each well and the plate incubated for 4 hours in the dark at 37°C within a 5% $CO_2$ atmosphere. The reaction mixture was collected, centrifuged for 5 minutes and supernatant frozen at –20 °C prior to the assay. The assay procedure was undertaken as follows:

- 1ml of direct bilirubin reagent (cat. no. 551-4) pipetted into tubes labelled Blank.
- 1ml of direct bilirubin assay reagent (formed by the addition of 30ml of direct bilirubin reagent with 1ml of Sodium Nitrite solution (cat. no.550-7)) was added to tubes labelled Test.
- 40µl of each cell culture supernatant were pipetted into the appropriate Test and Blank tubes.
- For the calibration 40µl of Bilirubin Calibrator (cat. no.8652) was added to Test and Blank tubes containing the appropriate reagent.
- After thorough mixing the sample was transferred immediately to a cuvet and absorbance (A) read on a spectrophotometer (Hewlett Packard model 8453). Absorbency readings were taken from specimen Test, specimen Blank, calibrator Test and calibrator Blank at 560nm against water as reference.

The level of bilirubin conjugation was estimated using the following calculation:

$$\text{Direct Bilirubin (mg/dl)} = \frac{\Delta A \text{ Specimen} \times \text{Bilirubin standard (mg/dl)}}{\Delta A \text{ Calibration}}$$

Where $\Delta A$ = Test – Blank or the change in absorbency

Bilirubin standard for each kit, was recorded in the manufacturer leaflet.

### 4.4.2 Assay for P 450 function by lignocaine metabolism

Lignocaine metabolism was chosen as a measure of P450 activity. Lignocaine is metabolised using the CYP3A4 isoenzyme, to produce monoethylglycinexylidide (MEGX) and glycinexylidide (GX). The particular oxidative reaction to form these products is N-dealkylation, and occurs rapidly, allowing a short exposure time.

To each well of cultured hepatocytes, 129µl of lignocaine (lignocaine injection BP 2%) was added, the mixture then incubated for 40 minutes, at 37°C. The medium was placed in a 2ml Eppendorf, centrifuged and supernatant stored at -20°C until required.

Prior to assaying the samples by HPLC, the metabolites were extracted from the culture medium as follows:

- 100µl of the thawed supernate, 100µl of working internal standard (100mgl/ml Tocainide (Astra Zeneca, Loughborough, U.K.)) and 100µl 1M sodium bicarbonate buffer (Appendix 2) were added to a test tube.
- A calibration sample was also made but a solution containing GX, MEGX (kindly donated by Astra Zeneca, Loughborough, U.K.) and Lignocaine at a concentration of 100µg/ml was added in place of the thawed standard.
- 2ml of ethyl acetate (BDH, cat. no.152486E) was added to test and control tubes and the solution vortexed.
- The tubes were then centrifuged for 5 minutes at 3000 rpm at 4°C.

- A calibration sample was also made but a solution containing GX, MEGX and Lignocaine at a concentration of 100µg/ml were added in place of the thawed standard.
- The upper organic layer following centrifugation was pipetted into 10ml tubes and evaporated to dryness using a centrifuge evaporator.
- The residue was reconstituted using 200µl of mobile phase (Appendix 2).

The solution was analysed using a Hewlett-Packard 1100 series HPLC and a Supelcoil™ LC-8-DB pore size 5um,120A (Supleco), injection volume of 20ul and a rate of 1ml/min. Wavelength analysis was undertaken at 214nm, analysis being completed by the use of Hewlett Packard Chem. Station B.02.05 software. An example of a typical trace is illustrated below (Figure 28). It shows the peaks of GX and MEGX as compared to the internal standard. The largest peak shown at 11.983 represents the residual lignocaine not metabolised.

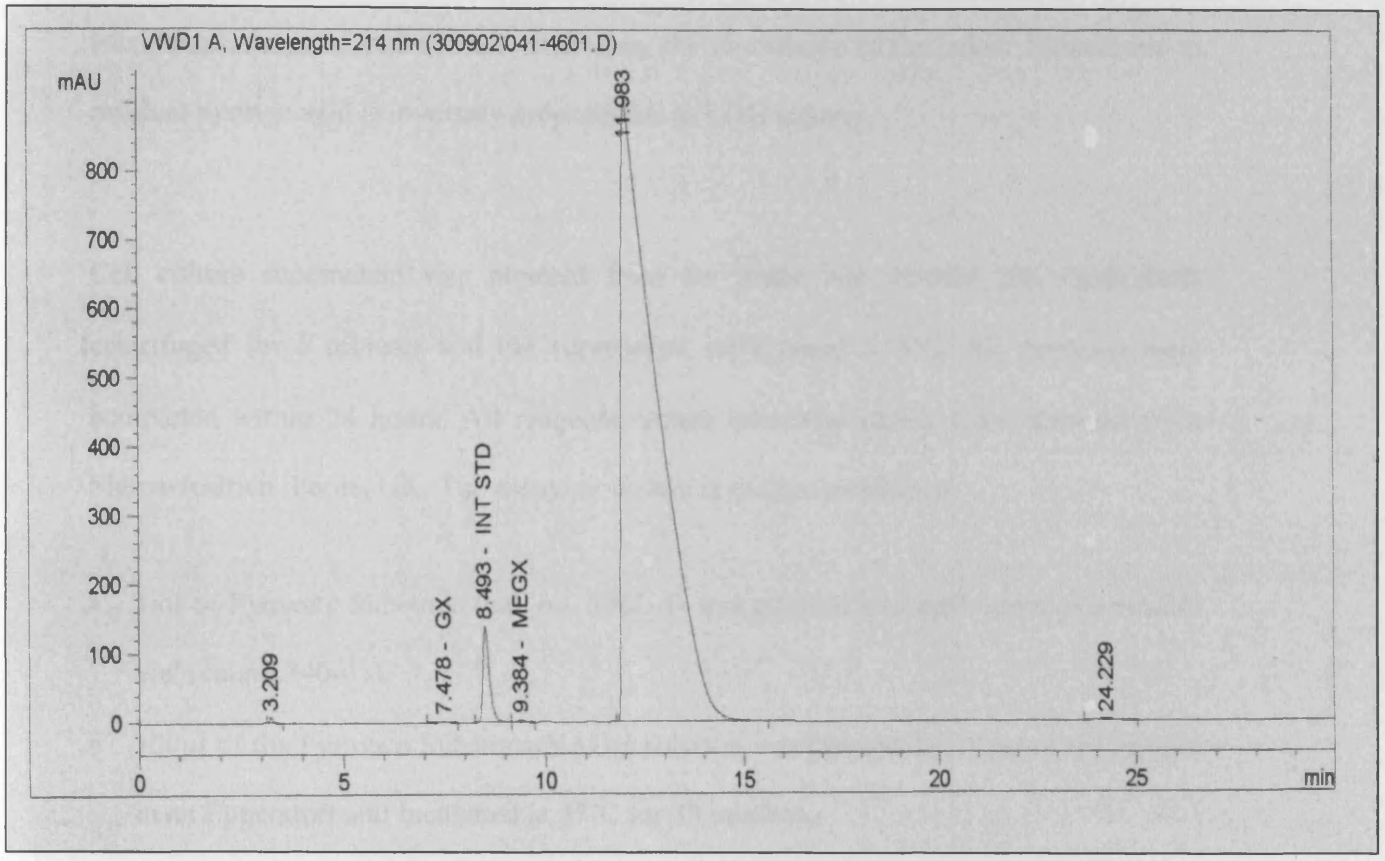
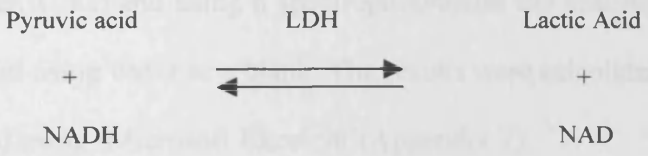



**Figure 28. Lignocaine metabolism as analysed by HPLC**

### 4.4.3 Lactate dehydrogenase leakage assay

The measurement of LDH leakage from cells whose membrane are not intact or permeable, can be related to cellular viability (Chen *et al.* 2001; Chesne *et al.* 1991). The assay is based on the ability of LDH to catalyse the following reaction.




The reaction equilibrium favours reduction of pyruvate to lactate at a rate proportional to the amount of LDH present. Pyruvic acid also reacts with 2,4-dinitrophenylhydrazine to form an intensely coloured hydrazone, which has a peak absorbance over the broad

wavelength range of 400-650nm. Therefore, the absorbance of the colour formed due to residual pyruvic acid is inversely proportional to LDH activity.

Cell culture supernatant was pipetted from the plates into labelled 2ml Eppendorfs centrifuged for 5 minutes and the supernatant refrigerated at 4°C. All reactions were completed within 24 hours. All reagents, unless otherwise stated, were obtained from Sigma-Aldrich, Poole, UK. The assay procedure is outline as follows:

- 1ml of Pyruvate Substrate (cat. no. 500L-1) was pipetted into the bottom of a NADH vial (cat.no.340-01).
- 100µl of the Pyruvate Substrate/NADH solution was pipetted into 10µl of the sample in an Eppendorf and incubated at 37°C for 30 minutes.
- 100µl of Sigma colour reagent (cat. no. 505-2) was then added and the Eppendorfs incubated at room temperature for 20 minutes.
- To each eppendorf 1ml of 0.40N Sodium Hydroxide (NaOH) solution was added to stop the reaction
- Each sample was then pipetted into 1.6ml cuvettes (cat. no. 67.742 Sarstadt, Leicester, U.K.) and using a spectrophotometer the absorbance read at a wavelength of 460nm using water as a blank. The results were calculated using the standard graph obtained using 'Microsoft Excel 98'(Appendix 2).

### 4.4.4 Protein assay

Following assessment of the initial studies it became evident that it was difficult to compare results between different isolations as the characteristics and attachment

efficiencies of the hepatocytes varied. Therefore, an assay to standardise the functional results was developed. By measuring the total amount of protein present a comparison between each experiment was possible and a comparative marker for the number of cells attached was thus obtained. This has been previously used in a number of published studies (Loretz *et al.*,1989; Lawrence and Benford, 1991; Alexandre *et al.* 2002 and personnel communication with Professor Richert, University of Besançon, France).

The protein assay (Bio-Rad) was based on the principle of colour change, measured by spectrophotometry. The dye (Bio. Rad., Hemel Hempstead cat. no. 500-0006) was added to the solution containing protein and the colour response measured by spectrophotometric analysis at a wavelength of 405nm. The results were compared to a standard protein curve and the volume of protein calculated.

The original culture supernatant was discarded to remove all unattached cells. Fresh tissue culture fluid was added and hepatocytes manually removed, using a cell scraper (Costar cat. no. 30100). The cell suspension was pipetted into labelled eppendorfs and centrifuged at 10,000rpm for 5 minutes. The supernatant was discarded cell pellet stored at 4°C.

The prepared protein pellets were dissolved in 1 ml of the urea solution (100ml of 6 M urea (Sigma, Poole, UK cat. no. U5378) solution prepared in water. Ten µls of each sample were pipetted into the wells of a 96 well plate and 200µl of the dye reagent solution added to the wells. Simultaneously, a series of protein standards were prepared

to produce a standard curve (Appendix 7). Protein estimations of each sample were compared with the standard curve.

## 4.5 Cryopreservation

### 4.5.1 Cryopreservation in suspension

For the purpose of this research project all cryopreservation was undertaken with hepatocytes in suspension. Hepatocytes were suspended in ice-cold DMEM at an appropriate concentration (see experimental chapters) and 20% (by final volume) foetal calf serum (Sigma, Poole, U.K cat. no. F4135), and placed on ice. A 10% (by final volume) solution of DMSO (Sigma cat. Poole, UK cat. no. D 879) was added over 1 minute, just prior to pipetting into 2ml cryovials. The cryovials were then subjected to the appropriate freezing protocols.

### 4.5.2 Cryopreservation systems

#### 4.5.2.1 Nalgene controlled freeze device

The Nalgene "Mr Frosty" propan-2-ol device (cat. no. 5100-0001) (Figure 29) was a device designed to allow a reduction in temperature of approximately 1°C/minute when placed in a -80°C freezer. The vials containing the cell suspensions were

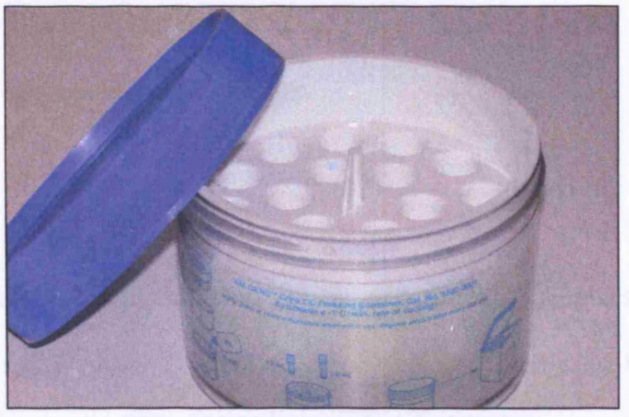


**Figure 29. Nalgene propan-2-ol device**

placed into the slots within the device, bathed in propan-2-ol. The device was left in the freezer for 4 hours before the cryovials were removed for storage in the gaseous phase of a liquid Nitrogen storage tank.

### 4.5.2.2 Programmable freezer

The Planer Cryo 10-16 series (Planer, Sunbury on Thames. UK.) (Figure 30) was used in some of the initial experiments this allowed a programmable reduction in temperature to be achieved. Full details of this are described in Chapter 6 .

**Figure 30. Planer Cryo 10-16**

### 4.5.2.3 Freezer method

Once the cryovials had been established with hepatocyte suspension they were placed in a clear plastic bag and stored in a –20°C refrigerator for 1 hour and then transferred to a -80°C refrigerator for 4 hours, before storage in liquid nitrogen.

## 4.6 Statistical analysis

The analysis for Chapter 5 is described within the chapter. For the experimental chapters mean comparisons were performed using either Student t-test or one way analysis of variance (ANOVA) according to the number of groups to be compared. Throughout the

analysis, a $p$ value $\leq 0.05$ was considered significant. The analysis was performed using the SPSS 9.0 statistical software.

# Chapter 5

## Effect of patient, operative and hepatocyte isolation factors on cellular yield, viability and function.

5.1 Introduction

5.2 Methods

5.3 Results

5.4 Discussion

5.6 Summary conclusions

## 5.1 Introduction

For bioartificial support and pharmacotoxicological research good quality human hepatocytes are desirable (Rozga *et al.*, 1993; Ballet *et al.*, 1984). The whole procedure from operative removal of the liver (surgically resected or MOD), through transport to the laboratory and the subsequent isolation process exposes the liver cells to a number of variables that could affect the final quality of the hepatocytes. The aim was to isolate the maximum number of hepatocytes with the greatest possible functional ability once cultured, and then to ensure that the maximum use is obtained from the donated tissue specimens.

Anecdotally the success, measured by hepatocyte viability and yield, of any liver isolation was difficult to predict. The potential for individual variation between patients, the influence of their medical history and other postulated influences such as the recent consumption of non prescription drugs on the subsequent quality and function of the liver has not been extensively investigated in the context of hepatocyte isolations (Alexandre *et al.*, 2003). However it could be important in determining the suitability of cells for proposed applications. Hepatocytes from certain subsets of patients may not be suitable for cryopreservation or it may be possible to predict that they will have a limited or inappropriate range of functions.

Currently there is some doubt about the consistent availability of human tissue that would be able to yield sufficient hepatocytes to support a clinical bioartificial liver programme. These questions are responsible for the parallel studies described here relating to the suitability of porcine hepatocytes as an alternate supply. The two sources of human liver

that are utilised are surgically resected tissue and whole organs from MODs. Surgically resected tissue has the advantage that hepatocytes can be isolated virtually immediately after removal from the patient, while MOD livers often have long cold ischaemic times while its suitability or otherwise is assessed for transplantation. Livers not suitable for transplantation are also generally of a poorer quality (the reason for their rejection). Most of the available literature has concentrated on MOD as a source of human hepatocytes, with minimal data being available on surgically resected specimens.

Little is known about the effect of cold ischaemic time on surgically resected tissue as measured by viability, yield and function. During these studies the cold ischaemic time was the period from perfusing ice cold Soltran in the operating theatre (Baxter Healthcare Ltd, Thetford, U.K. cat. no. B4708), to initiating the isolation process. For use in a bioartificial liver, tissue will almost certainly be required from other centres, possibly some considerable distance away. This can only be achieved if the liver does not significantly deteriorate during transport.

Although the isolation process is well established (Section 4.2.1) there are many aspects that have been identified as areas where there is potential for improvement. These improvements if studied together may lead to an optimised method of tissue isolation. It is unknown if prolonged exposure to collagenase during the isolation process causes a decrease in viability, whether there is an optimal size of tissue that will produce the highest yield or whether differing cannulation methods make a significant difference.

## 5.2 Methods

From October 2001 until the end of May 2003 a detailed record was kept of all patients undergoing liver resection and subsequent tissue donation. The liver tissue donated consisted of two types, resected tissue (Leicester and Manchester) and MOD. The procurement, isolation and handling of the tissue is described in the methods chapter (Chapter 4).

Parameters that were recorded during assessment included, age sex, Body Mass Index (BMI), smoking and alcohol history, pre-operative blood results (sodium, potassium, urea, creatinine, haemoglobin, white cell count, platelet count, bilirubin, ALT, alkaline phosphatase, albumin), Pringle time, operation undertaken, weight of donated tissue, number of cells isolated, viability, digestion assessment, transport time, digestion time and total isolation time. As part of the ongoing experiments on cultured hepatocytes, data was obtained on cellular function (LDH leakage, attachment, bilirubin conjugation, cytochrome P450 function (CYP3A4)), and the ability of the hepatocyte preparations to withstand cryopreservation. Differences in the two cannulation methods were also analysed (Section 4.2.1.1). Analysis was undertaken of these factors on yield, viability and function.

## 5.3 Results

Over the study period 161 patients were identified and approached about taking part in the study. If they were willing the process was described to them in detail and were consented. Of all the patients approached consent was not gained in 5 (3.1%) patients. Forty seven (33%) livers were inoperable leaving 88 liver resections and 11 MOD liver

donated. In total 11 MOD and 77 resected livers were utilised for hepatocyte isolations (Table 11). Of the remaining 29 resected specimens, on 5 occasions there were insufficient samples and 24 were snap frozen or donated directly to researchers. Some of the parameters were not analysed due to the lack of data recording.

Tables 12 and 13 present some of the basic statistics on various study parameters. The age of the patient is summarised by median and inter quartile range (IQR), owing to the large variability in the age pattern. It can be seen from Table 12 that the variation in the levels of cold time and weight of tissue isolated are highest in the MOD group. The average yield of hepatocytes is highest in the resected group as opposed to the MOD livers. The average viability of isolated hepatocytes is higher in the cells from Leicester but this is not statistically significantly higher compared to that from Manchester.

The patterns of variations in yield and viability between the three tissue sources are presented in the box-plots (Figure 31). It is evident from the box-plots for yield that the median levels are almost the same for Manchester and Leicester, the distribution patterns are quite different with few outlying observations in both sources. Obviously the yield pattern for MOD livers is quite different from the other sources. The median yield is much lower in the MOD group (median =1.8 hepatocytes $x10^6$/gram of tissue). For viability, the median level is higher for the Leicester group and lowest for MOD livers. The analysis of variance (ANOVA) suggests significant differences in the mean levels of viability ($p=0.01$) over the three sources. However, the yield levels do not differ significantly ($p=0.14$). The mean plots for yield and viability are presented in figure 32.

| Site | n isolated | Median Age (IQR) | Sex | | Cold ischaemic time/mins Mean±SD | Wweight of tissue /grams Mean±SD | Yield of cells $x10^6$/gram Mean±SD | Total Cells isolated $x10^9$ | Viability Mean±SD |
|---|---|---|---|---|---|---|---|---|---|
| | | | M | F | | | | | |
| Leic | 52 | 63 (17.5) | 35 | 17 | 40±25 | 188±159 | 5.1±4.3 | 39.9 | 83.9±8.9 |
| Manch | 25 | 67 (13.0) | 16 | 9 | 200±47 | 161±101 | 5.0±4.2 | 14.1 | 82.4±5.6 |
| MOD | 11 | 63 (14.8) | 6 | 5 | 1872±600 | 166±70 | 2.4±2.1 | 2.4 | 76.4±4.9 |

**Table 12. Summary of liver tissue received over study period**

| Variable | | Manchester n (%) | Leicester n (%) | MOD n (%) |
|---|---|---|---|---|
| Chemotherapy | Yes | 7 (35) | 18 (39.1) | - |
| | No | 13 (65) | 28 (60.9) | - |
| Fatty Liver | Yes | 4 (16) | 9 (17.3) | 2 (18.2) |
| | No | 21 (84) | 41 (78.8) | 7 (63.6) |
| Digestion | Good | 12 (48) | 18 (34.6) | 1 (9.1) |
| | Moderate | 9 (36) | 25 (48.1) | 4 (36.4) |
| | Poor | 1 (4) | 5 (9.6) | - |
| Cannulation Method | Glue | 22 (88) | 27 (52.9) | 11 (100) |
| | Venflon | 3 (12) | 24 (47.1) | - |

**Table 13. Table of comparisons of non linear variables between the three tissue sources**

| Pathology | Number |
|---|---|
| Colonic Secondary | 66 |
| Cholangiocarcinoma | 3 |
| Carcinoid | 2 |
| Hepatocellular tumour | 2 |
| Haemangioma | 1 |
| Unknown Primary | 1 |
| Hydatid Cyst | 1 |
| Lung Secondary | 1 |
| MOD | 11 |

**Table 14. Summary of underlying pathology necessitating resection or donation.**

| Operation | Number |
|---|---|
| Right Hepatectomy | 43 |
| Segmental resection | 10 |
| Left hepatectomy | 9 |
| Extended right | 5 |
| Local excision | 3 |
| MOD | 7 |

**Table 15. Operation performed.**

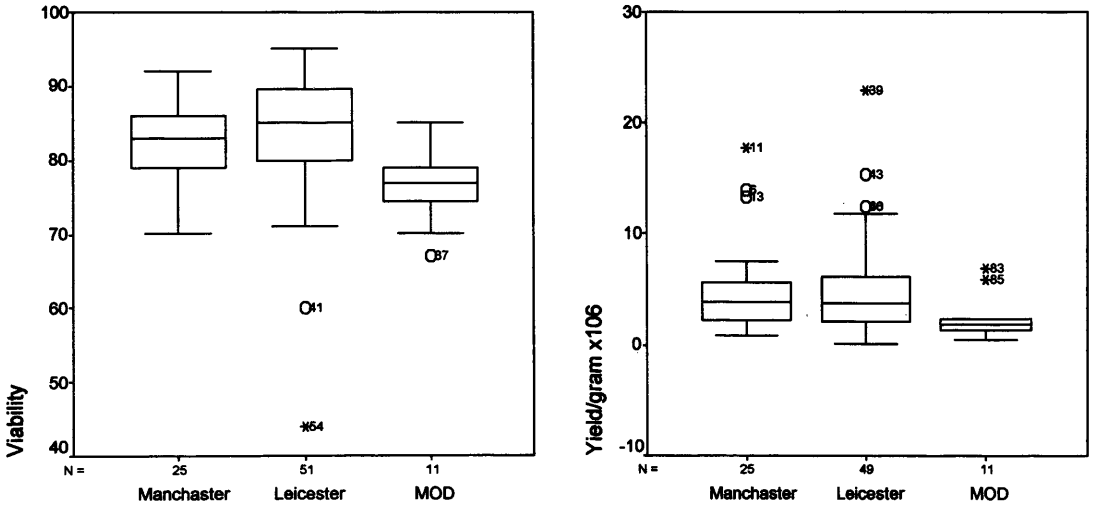



**Figure 31 Box-plot exploring the distribution patterns of end points between tissue sources. The yield and viability are significantly lower in the MOD livers.**

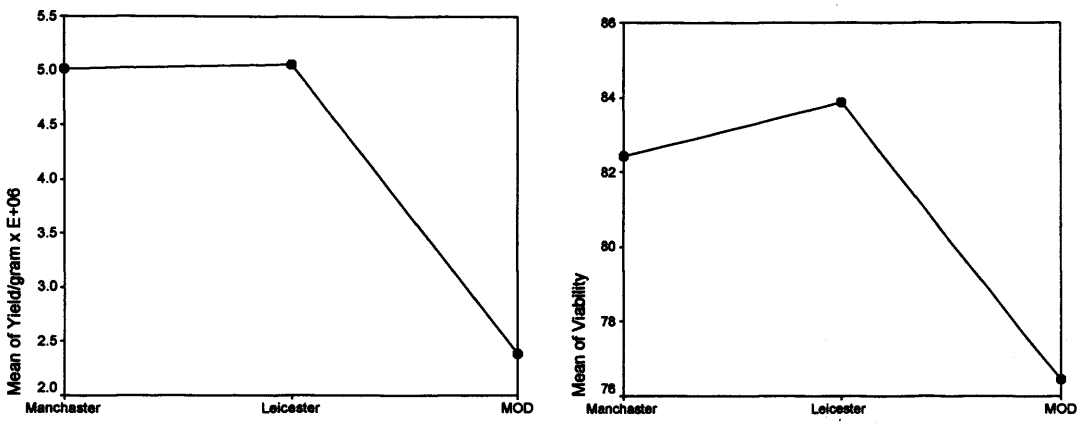



**Figure 32. Plots of means of yield and viability comparing tissue sources**

We have tested the differences in the average levels of various clinical parameters and endpoints over the levels of fatty liver. We have used the non-parametric Kruskal-Wallis test and the significance level was set at 5%. Only the viability was found to be significantly higher in livers determined as fatty (p=0.02).

To assess the impact of the various clinical and non-clinical factors on yield and viability, we have used both parametric and non-parametric multiple regression techniques. The multivariate robust regression technique was adopted in the final analysis, owing to the problem of non-normality of regression residuals and the evidence of the presence of heteroscedasticity in the general linear multiple regression fit. The factors significantly affecting the yield and viability were selected on the basis of "step-wise" selection procedure. The selection of covariates was set at 5% significance level.

The age and method of cannulation were found to strongly affect the hepatocyte viability (a decreasing viability with increasing age and the glue method superior to the cannulation method). The source of the tissue also influenced the viability, as evident from the regression estimate (p value) provided in Table 16. The univariate analysis,

presented earlier, was consistent with this finding. The regression model was fitted with logarithmic transformation of the response variable (viability). The model comparisons are based on the diagnostic criteria like adjusted $R^2$, Akaike Information Criteria (AIC), etc. The regression result is presented in Table 16, for viability only and not for yield, as no factor came out to be significantly affecting the yield.

| Parameter | Regression Coefficient | p-value |
|---|---|---|
| Age | -0.21 | 0.01 |
| Cannulation Method | 0.038 | <0.01 |
| Source of tissue | -0.02 | 0.01 |
| Adjusted $R^2$ | 0.58 | |
| AIC | 3.58 | |

**Table 16. The Robust Regression result for viability**

## 5.4 Discussion

There are few studies in the literature that have analysed the factors that might be important in the isolation of hepatocytes from surgically resected tissue (Alexandre *et al.*, 2003). Fischer *et al.* demonstrated that the quality of the liver has an impact in the subsequent functional ability (Fisher *et al.*, 2001). They reviewed 126 livers received as either rejection for transplantation or "for research livers". They found that the rejected livers had a higher functional ability and viability, and concluded that the method of removal (for transplant being different than for research) has a significant influence. They also considered the fat content of the liver (often the reason for rejection for transplantation) to be a factor that reduces the quality of the liver. The present study

inevitably must come to the opposite conclusion as it was observed that the fat content increased the viability of the isolated cells. This appears counterintuitive and possible explanations include the high fat content caused a density gradient while washing following the isolation, favouring the removal of cells. Another possibility is that the degree of fat content may interfere with the trypan blue uptake of the cells making the assessment of viability less reliable. However, the accuracy of the assessment must be questioned, as it was only a crude assessment by eye rather than a histological grading. Further work to clarify this anomaly would be required in further studies.

Analysis of patient and operative factors (pre-operative bloods, past medical history, smoking, alcohol intake, position of tissue removed, Pringle time,) showed that they do not appear to have any influence on yield or viability. Anecdotally, measurement of high serum bilirubin has accompanied poor cellular viability, although no such isolation was undertaken during the study period. This is consistent with the findings of Gomez-Lechon *et al.* who also commented on the damaging effect of bilirubin on small biopsy isolations (Gomez-Lechon *et al.*, 1990).

The transport or cold ischaemic times for surgically resected tissue was found not to have an influence on viability or yield whether it be from a local or more distant source. The observations suggest that surgically resected tissue could be transported with up to 5 hours of cold ischaemia. This would imply that tissue could be transported from anywhere in the UK to an isolation point. These findings are in agreement with Caruana *et al.* who found no significant decrease in viability with cold ischaemic time ranging from 175 to 360 minutes (Caruana *et al.*, 1999).

With only a limited number of MOD livers received over the study period, conclusions regarding the effective significance of cold ischaemic time on these livers cannot be drawn with any degree of certainty. From the studies it was observed that these livers produce a reduced cellular yield and viability when compared with surgically resected specimens. This reduced viability while confirmed by some other studies (Baccarani *et al.*, 2003) has not been observed by some groups who reported no significant deterioration in MOD livers with up to 24 hours of transport. (Ulrich *et al.*, 1998; Serrar *et al.*, 1999; Fisher *et al.*, 2001).

The isolation process itself cannot be compared to other studies as they all differ in methodology. The technique employed in this study has delivered cells from surgically resected specimens of a consistent yield and viability (Table 12). This is comparable with some studies, while others have achieved higher yields isolating from both smaller tissue samples and those of similar sizes (Ulrich *et al.*, 1998; Takahashi *et al.*, 1993; Gomez-Lechon *et al.*, 1990; David *et al.*, 1998). The two methods of cannulation did however produce a strongly significant difference in viability. When sewing the cannula in place the cut surface allowed free leakage of perfusion solution as opposed to the sealing effect of reforming Glissons capsule with adhesive. With this method a higher pressure within the tissue would occur with limited opportunity for the perfusion solution to leak out. This could damage the hepatocytes by exposing them to increased pressure and shear forces.

The time taken to digest the liver was also identified as a potential factor that may decrease the viability of the cells but possibly increase the yield. A prolonged digestion

phase may expose the hepatocytes to collagenase that could damage the cellular membrane; however this has to be balanced against a potential increase in yield. The difficulty with the present method is sometimes judging when a large piece of liver has digested sufficiently thus reducing the potential of viability decrease. The study has not identified this as a factor, which has a significant influence on yield or viability (mean time 28.4±9.8 minutes) suggesting either that this theory is incorrect or that the ability to judge the correct time to stop digestion is reasonably accurate and dependent upon experience.

The last significant result is in respect of the decrease in cellular viability with age. It has been suggested that tissue quality will decrease with age but that the effects of alcohol and chemotherapy, which can cause quite marked macroscopic changes, did not have the same effect (Dorko *et al.*, 1994). Although this trend was significant it should not prohibit the use of tissue resected from patients over 70 years of age.

Few significant findings were found in this study. It is possible that with the many variables a larger number of liver isolations would be required to identify any significant differences.

## 5.6 Summary conclusions

- Of the sixty resected livers studied, travel times of up to 4 hours in cold storage can be undertaken without a significant effect on the hepatocyte yield and viability.
- Current methods consistently produce hepatocytes at a reproducible viability.

- Decreasing age, increasing steatosis and the venflon method of tissue cannulation increased viability.
- In contrast to some other published studies steatotic livers produced hepatocytes with statistically higher viability.
- The present studies suggested that surgically resected liver tissue tended to produce hepatocytes with higher viability and yield compared to MOD's.
- Further investigation into pump pressures for perfusing collagenase requires additional evaluation and standardisation.
- In view of the volume of stored tissue a significantly higher number of hepatocytes could be potentially isolated.
- These studies need to continue to see whether further trends are revealed as numbers increase.

# Chapter 6

# Comparisons of cryopreservation freeze rates on human and porcine hepatocytes prior to storage in liquid nitrogen

## 6.1 Introduction

A critical step in the successful cryopreservation and recovery of hepatocytes is the rate of cooling prior to storage in liquid nitrogen. Three methods were evaluated: (1) the Nalgene propan-2-ol device (Device), (2) step-wise freezing utilising a programmable controlled rate machine Planer Cryo 10-16 series (Planer, Sunbury on Thames. UK.) (Planer) and (3) placing the cryovials into –20°C freezer for 1 hour, -80°C for 3 hours prior to liquid nitrogen storage (Freezer).

## 6.2 Method

Following hepatocyte isolation, three stock preparations each containing a total cell count of $1x10^8$ hepatocytes were washed twice in 50 ml of DMEM (Life Technologies, Paisley, Scotland. cat. no. 42430-027) and centrifuged at 100g to pellet the cells. The supernatant was removed and each stock pellet was first resuspended in 18ml of ice cold DMEM containing FCS (final concentration 20%). Once a homogeneous mixture was achieved, 2ml of DMSO was added over a period of 1 minute, making a final hepatocyte concentration of $5x10^6$ hepatocytes/ml. The final hepatocyte and cryo-formulated solutions were distributed in 1.8ml volumes into 2ml cryovials. The hepatocytes within the cryovials underwent one of the three cooling methods under investigation.

After 7-10 days of storage in liquid nitrogen the hepatocytes were removed, thawed, washed and cultured as previously outlined for cell viability and functional studies (Section 4.3.2 and 4.4).

## 6.3 Results

The results are limited for the studies using human hepatocytes, as protein assessment was not undertaken. It was realised following these studies that it was not known how the functional data was derived or whether it correlated with the number of hepatocytes that subsequently attached. Therefore, a protein assay was developed and implemented in later experiments. The attached hepatocytes were washed and scraped free from the plate. The amount of protein measured gave a comparative measurement of hepatocyte attachment (Section 4.4.4).

### 6.3.1 Assessment of cellular viability post cryopreservation

#### 6.3.1.1 Human hepatocytes

The percentage of viable hepatocytes recovered from the three experimental cryopreservation methods are illustrated in Figure 33. An isolation viability of 86±8.5% was consistently observed during the course of the studies. The Nalgene Device gave a higher mean return of viable hepatocytes (35±19.5%), which was marginally significant.

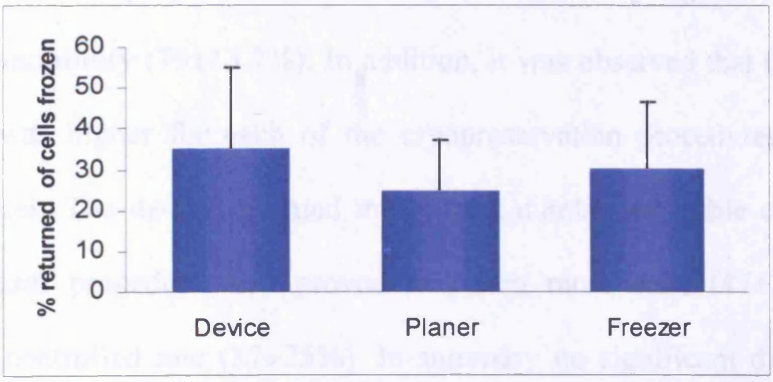



**Figure 33. Data represents % of human hepatocytes returned after cryopreservation using the three methods described in Section 4.5. There is no statistical difference between the methods (n=16).**

when compared to the Planer method (25.0±11.9%, p=0.21). The Freezer method returned 30.2±16.5% of the viable hepatocytes and was found not to be statistically different from the device method.

### 6.3.1.2 Porcine hepatocytes

The percentage viable hepatocytes recovered from the three experimental cryopreservation methods compared with those originally frozen are illustrated in Figure 34.

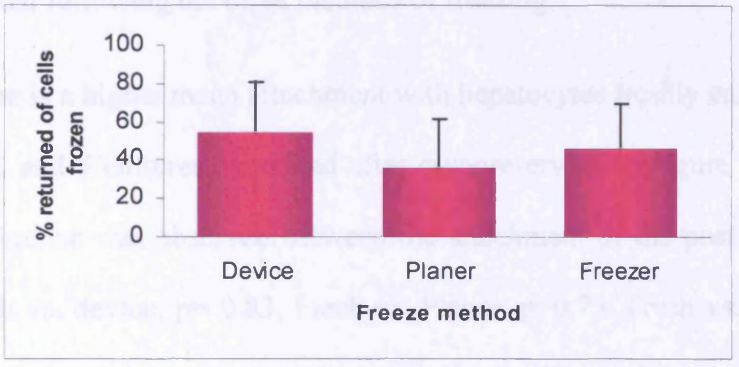



**Figure 34. Data represents % of porcine hepatocytes returned after cryopreservation using the three methods described in Section 4.5. There is no statistical difference between the methods (n=9).**

Unlike the human, the post isolation viability (measured by trypan blue exclusion) showed greater variability (79±13.7%). In addition, it was observed that the mean return of viable cells was higher for each of the cryopreservation procedures, compared to human hepatocytes. The device returned the highest number of viable cells (54±25%). The simple freezer procedure also proved to return more cells (47±23%) than the complex planer controlled rate (37±25%). In summary no significant difference in the recovery of viable porcine cells post cryopreservation was found when all three methods

for porcine hepatocytes were compared (Device vs. Planer p=0.22, Device vs. Freezer p=0.95, Planer vs. Freezer p=0.39).

### 6.3.2 Assessment of hepatocyte attachment post cryopreservation

#### 6.3.2.1 Porcine hepatocytes

The method described in (Section 4.3.4) measures the amount of hepatocytes attached through relative protein attached to the culture plates at day 2 and day 5. This measurement gave comparative attachment capabilities of fresh hepatocyte cultures and those undertaken following the three methods of freezing.

In general there is a higher mean attachment with hepatocytes freshly cultured as opposed to those day 2 and 5 cultures examined after cryopreservation (Figure 35). At day 2 no statistical difference was observed between the attachment of the post cryopreservation cultures (Fresh vs. device, p= 0.83. Fresh vs. Planer, p=0.73. Fresh vs. freezer p= 0.84) (Figure 35a). At day 5 there was a marked reduction in the attachment of the post (Figure 35b).

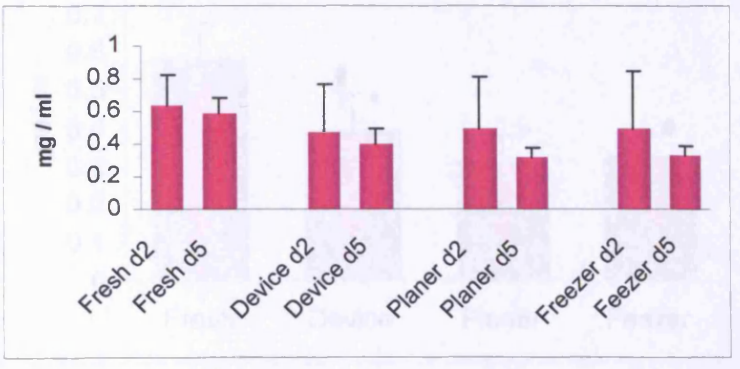



**Figure 35. Protein attachments measured as mg/ml values for fresh and post cryopreserved cultures using the 3 methods at 2 and 5 days (n=9).**

cryopreserved cells compared to the corresponding day 2 values and the fresh culture. The protein measured in the fresh hepatocytes was 0.59±0.09mg, which was statistically higher than that for the Device (0.40±0.09, p=0.005), the Planer method (0.32±0.07, p=0.0004) and the Freezer method (0.33±0.05, =0.0007).

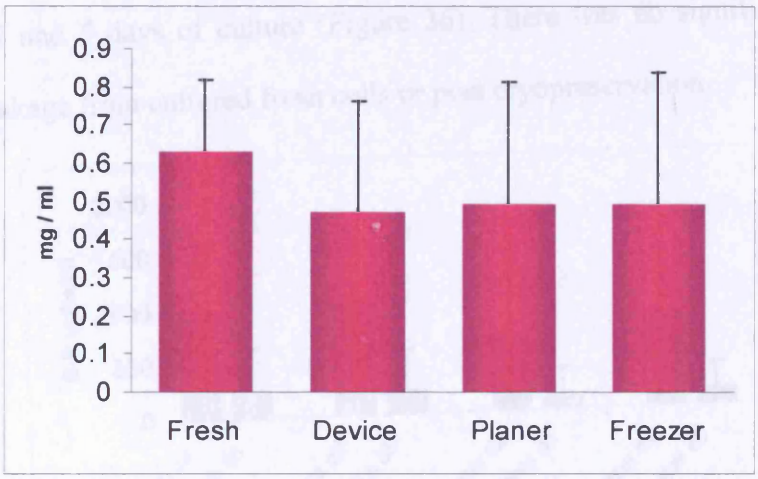



**Figure 35a. Protein attachment at day 2 only between fresh cells and cryopreserved.**

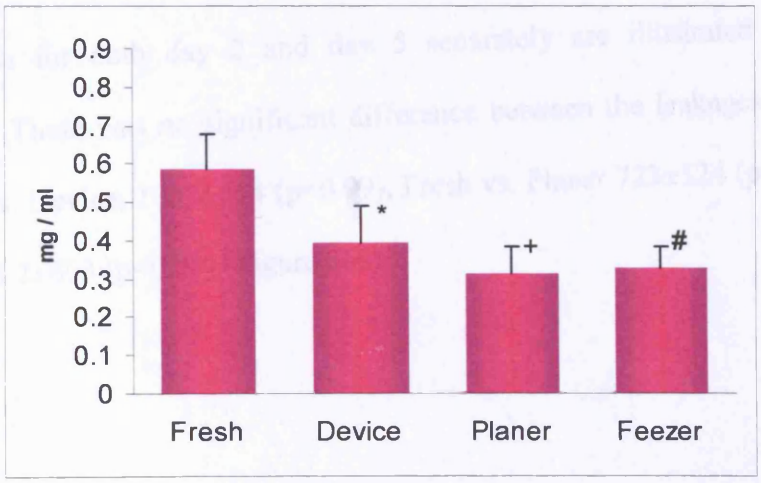



**Figure 35b Protein attachment at day 5 only between fresh cells and cryopreserved* p=0.0051, + p= 0.0004, # p=0.0007 when compared to the fresh value**

### 6.3.3 Assessment of Lactate Dehydrogenase (LDH) leakage

#### 6.3.3.1 Human hepatocytes

The lactate dehydrogenase leakage of the human hepatocytes in culture was undertaken as a measure of hepatocyte attachment (Section 4.3.4). The results express the mean LDH leakage after 2 and 5 days of culture (Figure 36). There was no significant difference between the leakage from cultured fresh cells or post cryopreservation.

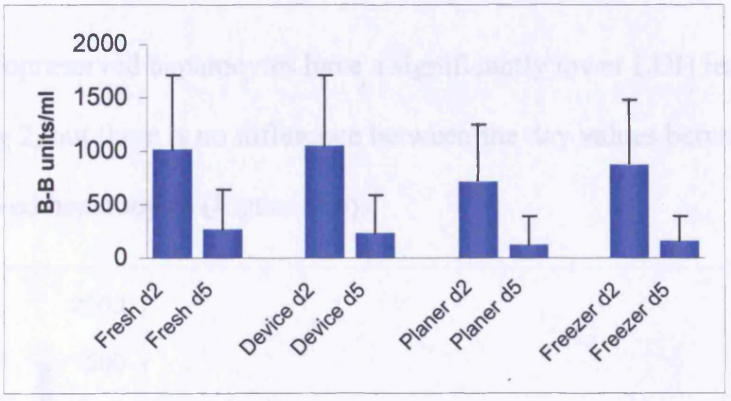



**Figure 36. LDH leakage of human hepatocyte cultures. Freshly cultured hepatocytes are illustrated with cultures post cryopreservation at 2 and 5 days (n=16).**

The leakages for both day 2 and day 5 separately are illustrated below to allow comparison. There was no significant difference between the leakages at day 2 (Fresh, 1017±696 vs. Device, 1052±664 (p=0.99), Fresh vs. Planer 723±524 (p= 0.63), Fresh vs. Freezer, 883.2±603 (p=0.95) (Figure 36a).

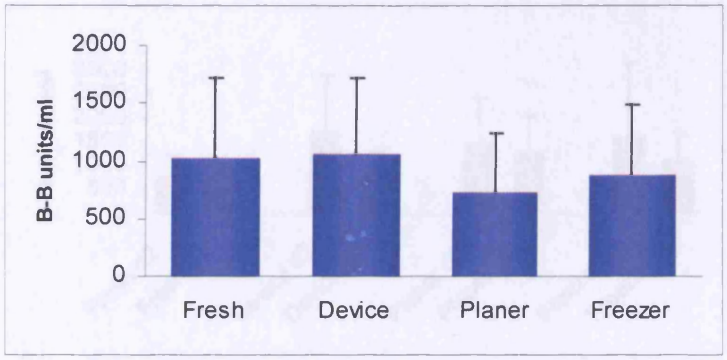



**Figure 36a LDH leakages at day 2, comparing fresh and post cryopreservation culture.**

At day 5 the cryopreserved hepatocytes have a significantly lower LDH leakage as compared to day 2, but there is no difference between the day values between fresh or post cryopreserved hepatocytes (Figure 36b).

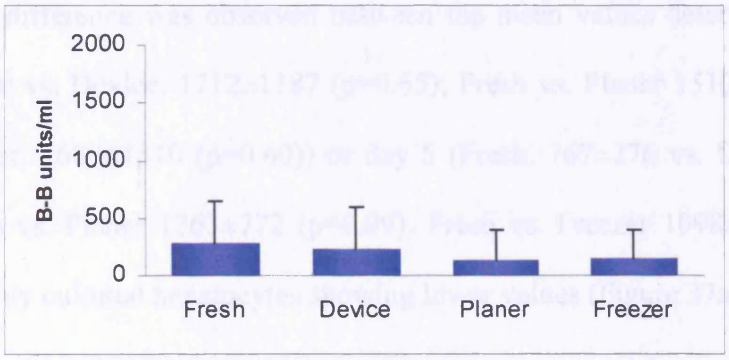



**Figure 36b. LDH leakages at day 5, comparing fresh and post cryopreservation culture.**

### 6.3.3.2 Porcine hepatocytes

The mean LDH leakage values observed for the freshly cultured porcine hepatocytes and post-cryopreserved cultures are illustrated in Figure 37. The cryopreserved cultures

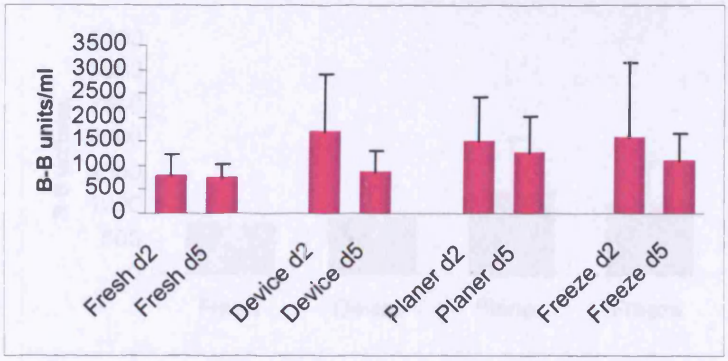



**Figure 37. LDH leakage of porcine hepatocyte cultures. Freshly cultured hepatocytes are illustrated with cultures post cryopreservation at 2 and 5 days (n=9).**

(2 and 5 days) show higher leakage values than those freshly cultured and represent the expected damage caused by cryopreservation.

No significant difference was observed between the mean values determined at day 2 (Fresh, 777±460 vs. Device, 1712±1187 (p=0.65); Fresh vs. Planer 1510±931 (p=0.40); Fresh vs. freezer, 1603±1540 (p=0.60)) or day 5 (Fresh, 767±276 vs. Device 874±454 (p=0.57); Fresh vs. Planer 1263±772 (p=0.99); Fresh vs. Freezer 1098±590 (p=0.82)); despite the freshly cultured hepatocytes showing lower values (Figure 37a and 37b).

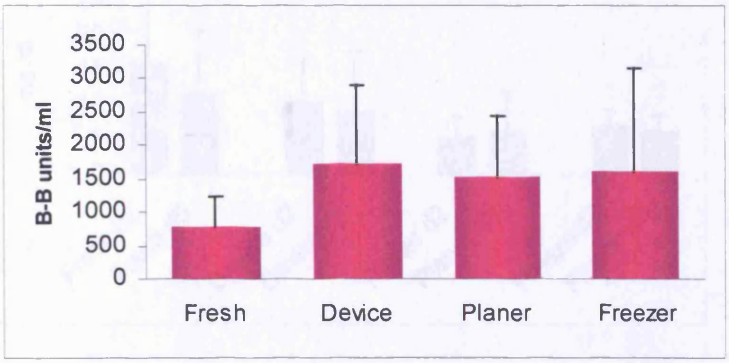



**Figure 37a. LDH leakages at day 2, comparing fresh and post cryopreservation culture.**

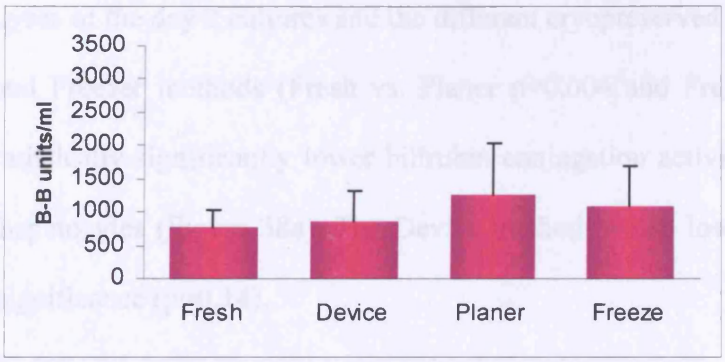



**Figure 37b. LDH leakages at day 5, comparing fresh and post cryopreservation culture.**

## 6.3.4 Assessment of Bilirubin conjugation

### 6.3.4.1 Human hepatocytes

Bilirubin conjugation studies were undertaken as described in section 4.3.1. The mean bilirubin conjugation values, expressed as mg/dl, for each day and method are illustrated in Figure 38. On preliminary investigation the results suggest that there was a higher functional activity in fresh hepatocyte cultures at day 2 (16.2±9.7) compared to the cryopreserved hepatocytes (Device 10.6±6.4; Planer 5.6±2.6; Freezer 7.1±2.2).

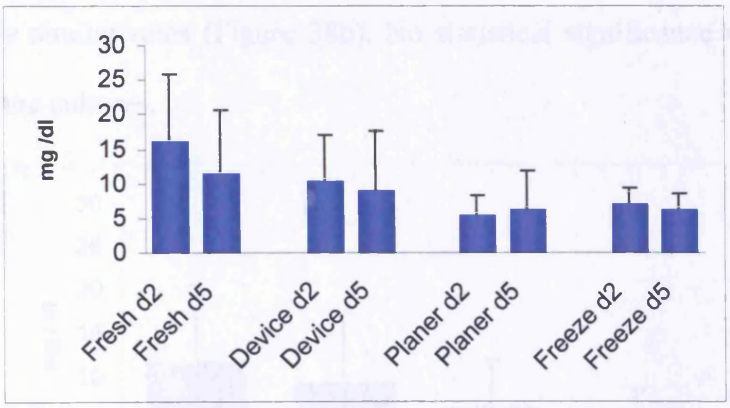



**Figure 38. Bilirubin conjugation of human hepatocytes for day 2 and 5 of fresh and post cryopreservation culture (n=16).**

Comparative analyses of the day 2 cultures and the different cryopreserved methods show that the Planer and Freezer methods (Fresh vs. Planer p=0.004 and Fresh vs. Freezer p=0.046) have statistically significantly lower bilirubin conjugation activity values than freshly cultured hepatocytes (Figure 38a). The Device method is also lower but is only tending towards significance (p=0.14).

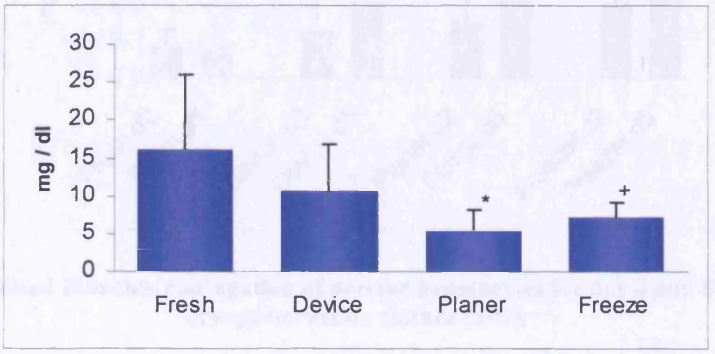



**Figure 38a Bilirubin conjugation at day 2. * p=0.004 when Planer is compared to Fresh. + p= 0.046 when freezer method is compared to fresh.**

Additionally, bilirubin conjugation activity at day 5 remains high in the freshly cultured hepatocytes. However the hepatocyte cultures derived from the Device method function at approximately similar rates (Figure 38b). No statistical significance was determined between any of the cultures.

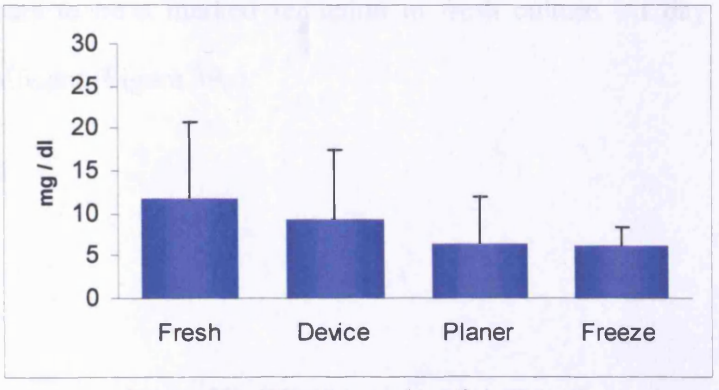



**Figure 38b. Bilirubin conjugation at day 5.**

### 6.3.4.2 Porcine hepatocytes

The bilirubin conjugation is normalised to the amount of attached protein and presented below (Figure 39). The freshly cultured cells have a lower mean bilirubin conjugation

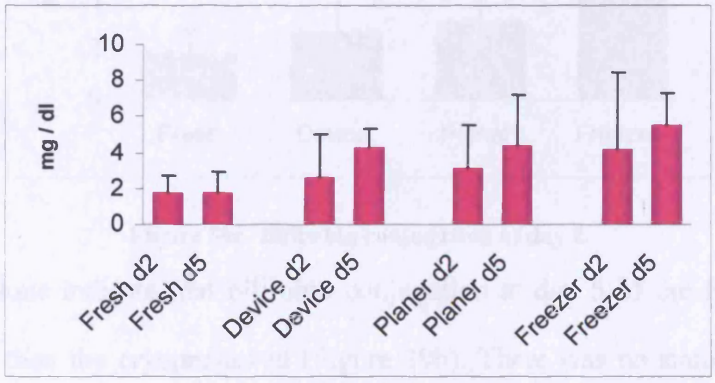



**Figure 39. Normalised Bilirubin conjugation of porcine hepatocytes for day 2 and 5 of fresh and post cryopreservation culture (n=9).**

ability (1.8±0.9) than the means for the cryopreserved cells (Device 2.6±2.4, Planer 3.1 ±2.4, Freezer 4.2±4.2). Unlike the human cells the ability to conjugate bilirubin increases as the cultures continue from day 2 to 5 in the cryopreserved hepatocytes but maintain about the same function in the fresh hepatocytes. This increase in function did not reach significance in any of the cryopreserved hepatocytes but tended towards significance in the Device cultures (day 2, 2.6±2.4 and day 5, 4.3±1.1 ($p=0.14$)). When considering each day, there appears to be a marked reduction in fresh culture. At day 2 this was not statistically significant (Figure 39a).

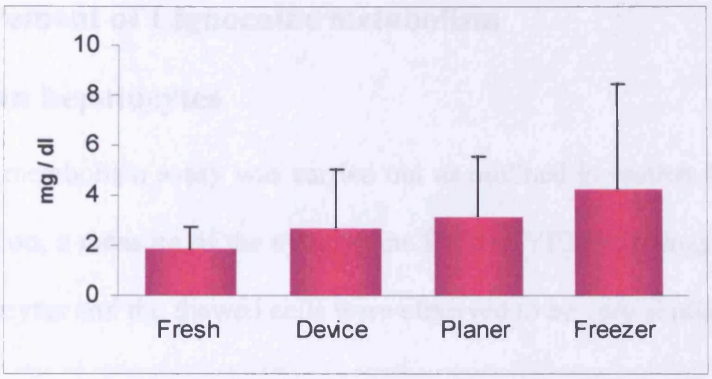



**Figure 39a. Bilirubin conjugation at day 2.**

Initial observations indicate that bilirubin conjugation at day 5 of the fresh hepatocyte cultures is less than the cryopreserved (Figure 39b). There was no statistical difference between the fresh hepatocytes (1.2±1.1) and the Device method (4.3±1.1) (p=0.28) or the Planer method (4.4±2.8) (p=0.27). However, the function of the hepatocytes cryopreserved using the Freezer method against the fresh was approaching significance (5.5±1.8) (p=0.09) in improved function.

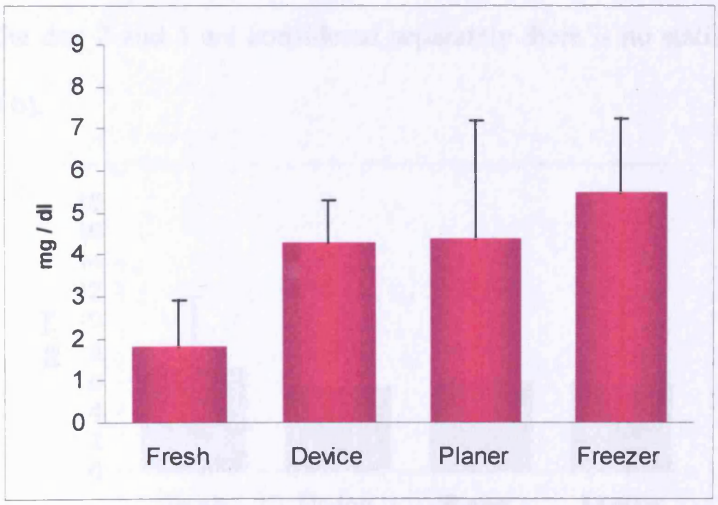



**Figure 39b. Bilirubin conjugation at day 5.**

### 6.3.5 Measurement of Lignocaine metabolism

#### 6.3.5.1 Human hepatocytes

The lignocaine metabolism assay was carried out as outlined in section 4.4.2. The mean MEGX production, a measure of the cytochrome P450 (CYP3A4 isoenzyme) activity for the fresh hepatocytes and the thawed cells were observed to be very similar (Figure 40).

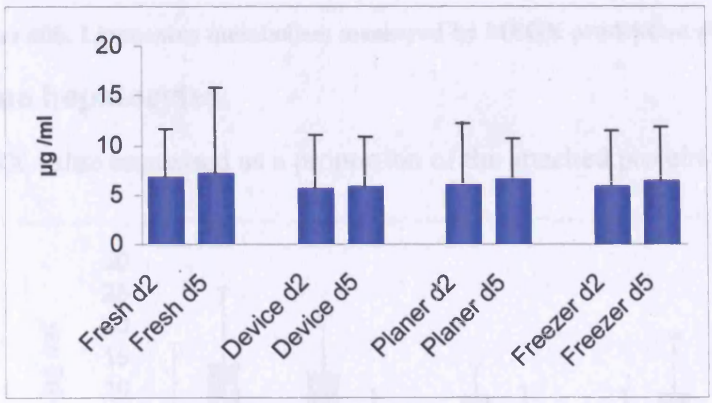



**Figure 40. Lignocaine metabolism measured by MEGX production for fresh and post cryopreservation human cultures at day 2 and 5 (n=16).**

When each of the day 2 and 5 are considered separately there is no statistical difference (Figure 40a and b).

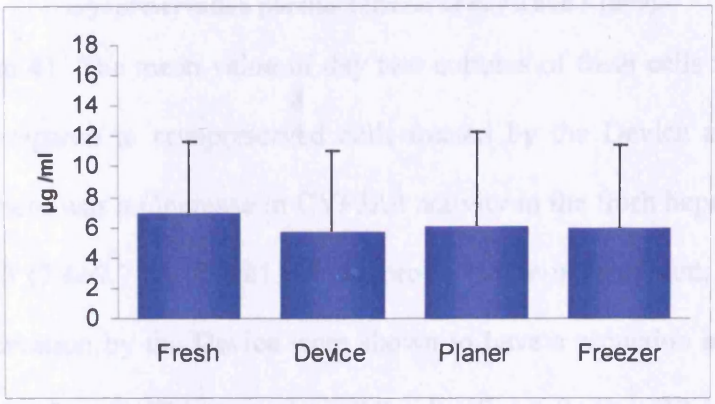



**Figure 40a. Lignocaine metabolism measured by MEGX production at day 2.**

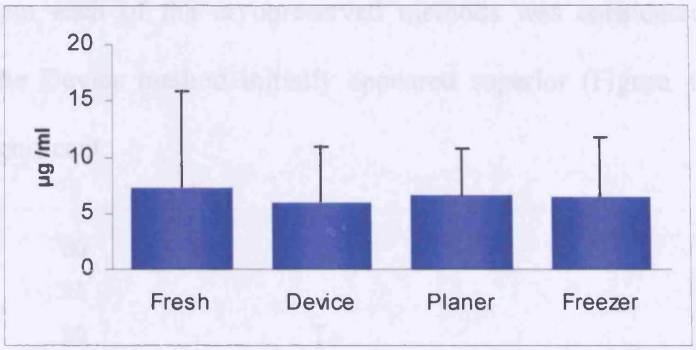



**Figure 40b. Lignocaine metabolism measured by MEGX production at day 5.**

### 6.3.5.2 Porcine hepatocytes

The mean MEGX value expressed as a proportion of the attached protein value is

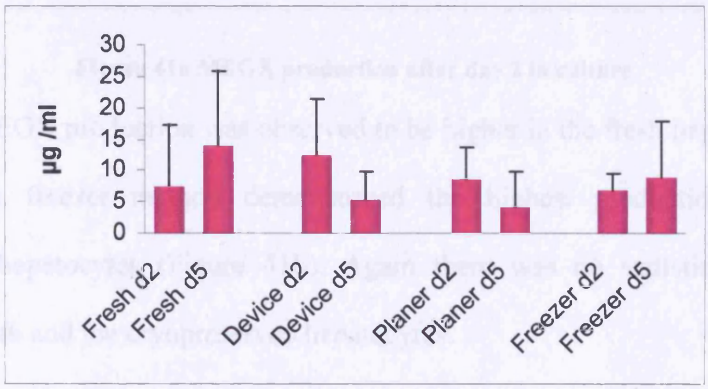



**Figure 41. Lignocaine metabolism measured by MEGX production for fresh and post cryopreservation porcine cultures at day 2 and 5 (n=9).**

shown in Figure 41. The mean value of day two cultures of fresh cells was found to be lower when compared to cryopreserved cells treated by the Device at day 2. It was observed that there was an increase in CYP3A4 activity in the fresh hepatocytes cultures from day 2 to 5 (7.4±9.7 vs. 13.9±11.9) but proved to be insignificant. Cells recovered from cryopreservation by the Device were shown to have a reduction in function when cultured from day 2 to 5. This proved a mildly significant finding (12.4±8.9 vs. 5.2±4.7 (p=0.08)). The Planer method also demonstrated a reduction in cellular function but proved not to be significant (8.7±4.8 vs. 4.1±5.6) (p=0.17). Comparative analysis of day

two cultures from each of the cryopreserved methods was considered in turn. This indicated that the Device method initially appeared superior (Figure 41a) but proved statistically insignificant.

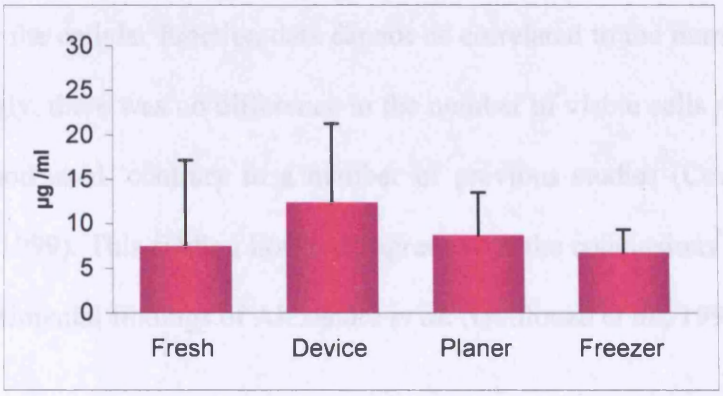



**Figure 41a MEGX production after day 2 in culture**

At day 5 the MEGX production was observed to be higher in the fresh hepatocytes, while cells from the freezer method demonstrated the highest production rate of the cryopreserved hepatocytes (Figure 41b). Again there was no statistical significance between the fresh and the cryopreserved hepatocytes.

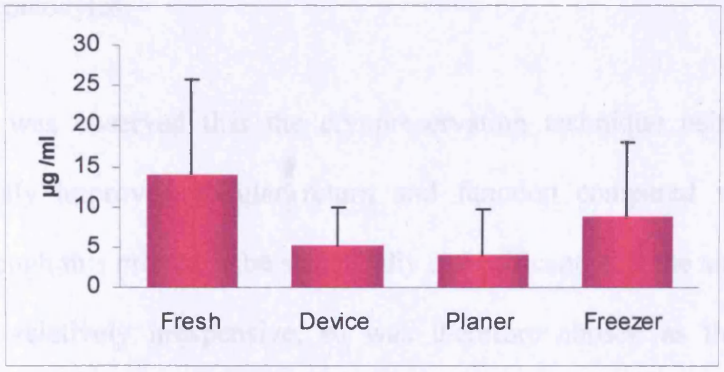



**Figure 41b MEGX production after day 5 in culture.**

## 6.4 Discussion

### 6.4.1 Human hepatocytes

Conclusions on the studies involving the cooling protocol on human hepatocytes are inconclusive, as the cellular function data cannot be correlated to the number of attached cells. Interestingly, there was no difference in the number of viable cells returned despite the freeze method used, contrary to a number of previous studies (Coundouris *et al.*, 1993; Li *et al.*, 1999). This finding however, agrees with the conclusions of Guillouzo *et al.* and the experimental findings of Alexandre *et al.* (Guillouzo *et al.*, 1999; Alexandre *et al.*, 2002).

Attachment measured by LDH leakage was difficult to interpret, as the values do not take into account how many cells were present. There was also no significant difference in the functional ability, as measured by bilirubin conjugation and lignocaine metabolism, with hepatocytes cryopreserved by the different methods used or between fresh and cryopreserved hepatocytes.

In summary, it was observed that the cryopreservation technique using the Device method marginally improved cellular return and function compared with the other procedures, although this proved to be statistically insignificant. It is the simplest method to perform and relatively inexpensive, so was therefore chosen as the method for subsequent studies.

### 6.4.2 Porcine hepatocytes

Like human hepatocytes there was statistically no difference in the return of porcine hepatocytes post -cryopreservation although there is a tendency for the Device to return a higher number of cells. There are generally a higher number of hepatocytes returned as a percentage of the number frozen in comparison too human. It has been previously published that an assessment of attachment of hepatocytes can be made by the estimation of protein in hepatocytes liberated from the culture plate (Loretz *et al.*1989; Lawrence and Benford, 1991; Alexandre *et al.* 2002, and personnel communication with Professor Richert, University of Besançon, France). The attachment data indicated that if similar numbers of viable cells were cultured, whether fresh or following cryopreservation, no statistical difference in the attachment after 2 days of culture was observed. After 5 days post cryopreservation hepatocyte cultures it was observed that there was a reduction in cellular attachment compared to freshly culture hepatocytes, irrespective of the cryopreserved method.

Analysis of the LDH leakage data indicates a higher level of leakage in cryopreserved cells, although this proved statistically insignificant. A higher initial value was expected, as this is the time period whereby the most damaged cells would be leaking. Between day 3 and 5 the leakage would be considerably lower as the hepatocytes left would be healthy and attached (Z. Chen *et al.* 2001, Lawrence and Benford, 1991)

MEGX production was lower at day 2 fresh than the comparative cryopreservation time points. This did not reach statistical significance. The finding that there was little difference between fresh and cryopreserved hepatocytes regarding P450 function has

previously been reported in the literature (Alexandre *et al.* 2002; McKay *et al.*, 2002; Chesne *et al.*, 1993; Diener *et al.*, 1993; Utesch *et al.*, 1992.). Although the full reason is yet to be elucidated, it has been proposed that it may represent de novo cytochrome P450 synthesis as part of a repair mechanism (Watt and Grant, 1996).

In summary porcine hepatocytes can be cryopreserved and despite a loss of cells over time they function as well as fresh hepatocytes. There is no statistical difference between any of the methods. Again the device was the easiest method of the three to perform and was therefore chosen, in the absence of any functional deterioration, for all subsequent studies.

## 6.5 Summary conclusions

- The study did not confirm that a controlled step-wise freeze rate was superior for both human and porcine hepatocytes.
- Both human and porcine hepatocytes function as measured by bilirubin, lignocaine metabolism, LDH leakage was not significantly impaired by cryopreservation, within the limits of the study.
- The simple cyopreservation procedure using the 'Device Method' had a tendency to return a higher number of viable cells although not statistically significant.
- Perhaps more isolations would have demonstrated a difference.

# Chapter 7

# Effect of cell concentration on human and porcine hepatocyte viability and function using the Nalgene propan-2-ol method of cryopreservation

7.1 Introduction

7.2 Method

7.3 Results

- 7.3.1 Assessment of cellular viability post cryopreservation
  - 7.3.1.1 Human hepatocytes
  - 7.3.1.2 Porcine hepatocytes
- 7.3.2 Attachment of hepatocyte cryopreservation post cryopreservation
  - 7.3.2.1 Human hepatocytes
    - 7.3.2.1 Porcine hepatocytes
- 7.3.3 Assessment of lactate dehydrogenase (LDH) leakage
  - 7.3.3.1 Human hepatocytes
  - 7.3.3.2 Porcine hepatocytes
- 7.3.4 Assessment of bilirubin conjugation
  - 7.3.4.1 Human hepatocytes
  - 7.3.4.2 Porcine hepatocytes
- 7.3.5 Measurement of lignocaine metabolism
  - 7.3.5.1 Human hepatocytes
  - 7.3.5.2 Porcine hepatocytes

7.4 Conclusion

- 7.4.1. Human hepatocytes
- 7.4.2. Porcine hepatocytes

7.5 Summary Conclusions

## 7.1 Introduction

The aim of this study was to determine the optimal concentration of hepatocytes within the cryopreservation solution during the freeze process. Following the findings of the previous chapter the cryopreservation was carried out using the Nalgene propan-2-ol device.

## 7.2 Method

Hepatocytes were isolated and cultured by the methods previously described for humans and for porcine livers (Section 4.2.1.and 4.2.2.). A range of cell concentrations ($2.5x10^6$, $5x10^6$, $1x10^7$ and $2x10^7$) cells/ml of solution were evaluated. The content of each cryopreservation solutions is outlined in Table 17. The cryovials were placed into the Nalgene device and placed into a –80°C freezer for 4 hours before being transferred into liquid nitrogen storage.

After 7-10 days of storage in liquid nitrogen the hepatocytes were removed, thawed, washed and cultured as previously outlined for cell viability and assessment of hepatocyte function (Section 4.3.1).

| | $2.5x10^6$ | $5x10^6$ | $1x10^7$ | $2x10^7$ |
|---|---|---|---|---|
| DMEM | 28 ml | 14 ml | 7 ml | 3.5 ml |
| FCS | 8 ml | 4 ml | 2 ml | 1 ml |
| DMSO | 4 ml | 2 ml | 1 ml | 0.5 ml |

**Table 17. Composition of cryopreservation solutions for assessment of effect of cell concentration on survival and function of hepatocytes post-cryopreservation.**

# 7.3 Results

## 7.3.1 Assessment of cellular viability post cryopreservation

### 7.3.1.1 Human hepatocytes

This study utilised hepatocytes isolated from 16 separate donations (mean initial viability 85.3±6.6%). The mean percentage of viable cells returned is tabulated in Figure 49. The highest mean return and hence optimal concentration of hepatocytes through the cryopreservation process was in the $5x10^6$ group. The only significant cellular return was observed from studies of the $5x10^6$ sample (35.2±14.8%), which was statistically significant compared with the $2x10^7$ sample (15.5±11.1%) ($p=0.01$). Comparative analysis of the $2.5x10^6$ (23.3±3.2) and $5x10^6$ sample concentrations approached statistical significant ($p=0.22$).

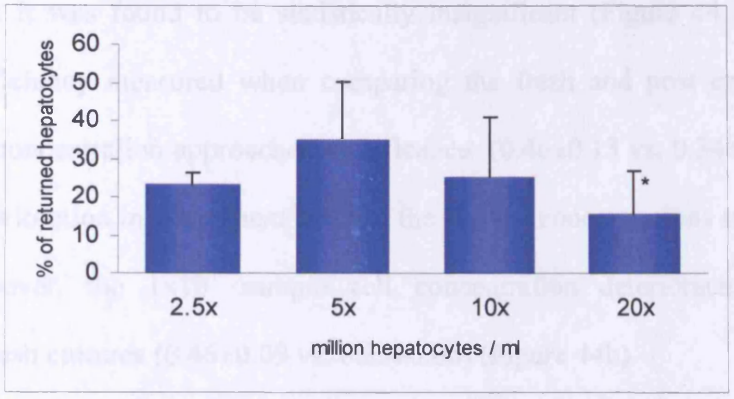



**Figure 42 The percentage of returned viable human hepatocytes following cryopreservation at different concentrations (n= 16).*p=0.01 compared with $5x10^6$ concentration.**

### 7.3.1.2 Porcine hepatocytes

The viability of the isolated cells was 79±14%. The hepatocytes return was consistent across all concentrations but no difference was found statistically (Figure 43).

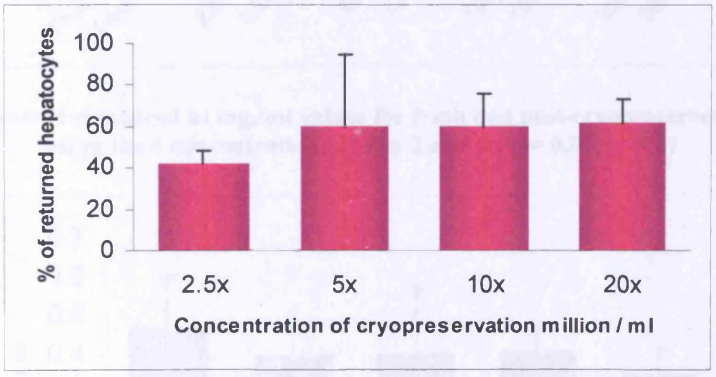



**Figure 43 The percentage of returned viable porcine hepatocytes following cryopreservation at different concentrations (n=6)**

## 7.3.2 Assessment of hepatocyte attachment post cryopreservation

### 7.3.2.1 Human hepatocytes

The attachment of fresh hepatocytes was found to be more efficient than that observed for post cryopreserved thawed hepatocyte cultures. This could not be shown in Day 2

cultures where it was found to be statistically insignificant (Figure 44 and 44a). The attachment efficiency measured when comparing the fresh and post cryopreservation $2x10^7$ sample concentration approached significance (0.46±0.13 vs. 0.34±0.06, p=0.21). There was deterioration in attachment in all of the thawed concentrations studied in day 5 cultures. However, the $1x10^7$ sample cell concentration deteriorated significantly compared to fresh cultures (0.46±0.09 vs. 0.33±0.06) (Figure 44b)

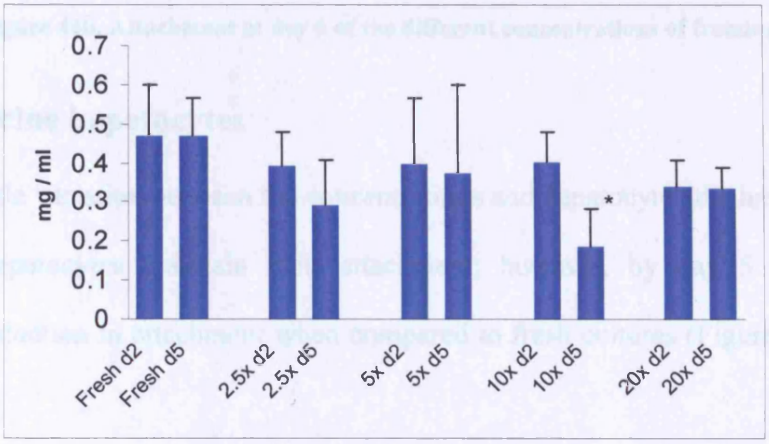



**Figure 44 Attachments measured as mg./ml values for fresh and post-cryopreserved human cultures using the 4 concentrations at day 2 and 5 (*p= 0.01; n =16)**

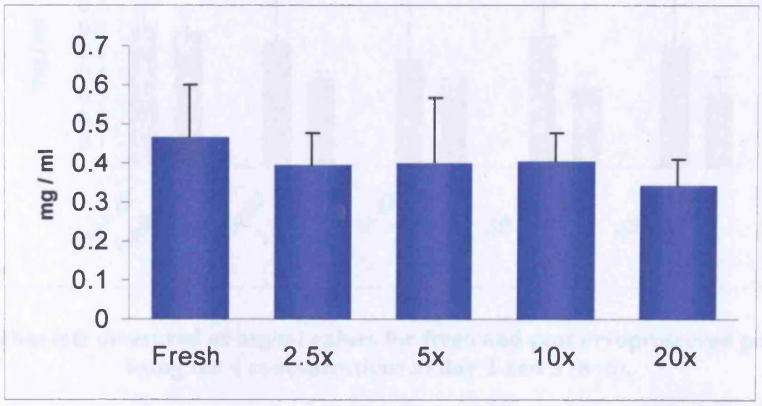



**Figure 44a Attachment at day 2 of the different concentrations of freezing**

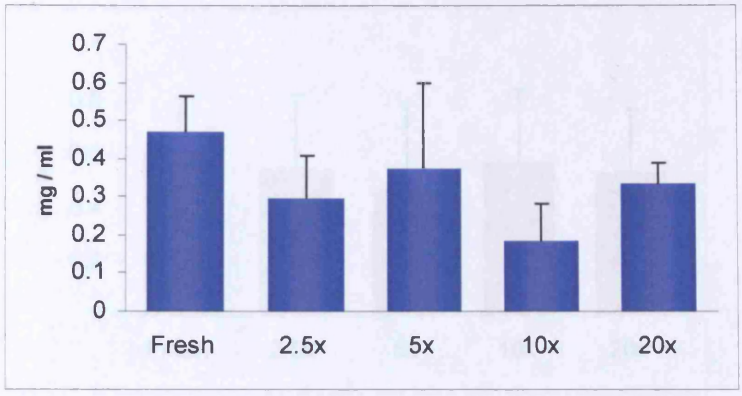



**Figure 44b. Attachment at day 5 of the different concentrations of freezing.**

### 7.3.2.2 Porcine hepatocytes

There was little variation between the concentrations and hepatocyte attachment at day 2. The fresh hepatocytes maintain their attachment; however, by day 5 there was a significant reduction in attachment when compared to fresh cultures (Figure 45, 45a and b).

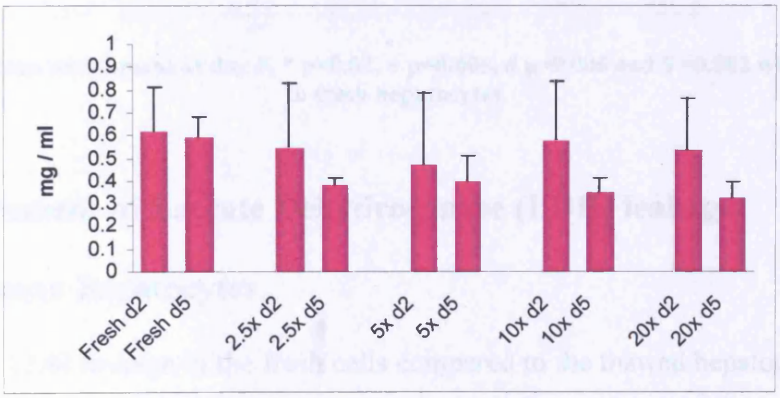



**Figure 45 Attachments measured as mg/ml values for fresh and post cryopreserved porcine cultures using the 4 concentrations at day 2 and 5 (n=6).**

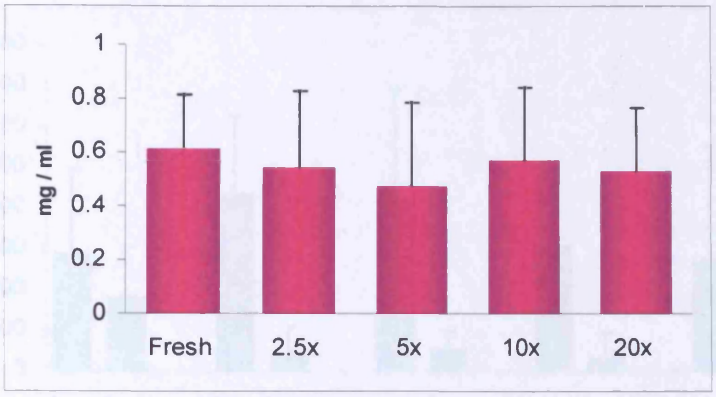



**Figure 45a Mean attachments at day 2**

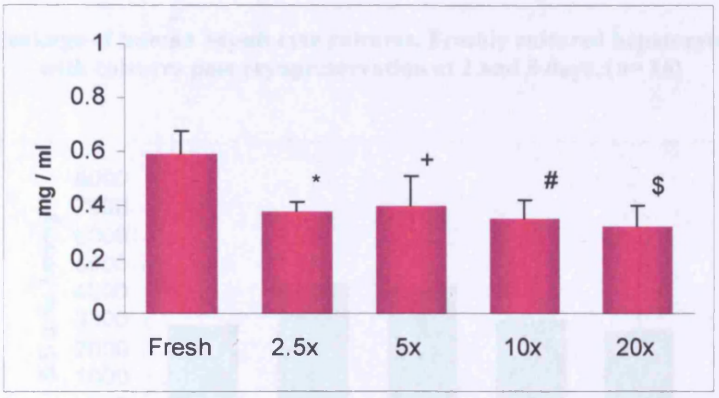



**Figure 45b Mean attachment at day 5, * p=0.02, + p=0.008, # p=0.005 and $ =0.002 when compared to fresh hepatocytes.**

## 7.3.3 Assessment of Lactate Dehydrogenase (LDH) leakage

### 7.3.3.1 Human hepatocytes

There is less LDH leakage in the fresh cells compared to the thawed hepatocytes. By day 5 there was a significant reduction in LDH leakage from the post-cryopreserved hepatocytes but not in the fresh cultures. There was no statistical difference between the LDH leakage from the thawed cells and from any of the concentrations frozen (Figure 46, 46a and b).

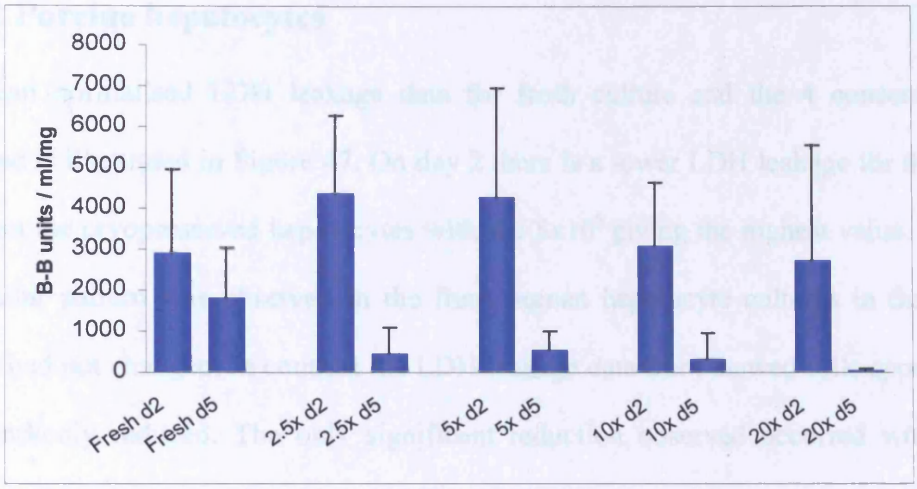



**Figure 46. LDH leakage of human hepatocyte cultures. Freshly cultured hepatocytes are illustrated with cultures post cryopreservation at 2 and 5 days. (n= 16)**

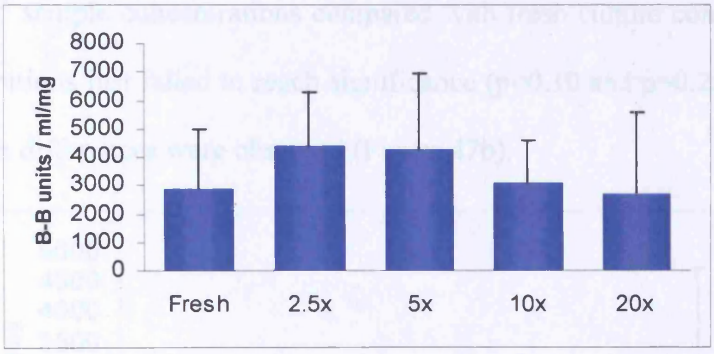



**Figure 46a Difference in LDH leakage between the concentrations at day 2**

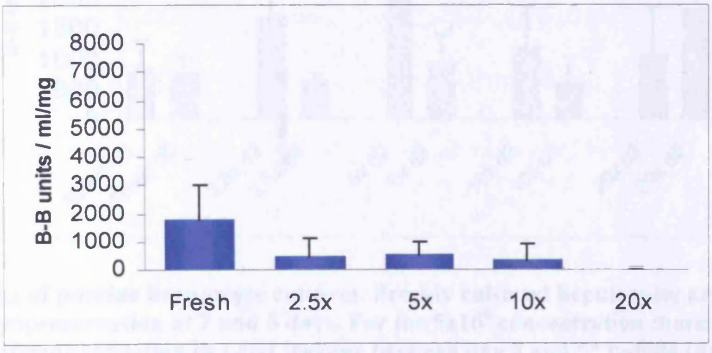



**Figure 46b Difference in LDH leakage between the concentrations at day 5**

### 7.3.3.2 Porcine hepatocytes

The mean normalised LDH leakage data for fresh culture and the 4 concentrations examined is illustrated in Figure 47. On day 2 there is a lower LDH leakage for the fresh cells than the cryopreserved hepatocytes with the $5x10^6$ giving the highest value. By day 5 a similar pattern was observed in the fresh human hepatocyte cultures in that LDH leakage had not changed. In contrast the LDH leakage data from thawed cells appeared to have markedly reduced. The only significant reduction observed occurred within the $5x10^6$ ($p=0.04$) sample cell concentration studies. Analysis of the day 2 and 5 culture LDH leakage values was shown to be statistically insignificant. At day 2 the observed values for $5x10^6$ sample concentrations compared with fresh culture controls and $2x10^7$ sample concentrations just failed to reach significance ($p=0.10$ and $p=0.25$) (Figure 47a). At day 5 no such differences were observed (Figure 47b).

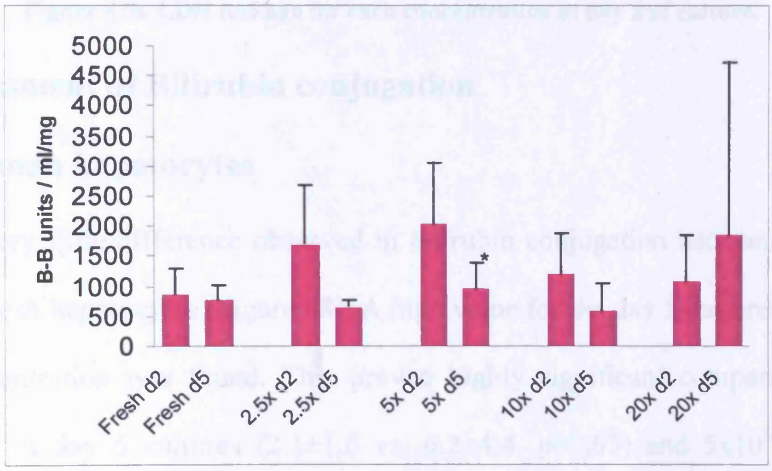



**Figure 47. Leakage of porcine hepatocyte cultures. Freshly cultured hepatocytes are illustrated with cultures post cryopreservation at 2 and 5 days. For the $5x10^6$ concentration there is a statistically significant reduction in LDH leakage between day 2 and 5* p=0.04 (n=6).**

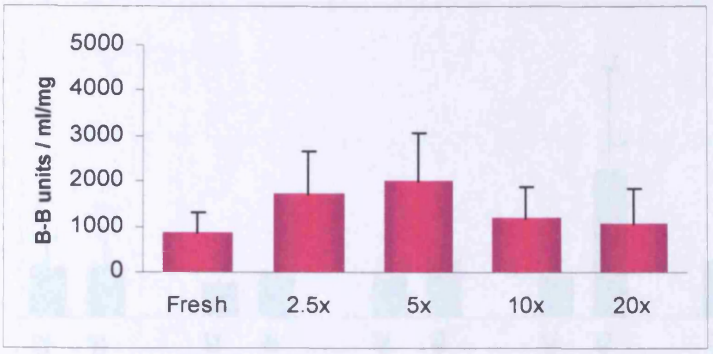



**Figure 47a. LDH leakage for each concentration at day 2 of culture.**

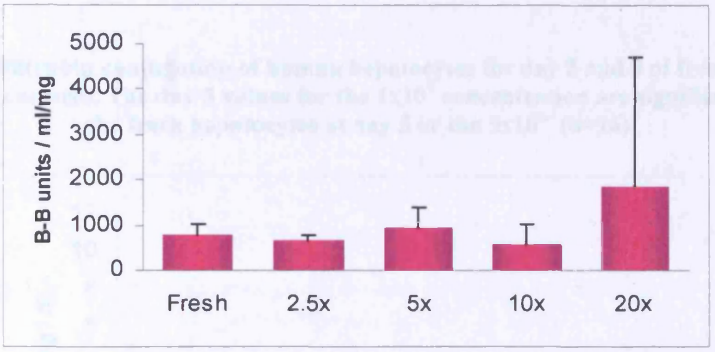



**Figure 47b. LDH leakage for each concentration at day 5 of culture.**

## 7.3.4 Assessment of Bilirubin conjugation

### 7.3.4.1 Human hepatocytes

There was very little difference observed in bilirubin conjugation between day 2 and 5 cultures of fresh hepatocytes (Figure 48). A high value for the day 5 cultures of the $1x10^7$ sample concentration was found. This proved highly significant compared with fresh control cells in day 5 cultures (2.1±1.6 vs. 6.2±4.4, p=0.03) and $5x10^6$ (6.2±4.4 vs. 2.4±2.0) (Figure 48a and 48b)

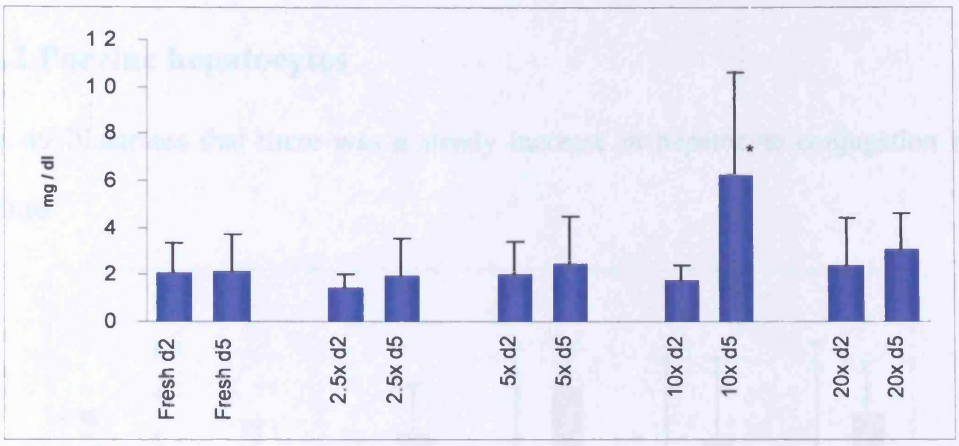



**Figure 48 Bilirubin conjugation of human hepatocytes for day 2 and 5 of fresh and post cryopreservation cultures. The day 5 values for the $1x10^7$ concentration are significantly higher than the fresh hepatocytes at day 5 or the $5x10^{6.}$ (n=16)**

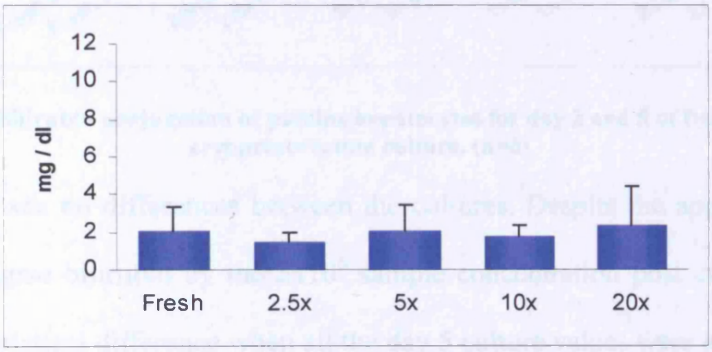



**Figure 48a. Bilirubin conjugation at day 2.**

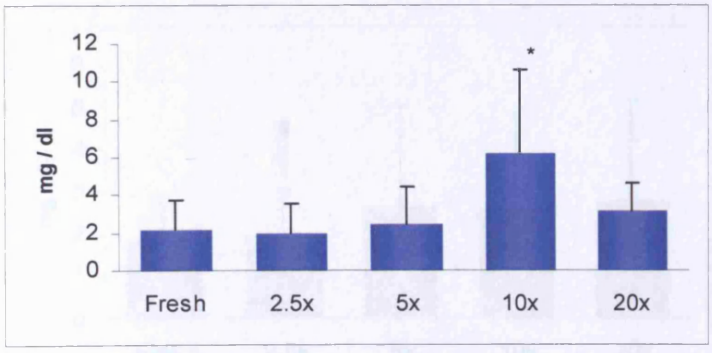



**Figure 48b. Bilirubin conjugation at day 5.**

### 7.3.4.2 Porcine hepatocytes

Figure 49 illustrates that there was a steady increase in hepatocyte conjugation ability with time.

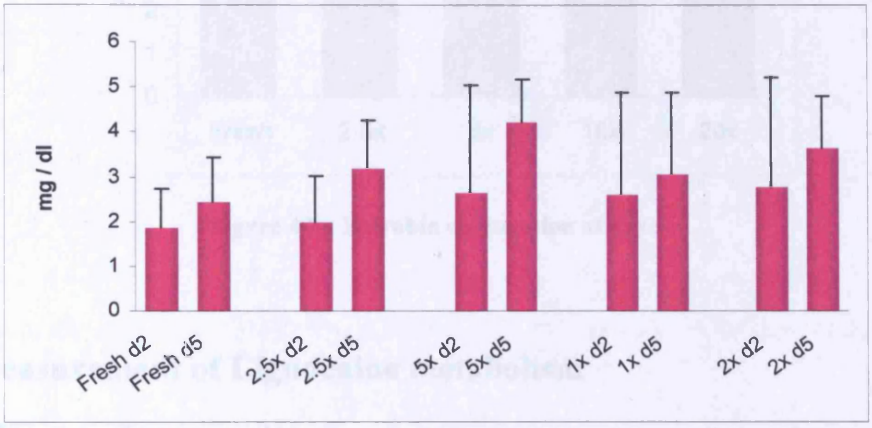



**Figure 49. Bilirubin conjugation of porcine hepatocytes for day 2 and 5 of fresh and post cryopreservation culture. (n=6)**

At day 2 there was no differences between the cultures. Despite the apparent increased ability to conjugate bilirubin by the $5x10^6$ sample concentration post cryopreservation, there was no statistical difference when all the day 5 culture values were analysed (Figure 49 a and b).

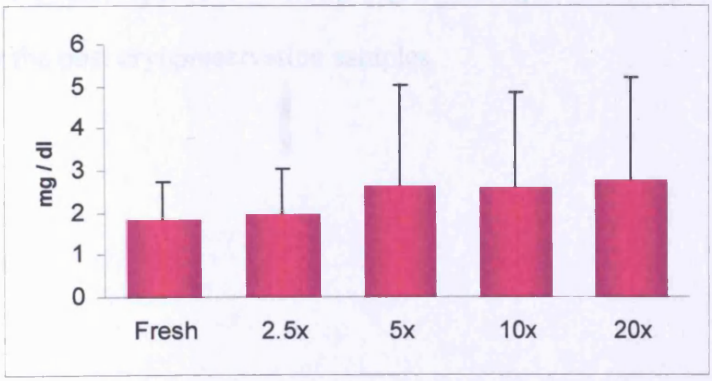



**Figure 49a. Bilirubin conjugation at day 2**

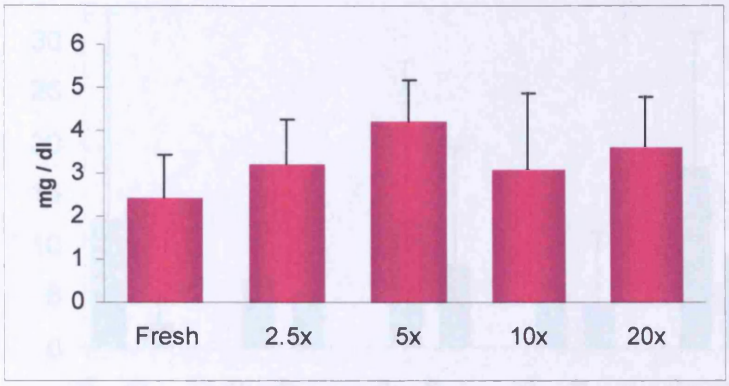



**Figure 49b. Bilirubin conjugation at day 5.**

## 7.3.5 Measurement of Lignocaine metabolism

### 7.3.5.1 Human hepatocytes

Lignocaine metabolism proved highly variable across the 4 concentrations studied with the $2x10^7$ sample producing the most MEGX at day 2 and 5 cultures (Figure 50). Despite the variation there was no statistical significance observed between any of the peaks (Figure 50a and b). The reduction of function from day 2 to day 5 in the fresh sample (12.5±20.0 vs. 2.3±2.8) approaches being significant (p=0.2) but proved statistically insignificant for the post cryopreservation samples.

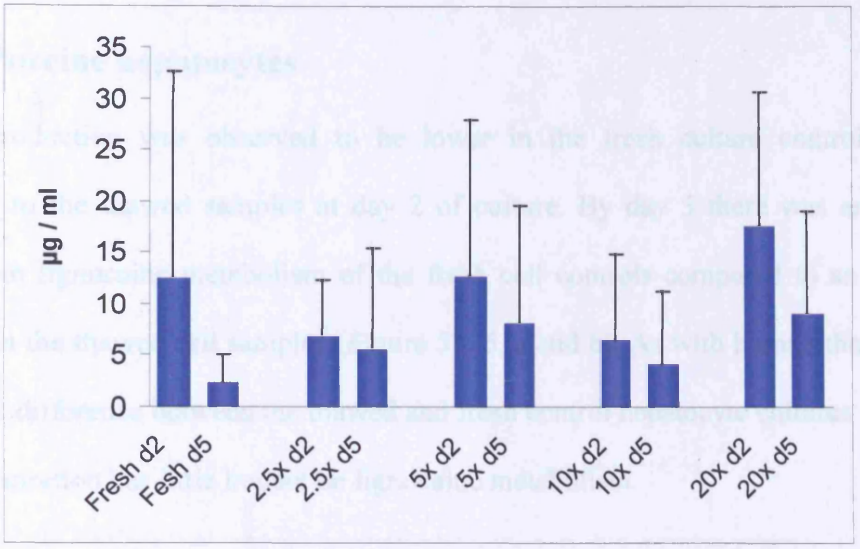



**Figure 50. Lignocaine metabolism measured by MEGX production for fresh and post cryopreservation human cultures at day 2 and 5 (n=16).**

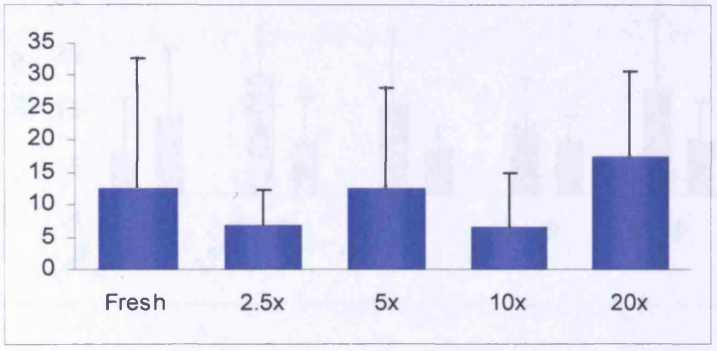



**Figure 50a.. Effect of concentration on lignocaine metabolism at day 2.**

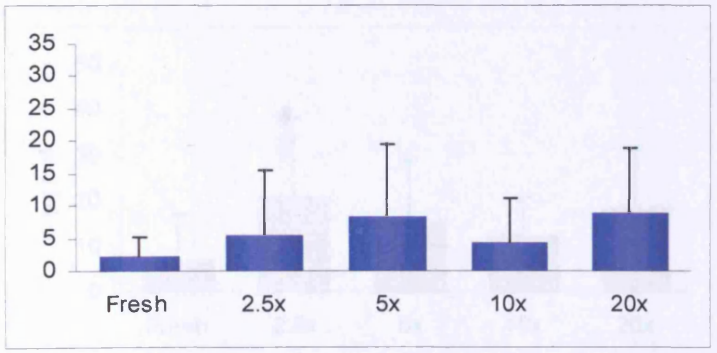



**Figure 50b. Effect of concentration on lignocaine metabolism at day 5.**

### 7.3.5.2 Porcine hepatocytes

MEGX production was observed to be lower in the fresh culture control samples compared to the thawed samples at day 2 of culture. By day 5 there was an increase observed in lignocaine metabolism of the fresh cell controls compared to an observed decrease in the thawed cell samples (Figure 51, 51a and b). As with human there was no significant difference between the thawed and fresh control hepatocyte cultures indicating that concentration has little impact on lignocaine metabolism.

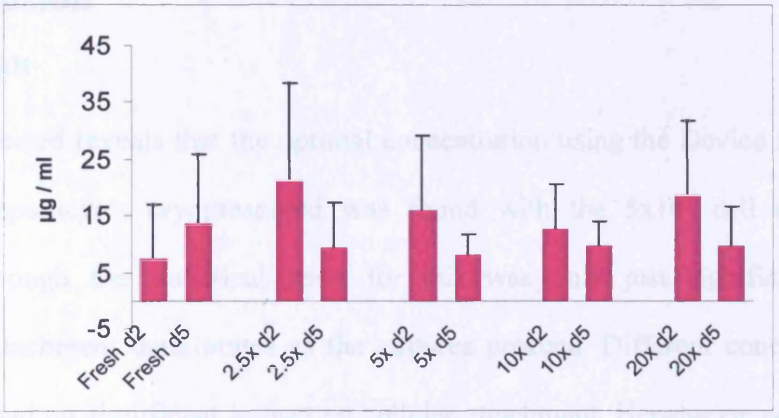



**Figure 51. Lignocaine metabolism measured by MEGX production for fresh and post cryopreservation porcine cultures at day 2 and 5 (n=6).**

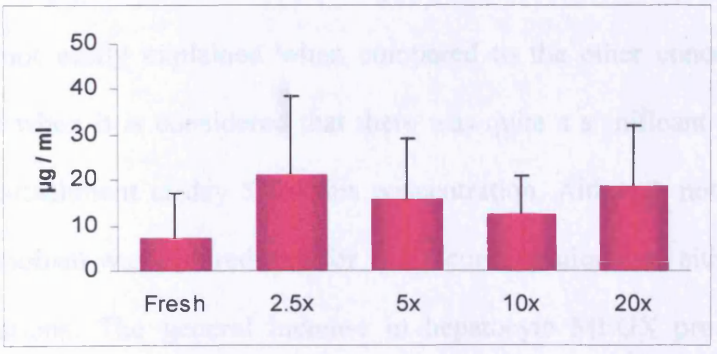



**Figure 51a. Effect of concentration on lignocaine metabolism at day 2.**

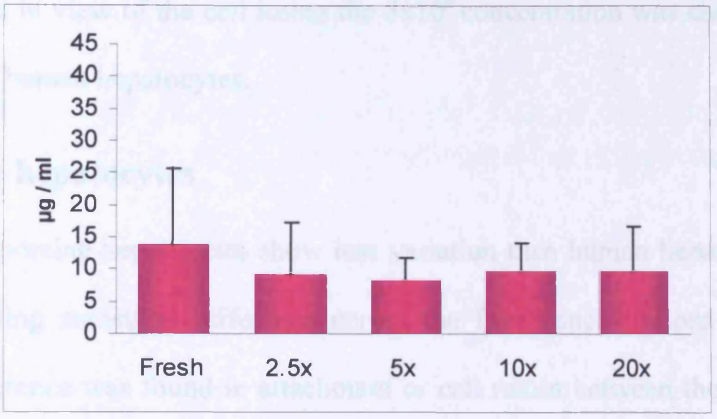



**Figure 51b. Effect of concentration on lignocaine metabolism at day 5.**

## 7.4 Conclusion

### 7.4.1 Human

The data collected reveals that the optimal concentration using the Device for the return of human hepatocytes cryopreserved was found with the $5x10^6$ cell concentration samples, although the statistical basis for this was only just significant. Thawed hepatocyte attachment deteriorates as the cultures proceed. Different concentrations of hepatocytes had no significant impact on cellular attachment. Hepatocyte concentrations were found to have no impact on cellular function in post cryopreservation cultures. The particularly high day 5 values for bilirubin conjugation in the $1x10^7$ is possibly a spurious result, as it is not easily explained when compared to the other concentrations. It is particularly odd when it is considered that there was quite a significant deterioration in the hepatocyte attachment at day 5 for this concentration. Although not significant the lignocaine metabolism was also reduced for $1x10^7$ concentration than either the $5x10^6$ or $2x10^7$ concentrations. The general increase in hepatocyte MEGX production despite cryopreservation was not statistically significant, but is in agreement with published literature (discussed in previous chapter). The functional data does not reach statistical

significance and in view of the cell losses the $5x10^6$ concentration was chosen for further experiments on human hepatocytes.

### 7.4.2 Porcine hepatocytes

Cryopreserved porcine hepatocytes show less variation than human hepatocytes with no parameter meeting statistical difference across the four concentrations examined. No significant difference was found in attachment or cell return between the concentrations studied. Some concentrations demonstrated higher mean results for the functional parameters measured but these did not reach statistical significance.

The optimal concentration is difficult to conclude on the basis of this study. The data did not reveal an optimal concentration, however the $5x10^6$ concentration was chosen for the remaining experiments. This was partly to avoid confusion and potential error by using the same concentration as the human experiments that were being performed in parallel.

## 7.5 Summary conclusions

- The optimum cell concentration of $5x10^6$ human hepatocytes / ml was found to return statistically significant level of viable cells post cryopreservation.
- Human and Porcine hepatocyte concentration was found to have no impact on cellular function in post cryopreservation cultures.
- For consistency in all subsequent comparative hepatocyte studies human and porcine cryopreservation was undertaken at a concentration of $5x10^6$ hepatocytes/ml.

# Chapter 8

## The effect of pre-incubation on human and porcine hepatocytes cultured before and after cryopreservation

8.1 Background

8.2 Method

8.3 Results

8.3.1 Assessment of cellular viability post cryopreservation
- 8.3.1.1 Human hepatocytes
- 8.3.1.2 Porcine hepatocytes

8.3.2 Assessment of hepatocyte attachment post cryopreservation
- 8.3.2.1 Human hepatocytes
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8.3.3 Assessment of lactate dehydrogenase (LDH)leakage
- 8.3.3.1 Human hepatocytes
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8.3.5 Measurement of lignocaine metabolism
- 8.3.5.1 Human hepatocytes
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8.4 Conclusion

8.4.1 Human hepatocytes

8.4.2 Porcine hepatocytes

8.5 Summary Conclusion

## 8.1 Introduction

Recent publications have demonstrated that immediate pre-incubation of freshly isolated hepatocytes in tissue culture growth medium, improves functional recovery and the ability to withstand cryopreservation (Darr and Hubel, 2001; Silva *et al.*, 1999; Chen *et al.*, 2001; Alexandre *et al.*, 2002). The methods for performing this pre-incubation step are varied. Darr and Hubel demonstrated that pre-incubation showed improved albumin production when performed for up to 24 hours prior to culture. Others have pre-incubated for 30 minutes (Silva *et al.*, 1999; Alexandre *et al.*, 2002). As the data are limited and inconclusive with no consensus as to the optimal method, a protocol was required for our laboratory. The equipment that was chosen to enable pre-incubation was a cell spinner (Cellspin, Integra Biosciences) as this was already available in the laboratory.

The times selected were based upon practical and logistic considerations together with information from a review of the literature. A short time of 1 hour was chosen which fits with the practical arrangements following the isolation, and other experiments being undertaken. The majority of isolations took place in the evening and late at night due to the time of acquisition. It was therefore decided to select a time that would allow the experiment to continue during working hours to minimise human error from logistical difficulties. Sixteen hours of pre-incubation facilitated this and was therefore the preferred time period.

Previous experiments reported in the literature utilised different speeds of spinning and a variety of equipment (Darr and Hubel, 2001; Silva *et al.*, 1999; Chen *et al.*, 2001; Alexandre *et al.*, 2002). Preliminary studies were undertaken in house to determine an

appropriate spinning speed (not reported). The speed chosen, which did not allow the hepatocytes to sediment was 30 rpm.

Following these steps the effect of pre incubation for 1 hour and 16 hours on cells cultured before cryopreservation and following cryopreservation was evaluated.

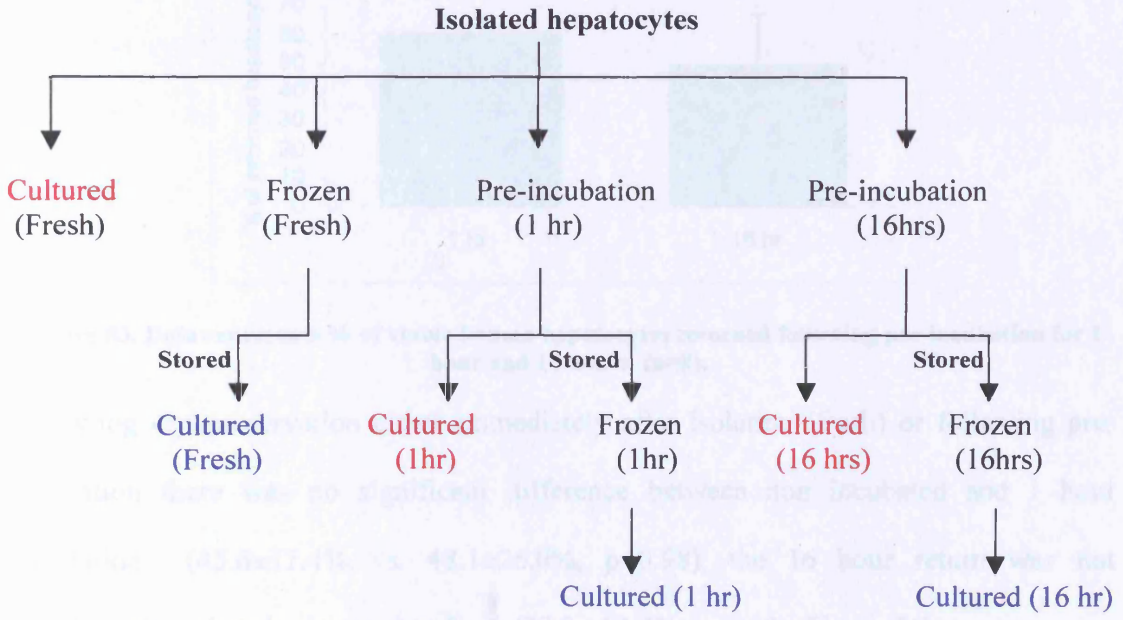
## 8.2 Method

After hepatocyte isolation, four stock preparations were prepared, 2 containing a total cell count of $1.5x10^8$ hepatocytes, one containing $1x10^8$ hepatocytes and 1 containing $3x10^7$ hepatocytes. Each tube was washed twice in 50ml of DMEM supplemented with 4.5g/l glucose (Life Technologies, Paisley, Scotland. cat. no. 42430-027) and centrifuged at 100g for 5 minutes, to form a pellet of cells.

For the tubes containing $1.5x10^8$ hepatocytes, the supernatant was pipetted and the cell pellet resuspended in 10ml of culture medium (Hepatozyme for human and Koebe culture media for porcine, section 4.3) and then pipetted into 80ml of pre-warmed Hepatozyme within the spinner flask in a 37°C incubator (5% $CO_2$). A further 10ml of culture media was used to wash the tube to ensure suspension of all cells. The spinner flask (Cellspin, Integra Biosciences) was set to rotate at 30rpm and left for 1 hour and 16 hours respectively. After incubation the cells were transferred to a 150ml tube, centrifuged at 100g and 4°C, for 5 minutes, to pellet the cells. Following trypan blue exclusion cell count, $2.5x10^7$ viable hepatocytes were cultured as outlined in section 4.3. The remainder of the viable cells were cryopreserved in solution at a concentration of $5x10^6$ cells /ml in the Nalgene propan-2-ol device as outlined in section 4.5.2.1. and 7.2. After 7-10 days of

storage in liquid nitrogen the hepatocytes were removed, thawed, washed and cultured as previously outlined (section 4.3 and 4.4) for cell viability and functional studies.

The remaining original stock preparations ($1.0x10^8$ and $3x10^7$ hepatocyte tubes) were either cryopreserved or cultured. The $1.0x10^8$ hepatocyte pellet, following washing was cryopreserved in the Nalgene propan-2-ol device to act as a control (Section 4.5.2.1). The $3x10^7$ pellet was cultured on three 12 well plates, to act as a fresh control (Section 4.3). Figure 9.1 illustrates a flow diagram of the experimental protocol




Key: Red = control cells, Blue = Cryopreserved cells

**Figure 52 Flow diagram of pre-incubation experiment for both human and porcine hepatocytes**

## 8.3 Results

### 8.3.1 Assessment of cellular viability post cryopreservation

#### 8.3.1.1 Human hepatocytes

Following pre-incubation in the spinner flasks there was a reduction in the return of viable cells. Incubating for 1 hour will reduce the returned viable hepatocytes to 61±6% and for 16 hours gives a return of 50±18% of the number of viable cells originally added (Figure 53).

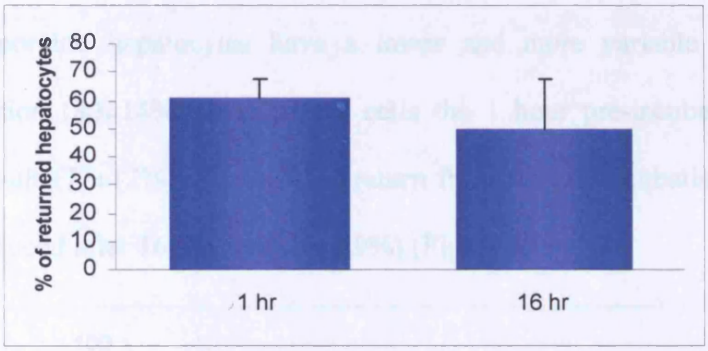



**Figure 53. Data represents % of viable human hepatocytes returned following pre-incubation for 1 hour and 16 hours. (n=8).**

Following cryopreservation either immediately after isolation (fresh) or following pre-incubation there was no significant difference between non incubated and 1 hour incubation (45.6±17.4% vs. 48.1±26.0%, p=0.98), the 16 hour return was not significantly reduced compared to fresh (30.3 ±14.3%, p=0.23) (Figure 54).

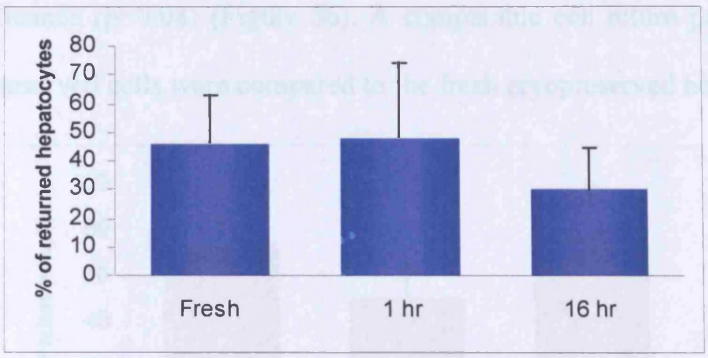



**Figure 54. Percentage of returned viable human hepatocytes following cryopreservation. Data illustrates the effect of pre-incubation on the return of viable hepatocytes**

### 8.3.1.2 Porcine hepatocytes

Although the porcine hepatocytes have a lower and more variable mean viability following isolation (80±14%) than human cells the 1 hour pre-incubation returned a higher mean result (77±12%). The cellular return from the pre incubation was however significantly reduced after 16 hours (17.9±8.9%) (Figure 55).

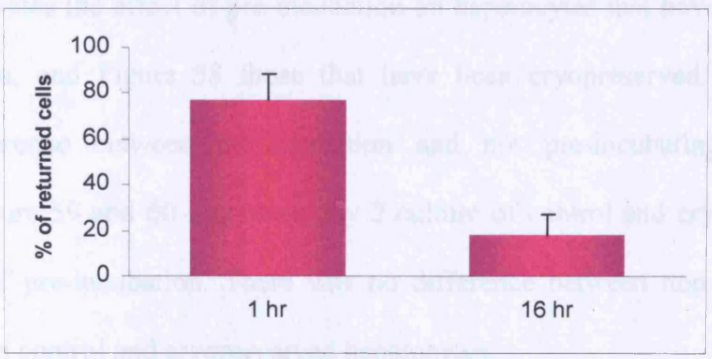



**Figure 55. Data represents % of viable porcine hepatocytes returned following pre-incubation for 1 hour and 16 hours. (n=6).**

After cryopreservation the effect of pre-incubating for 1 hour did not significantly improve the hepatocyte return, and although there seemed to be an improved return by incubating for 16 hours (81.3±28% vs. 50.5±22.1% for 1 hour) it did not quite reach

statistical significance (p=0.08) (Figure 56). A comparable cell return pattern was seen when the cryopreserved cells were compared to the fresh cryopreserved hepatocytes.

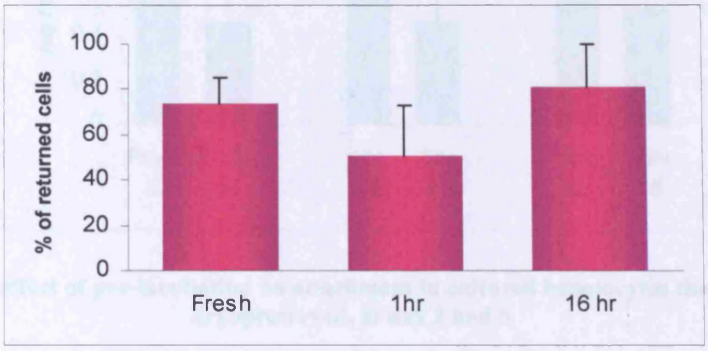



**Figure 56 Percentage of returned hepatocytes following thawing on hepatocytes that had not been pre-incubated or incubated for 1 hour and 16 hours**

## 8.3.2 Assessment of hepatocyte attachment post cryopreservation and pre-incubation

### 8.3.2.1 Human hepatocytes

Figure 57 illustrates the effect of pre-incubation on hepatocytes that have not undergone cryopreservation, and Figure 58 those that have been cryopreserved. There was no statistical difference between pre-incubation and not pre-incubating in terms of attachment. Figure 59 and 60 illustrates day 2 culture of control and cryopreserved and the influence of pre-incubation. There was no difference between non-incubation and incubation, a0nd control and cryopreserved hepatocytes.

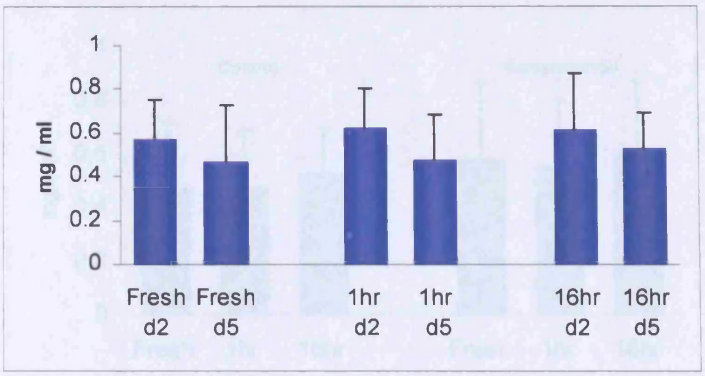



**Figure 57. The effect of pre-incubation on attachment in cultured hepatocytes that have not been cryopreserved, at day 2 and 5.**

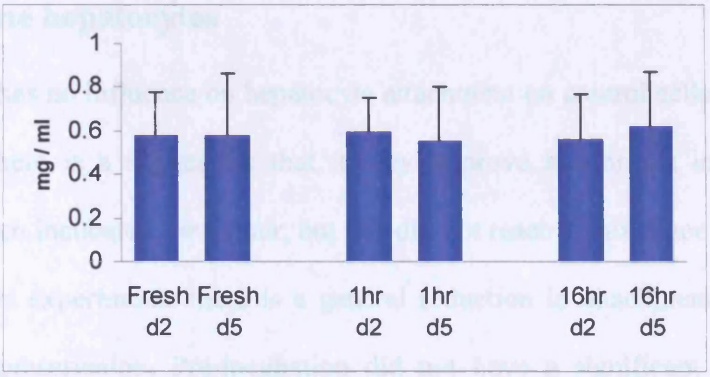



**Figure 58. Effect of pre-incubation on cryopreserved hepatocytes**

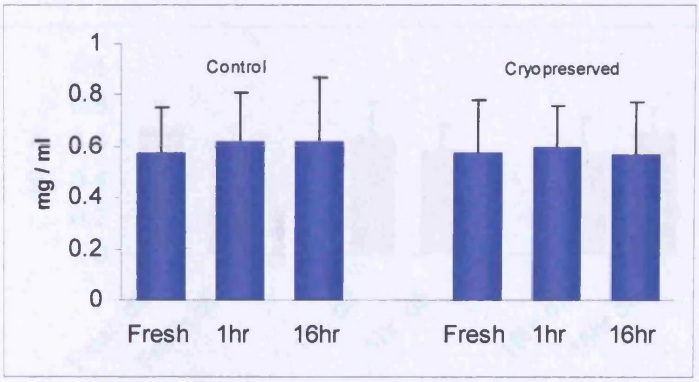



**Figure 59. Day 2 attachments for cells not cryopreserved and cryopreserved and the influence of..pre-incubation**

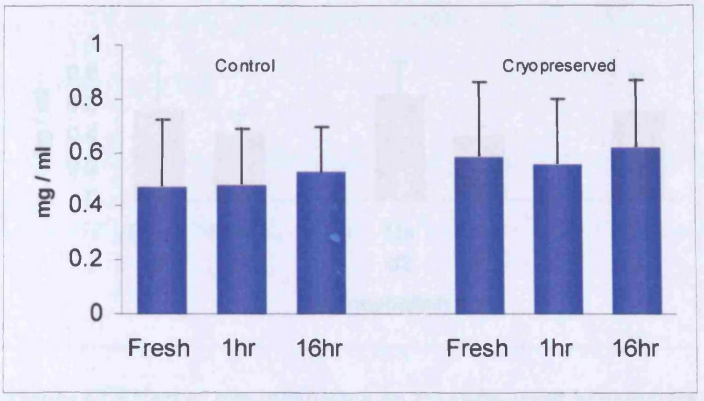



**Figure 60. Day 5 attachments for cells control and cryopreserved and the influence of pre-incubation**

### 8.3.2.2 Porcine hepatocytes

Pre-incubation has no influence on hepatocyte attachment on control cells (Figure 61, 62, 63 and 64). There is a suggestion that it may improve attachment in cryopreserved hepatocytes when incubated for 1 hour, but this did not reach significance (Figure 62). As seen in previous experiments there is a general reduction in attachment when cultured following cryopreservation. Pre-incubation did not have a significant impact on this decline.

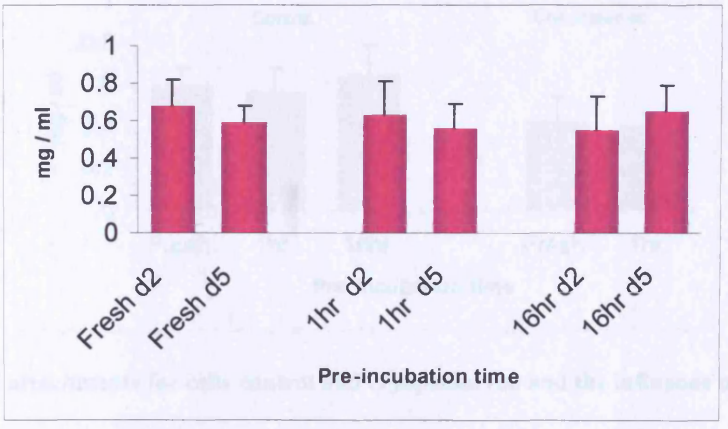



**Figure 61 The effect of pre-incubation on attachment in cultured hepatocytes that have not been cryopreserved, at day 2 and 5.**

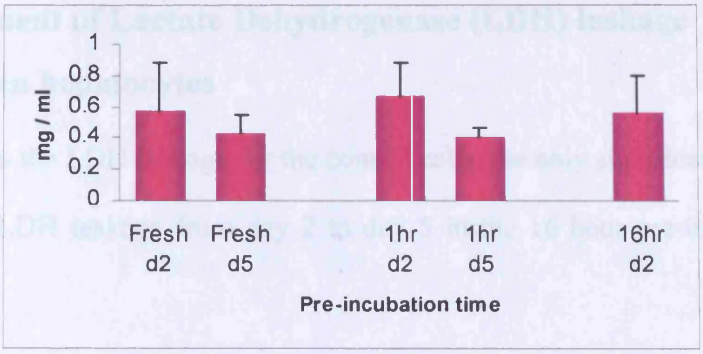



**Figure 62 Effect of pre-incubation on cryopreserved hepatocytes**

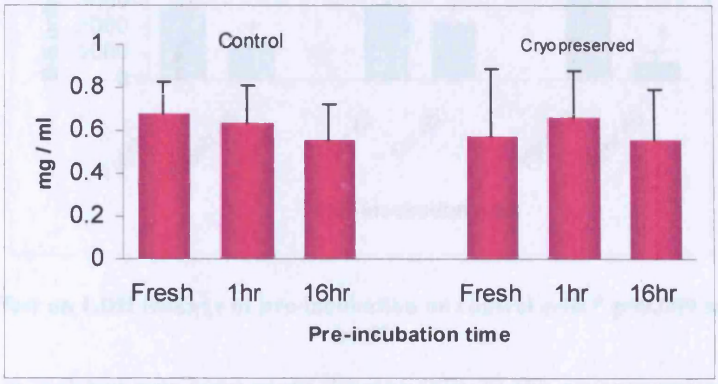



**Figure 63. Day 2 attachments for cells control and cryopreserved and the influence of pre-incubation**

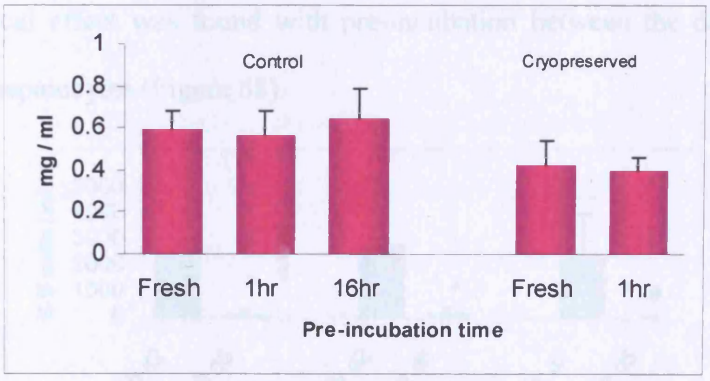



**Figure 64. Day 5 attachments for cells control and cryopreserved and the influence of pre-incubation**

### 8.3.3 Assessment of Lactate Dehydrogenase (LDH) leakage

#### 8.3.3.1 Human hepatocytes

Figure 65 shows the LDH leakage for the control cells, the only significant finding being a reduction in LDH leakage from day 2 to day 5 in the 16 hour pre-incubation group (p=0.009).

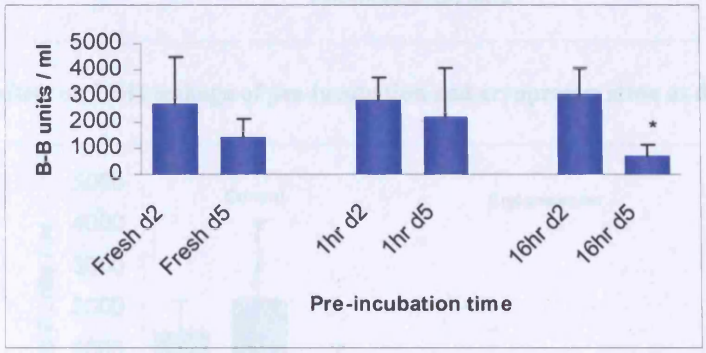



**Figure 65 The effect on LDH leakage of pre-incubation on control cells * p=0.009 as compared to 16 hr d2**

This reduction in leakage was seen more dramatically in the cryopreserved group (Figure 66) although the pre-incubation did not affect the level that this occurred at day 5 (Figure 67). No statistical effect was found with pre-incubation between the day 2 control or cryopreserved hepatocytes (Figure 68).

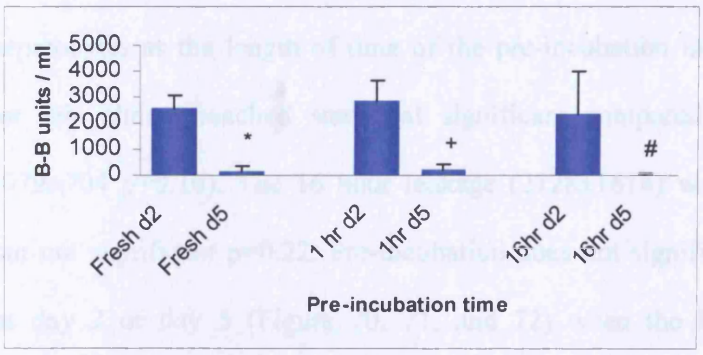



**Figure 66. The effect on LDH leakage of pre-incubation on cryopreserved cells. * p=<0.001 compared to Fresh d2, + p=0.001 as compared to 1 hr d2 and # p=0.01 as compared to 16hr d2.**

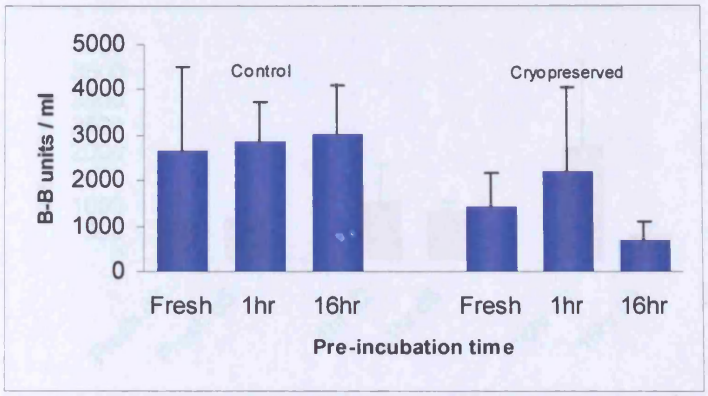



**Figure 67 The effect on LDH leakage of pre-incubation and cryopreservation at day 2 of culture.**

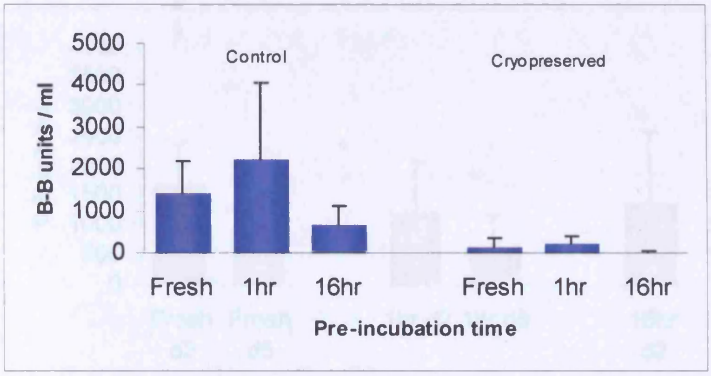



**Figure 68. The effect on LDH leakage of pre-incubation and cryopreservation at day 5 of culture.**

### 8.3.3.2 Porcine hepatocytes

The mean LDH leakage for the pre-incubation of porcine hepatocytes increased over time in the control hepatocytes as the length of time of the pre-incubation increased (Figure 69). For 1 hour this almost reached statistical significant compared to fresh cells (786±471 vs. 1079±704 p=0.10). The 16 hour leakage (2128±1614) was much higher than the fresh but not significant p=0.22. Pre-incubation does not significantly alter the LDH leakage at day 2 or day 5 (Figure 70, 71, and 72) when the hepatocytes are cryopreserved or not cryopreserved.

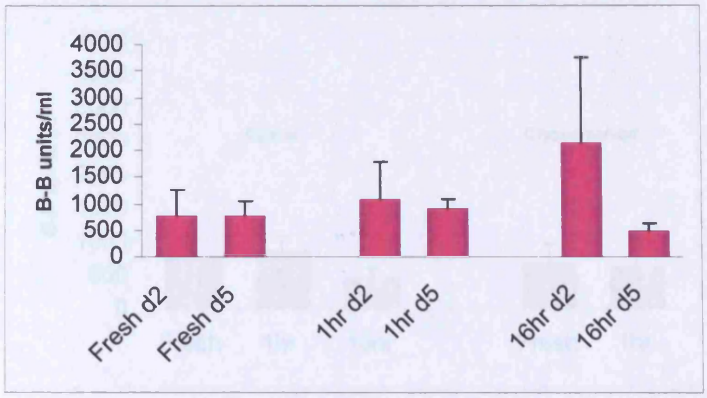



**Figure 69. Effect of pre–incubation on LDH leakage on control hepatocytes.**

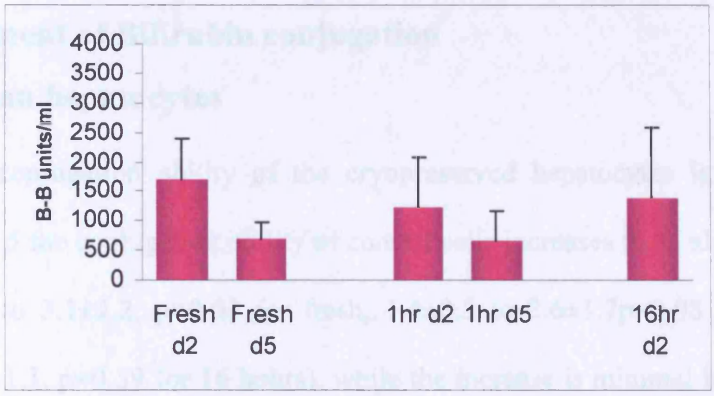



**Figure 70. Effect of pre-incubation on LDH leakage on cryopreserved cells.**

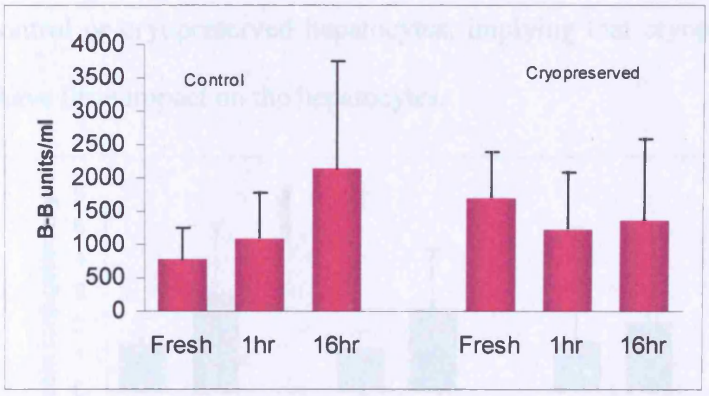



**Figure 71. Effect of pre-incubation on LDH leakage on control and cryopreserved hepatocytes at day 2.**

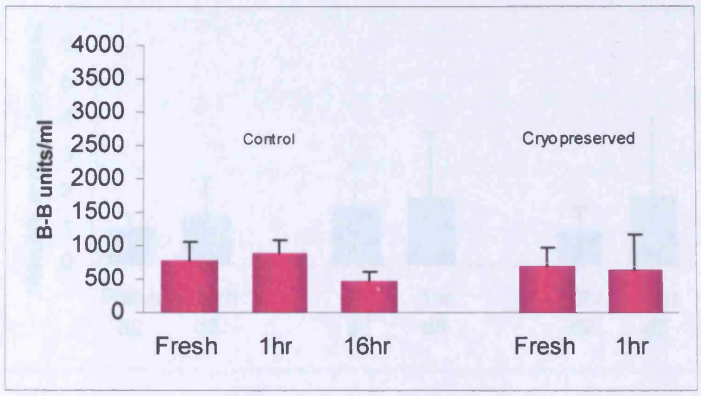



**Figure 72. Effect of pre-incubation on LDH leakage on control and cryopreserved hepatocytes at day 5**

## 8.3.4 Assessment of Bilirubin conjugation

### 8.3.4.1 Human hepatocytes

The bilirubin conjugation ability of the cryopreserved hepatocytes is comparable to control. At day 5 the conjugation ability of control cells increases to an almost significant level (1.5±0.8 to 3.1±2.2, p=0.07 for fresh, 1.4±0.5 to 2.6±1.7p=0.08 for 1 hour and 1.5±1.0 to 2.2±1.1, p=0.39 for 16 hours), while the increase is minimal in cryopreserved cells (Figure 73, 74, 75 and 79). There was no significant difference between pre-incubation in control or cryopreserved hepatocytes, implying that cryopreservation and pre-incubation have little impact on the hepatocytes.

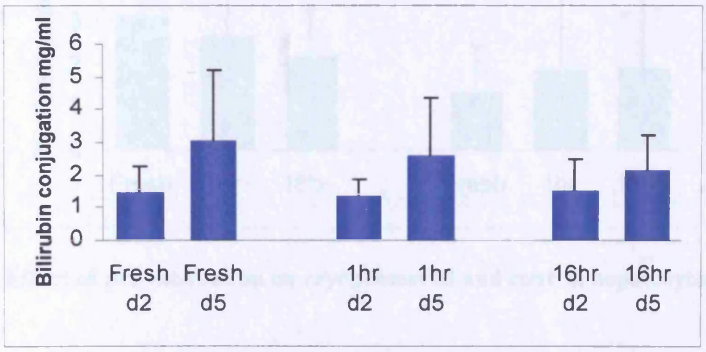



**Figure 73. Bilirubin conjugation following pre-incubation on control hepatocytes.**

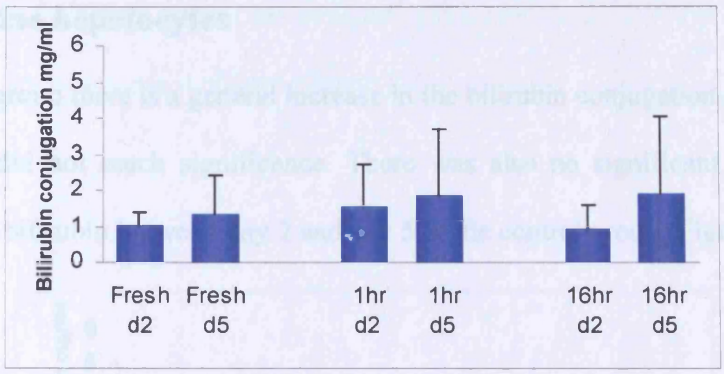



**Figure 74. Effect of bilirubin conjugation on cryopreserved hepatocytes.**

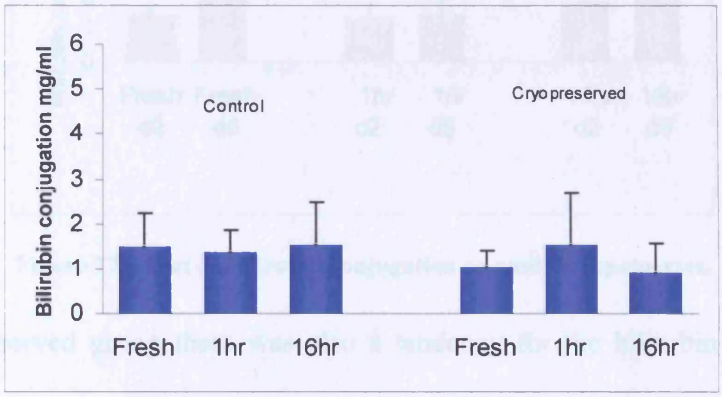



**Figure 75. Effect of pre-incubation on cryopreserved and control hepatocytes at day 2.**

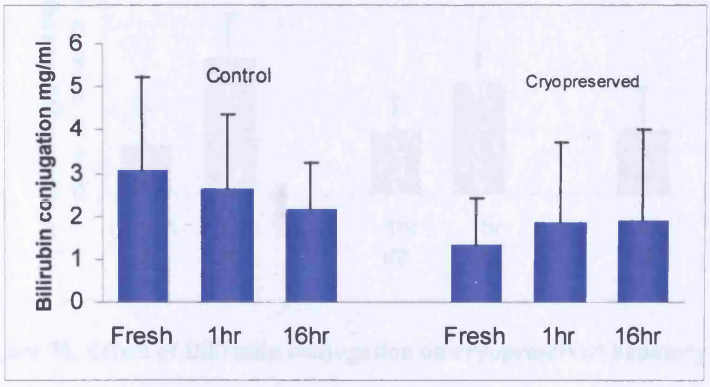



**Figure 76 Effect of pre-incubation on cryopreserved and control hepatocytes at day 5.**

### 8.3.4.2 Porcine hepatocytes

In the control group there is a general increase in the bilirubin conjugation ability at day 2 however this did not reach significance. There was also no significant change in the conjugation of bilirubin between day 2 and day 5 in the control group (Figure 77).

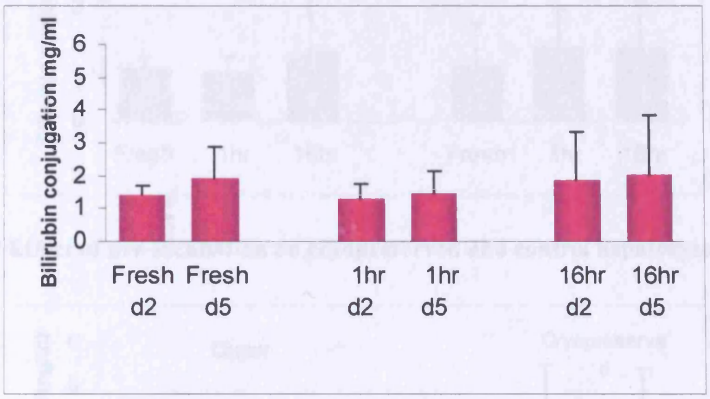



**Figure 77 Effect of Bilirubin conjugation on control hepatocytes.**

In the cryopreserved group there was also a tendency for the bilirubin conjugation to increase as the pre-incubation time increases and also from day 2 to day 5 (Figure 78).

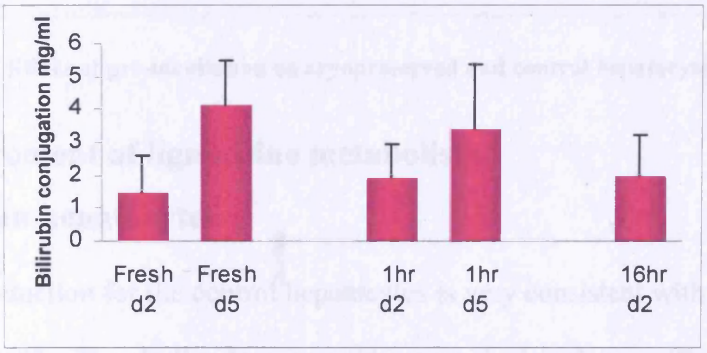



**Figure 78. Effect of Bilirubin conjugation on cryopreserved hepatocytes.**

The increase in the fresh cells (cryopreserved) from day 2 to day 5 was significant (1.5±1.1 vs. 4.1±1.4 p=0.01). The increase at 1 hour was not significant (2.0±1.0 vs.

3.4±2.0 p=0.23). However there is no statistical improvement in bilirubin conjugation by pre-incubating the hepatocytes (Figure 79 and 80).

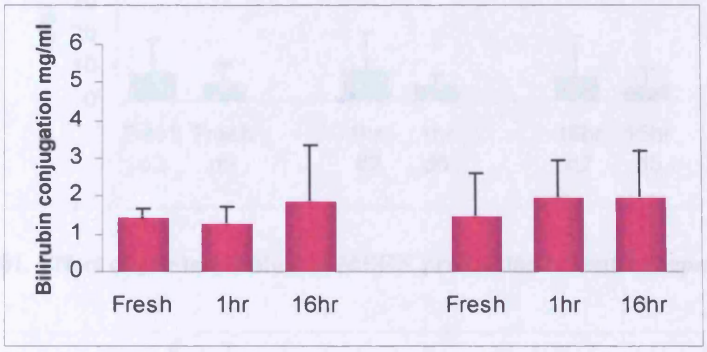



**Figure 79 Effect of pre-incubation on cryopreserved and control hepatocytes at day 2**

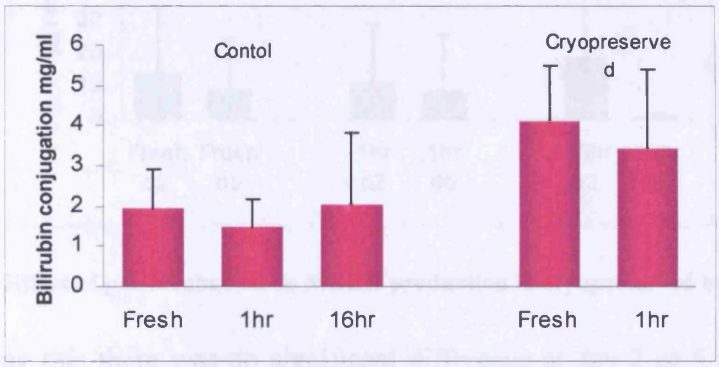



**Figure 80 Effect of pre-incubation on cryopreserved and control hepatocytes at day 5**

## 8.3.5 Measurement of lignocaine metabolism

### 8.3.5.1 Human hepatocytes

The MEGX production for the control hepatocytes is very consistent with a slight decline at day 5 (Figure 81). The decline however did not reach significance. The cryopreserved hepatocytes showed a wider range of function (Figure 82).

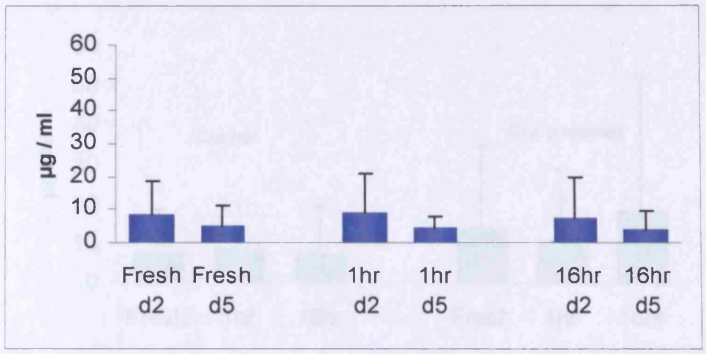



**Figure 81. Effect of pre-incubation on MEGX production in control hepatocytes.**

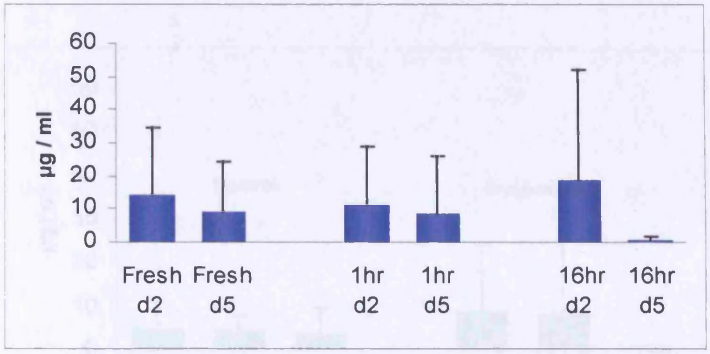



**Figure 82. Effect of pre-incubation on MEGX production in cryopreserved hepatocytes.**

However, despite this there was no significant difference at day 2 or 5 (Figure 83, 84) between fresh and either of the two pre-incubation times. Pre-incubation appears not to influence MEGX production in control or cryopreserved hepatocytes.

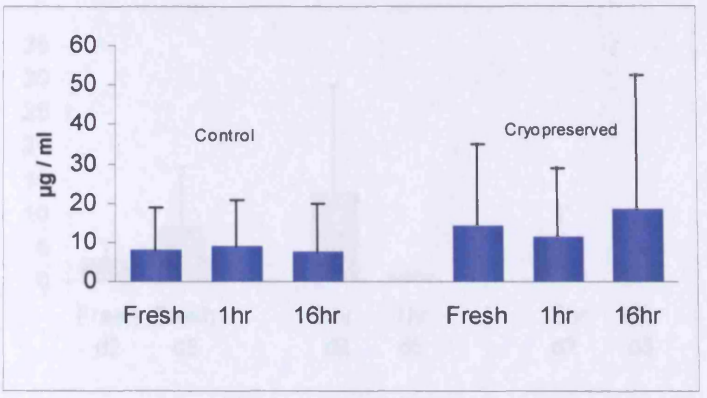



**Figure 83. Effect of pre-incubation on MEGX production, and cryopreservation at day 2 of culture.**

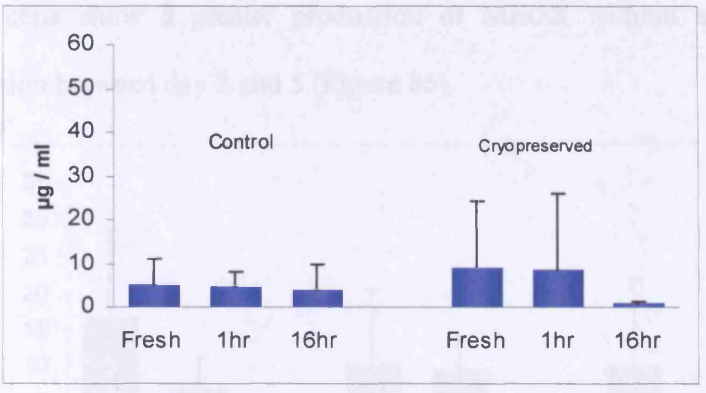



**Figure 84. Effect of pre-incubation on MEGX production, and cryopreservation at day 5 of culture.**

### 8.3.5.2 Porcine hepatocytes

The MEGX production for day 2 pre-incubation shows an increase for 1 hour, but decreases dramatically at day 5 while the fresh cells increase MEGX production (Figure 85).

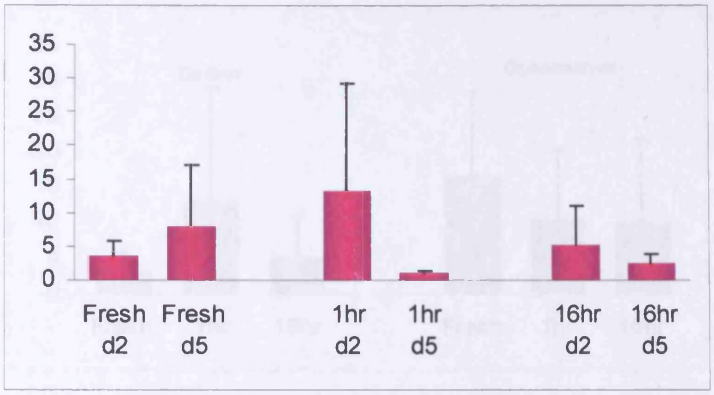



**Figure 85. Effect of pre-incubation on MEGX production in control hepatocytes.**

Cryopreserved cells show a greater production of MEGX without such a marked decrease in function between day 2 and 5 (Figure 86).

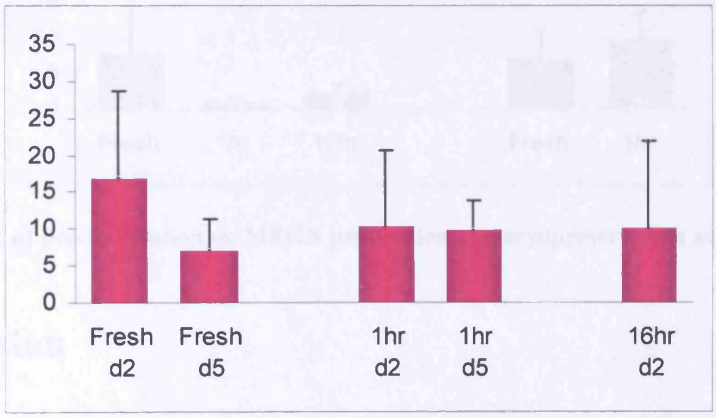



**Figure 86. Effect of pre-incubation on MEGX production in cryopreserved hepatocytes.**

Statistically there is no significance between any of the results indicating that cryopreserved cells are as good as control and that pre-incubation makes a negligible difference in increasing hepatocyte function (figure 87, 88).

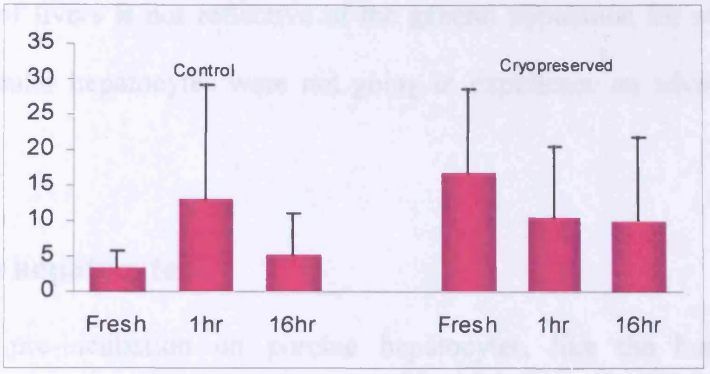



**Figure 88. Effect of pre-incubation on MEGX production and cryopreservation at day 2 of culture.**

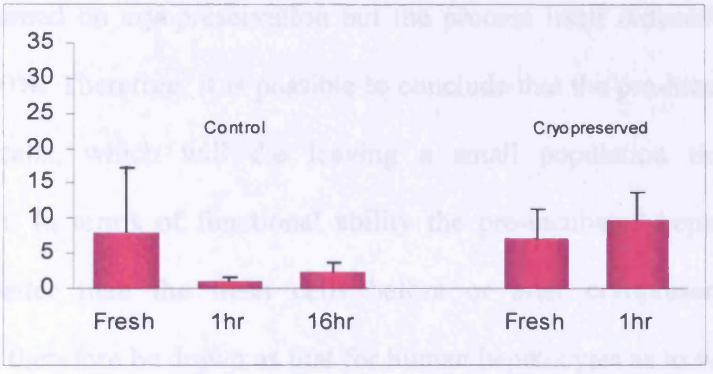



**Figure 89. Effect of pre-incubation on MEGX production and cryopreservation at day 5 of culture.**

# 8.4 Conclusion

## 8.4.1 Human hepatocytes

The effect of pre-incubation on human hepatocytes was disappointing. The data suggested a slight improvement in lignocaine metabolism and bilirubin conjugation in hepatocytes that had been pre-incubated and then cryopreserved, but this was not statistically significant. There was also a considerable reduction in viable hepatocytes during the pre-incubation process. Reasons why this may occur could be too high spin rate for the equipment used and thus detrimental shear forces. It is also possible that these cells were going to die anyway and would not have attached. The third possibility is that

the population of livers is not reflective of the general population for some reason and that these particular hepatocytes were not going to experience an advantage with pre-incubation.

### 8.4.2 Porcine hepatocytes

The effect of pre-incubation on porcine hepatocytes, like the human, was also disappointing. The pre-incubation for 16 hours did improve the percentage of viable hepatocytes returned on cryopreservation but the process itself reduced the viable cell population by 80%. Therefore, it is possible to conclude that the pre-incubation removes many of the cells, which will die leaving a small population that can survive cryopreservation. In terms of functional ability the pre-incubated hepatocytes did not perform any better than the fresh cells before or after cryopreservation. Similar conclusions can therefore be drawn as that for human hepatocytes as to why these results differ from others in the literature.

# Chapter 9

# Summary Conclusions

9.1 Hepatocyte recovery following cryopreservation

9.2 Functions following cryopreservation

9.2.1 Pre-incubation

9.2.2 Concentration and cryopreservation

9.2.3 Phase I and I I metabolism and cryopreservation

9.3 Inter species and inter individual differences

9.4 Discussion of Experimental Methods

9.4.1 Viability

9.4.2 Attachment

9.4.3 LDH

9.4.4 P450

## 9.1 Hepatocyte recovery following cryopreservation

The aim of this study was to determine the efficacy and reproducibility of current methodology on the survival and function of human and porcine hepatocytes, for use in bioartificial liver systems. Efforts centred around the development of a methodology that would be simple, reproducible and take into account logistical factors surrounding hepatocyte acquisition currently at our centre e.g. time of arrival, equipment available, personnel availability. The study primarily examined three main areas that influenced the condition of hepatocytes namely freeze rate concentration of storage and pre-incubation prior to cryopreservation. It was also methodologically limited due to the physical practicalities of performing the whole experimentation myself.

The studies undertaken illustrated that both human and porcine hepatocytes can, in most instances, be cryopreserved and thawed with functions that are comparable to freshly isolated hepatocytes. The concern is the large number of hepatocytes that are lost through the cryopreservation process. Surprisingly, the rate of cooling did not have a significant impact on the number of viable cells returned using the chosen methods. This has been previously identified as important in preserving hepatocytes especially utilising a stepwise temperature reduction programme (Wu *et al.*, 2000; Chesne *et al.*, 1993; Li *et al.*, 1999; Diener *et al.*, 1993; Sun *et al.*, 1990; Utesch *et al.*, 1992; Chen *et al.*, 2001). Most of these studies have been performed on rat hepatocytes with little data being produced on either porcine or human hepatocytes. It is reasonable to hypothesise that many of the early assumptions concerning hepatocyte cryopreservation have been based on studies on a range of vertebrate species having different characteristics, while parallel

human hepatocyte studies are limited. The results of this study are similar to those reported by Alexandre *et al.* using comparable methods. Human hepatocytes studies also support the assertion by Guillouzo *et al.* that there is no convincing evidence that complex freeze rates significantly effect post cryopreservation function (Guillouzo *et al.*, 1999; Alexandre *et al.*, 2002).

The differing definitions of viability following cryopreservation compound the problems encountered when comparing studies. Preliminary experiments that were undertaken indicate that the cryopreservation process resulted in the loss of many cells that were presumably totally destroyed and essentially "disappear" from the cell count on thawing. Of the papers reviewed herein most quote the viability of the remaining hepatocytes, and do not discuss in detail the phenomenon of cell loss. These accounts do not give any indication of how successful their procedure was in the total return of viable hepatocytes.

In human hepatocyte cryopreservation the concentration of cells for freezing did make a significant difference on the return of hepatocytes. It was found that both low ($2.5x10^6$ cells/ml) and high concentrations (up to $2x10^7$) resulted in reduction of hepatocyte return. It was noted that a peak of $5x10^6$ was found to be the optimal level with the greatest return of hepatocytes. In porcine hepatocytes the concentration did not have as significant an impact. The results for human hepatocytes broadly fit the hypothesis of De Loecker *et al.* that hepatocytes should not be stored too densely as the membrane to membrane contact can cause cellular damage (Diener *et al.*, 1993; Kasai and Mito, 1993). It is difficult to draw any further conclusions as our methods utilised different species and cooling methods. However, it can be concluded that the return of human hepatocytes is

highest when cryopreserved at $5x10^6$ hepatocytes/ml. No studies were identified that compare different concentrations of porcine cryopreservation, despite this being the major tissue source for current bioartificial livers. The cell return was remarkably consistent and it is possible that the cell clumping that occurs is providing some protection, or that cell to cell contact is not a factor for porcine hepatocytes.

Pre-incubation also aided an increased return of cells following thawing, but not without a 40% loss with human and 60% loss with porcine hepatocytes through the spinning process. Therefore despite the interventions, at best a mean of 30-40% of viable human hepatocytes cryopreserved and 60% of porcine hepatocytes could be returned. This has important implications for both banking and clinical application. The number of cells that have been isolated over the study period amounts to $5.1x10^{10}$. Approximately 22kg of tissue was not isolated due to lack of personnel or to too much tissue being donated on a particular day. If a similar yield of cells could be extracted from this 22kg then approximately $1.4x10^{11}$ hepatocytes could be made available. The lowest clinical estimate for hepatocytes in a bioartificial liver is $6x10^9$ (approximately 2% of the native liver). Therefore if all the cells from the 7.7kg of tissue donated were used in BAL construction as soon as they were isolated, then approximately 8 BALs could have been constructed. If the same yield could be obtained from the full 22kg then this would increase to approximately 24. Unfortunately, the current study confirmed that following cryopreservation only 30% of this volume could be returned, thus only 8 BALs could realistically be constructed. If pre-incubation was to be employed it would have to be accepted that the cell population would decrease. To compensate for this the method would have to result in a substantial improvement in functional ability.

The donated liver tissue came from one UK hepatobilliary centre and a second for 12 months of the study, with 11 whole livers donated from brain stem dead multiple organ donors. Many other centres in the UK undertake this kind of surgery and although some have arrangements with research establishments, it is probably common to have excess tissue and hepatocytes that could be made available. A national and possibly European co-ordination of all tissue could help to obtain hepatocytes for clinical use (Anderson *et al.*, 2001; Orr *et al.*, 2002). Clearly further work is required into the reasons and methods to prevent cell loss during freezing (Chapter 10).

## 9.2 Functions following cryopreservation

Throughout the study the function of the majority of recovered hepatocytes from most of the cryopreservation methods are comparable to the fresh cultures in both pigs and humans. Phase 1 and 2 reactions were assessed throughout the study with cryopreservation, concentration and pre-incubation having little significant effect.

### 9.2.1 Pre-incubation

Darr and Hubel demonstrated significant improvements in albumin excretion following pre-incubation for up to 24 hours in a spinner vessel, using a magnetic stirring plate set at a speed of 90rpm. No other parameters were assessed and no comment was made on the number of cells recovered. Other studies using pre-incubation have found an improvement in attachment, no improvement in cell return, but a minimal impact on function following cryopreservation, thawing and culture (Darr and Hubel, 1997; Alexandre *et al.*, 2002). Similar results were found in the current studies, namely no

significant impact in the number of viable hepatocytes recovered and no statistically significant improvement in the functional data. However in contrast with published works no improvement in cellular attachment with pre-incubation was evident. Whether pre-incubation is a beneficial technique that can improve hepatocytes attachment cannot be concluded. It is possible that the method of assessing attachment in this study is not sensitive enough to detect changes in attachment. The method chosen in these studies do not offer any advantages or have significant impact on hepatocyte function.

### 9.2.2 Concentration and cryopreservation

Published work has shown that the density of the hepatocytes within the cryopreservation solution can influence the post thaw viability and functional ability. These studies were mainly conducted in rats (Hengstler *et al.*, 2000; Diener *et al.*, 1993; De Loecker *et al.*, 1998). Comparing the storage of different concentrations of human and porcine hepatocytes indicated that variations of cell populations had no statistical impact on the functional parameters studied. However, it was observed that as the concentration increases above $5x10^6$ hepatocytes/ml there is a reduction in the number of returned viable human hepatocytes. Most of the published studies in cryopreservation have chosen this concentration, and it appears to be the density of choice for human hepatocyte cryopreservation (Hengstler *et al.*, 2000; De Sousa *et al.*, 1991; Chen *et al.*, 2001). For porcine hepatocytes the concentration appears to make no significant difference, and no studies could be found that have compared this directly.

### 9.2.3 Phase I and I I metabolism and cryopreservation

In general the cryopreservation of porcine and human hepatocytes was found not to alter their functional activity significantly, in comparison to freshly cultured hepatocytes. The results are comparative means of a number of different livers and therefore reflect a random population. This is a very encouraging result for the prospect of successful hepatocyte banking and use for research and clinical interventions. It has been previously reported that cryopreserved human hepatocytes can be used as a model for pharmacotoxicological research, with the main interest being in phase I cytochrome P450 activity (Li, 1999; Loretz *et al.*, 1989; Li and Jurima-Romet, 1997). Other published studies have found that cytochrome P450 (3A4) recovers well from cryopreservation and can express functional values similar to the fresh hepatocytes (Roymans et al., 2004; Alexandre *et al.* 2002; McKay *et al.*, 2002; Chesne *et al.*, 1993; Diener *et al.,* 1993; Utesch *et al.*, 1992.).

## 9.3 Inter species and inter individual differences

One of the aims was to develop a protocol that could be applied to both porcine and human hepatocytes. As the experiments progressed it was evident that each species has different characteristics that can affect optimal freezing and culture conditions. Porcine hepatocytes tended to aggregate during the isolation process which made numerical assessment difficult and introduced operator dependent error. This may have resulted in more cells being frozen than the stated concentration thus adding to the variation of the results obtained. Porcine hepatocytes have been extensively used in bioartificial design but few studies have compared their functional efficacy with human hepatocytes. Vilei *et*

*al.* compared human and porcine hepatocytes in culture and although no difference was found in urea synthesis, porcine hepatocytes possessed superior diazepam metabolism (cytochrome P450) and were able to conjugate bilirubin more efficiently after 24 hours of culture (Villei *et al.*, 2001). Donata *et al.* characterised porcine cultures against rat, dog and human, specifically looking at phase I and phase II functions in fresh culture (Donata *et al.*, 1999). They found that porcine cultures closely resemble those of humans and concluded that it was the cell source that most represented the human. Current studies demonstrate that the main functional markers, MEGX and bilirubin conjugation, showed comparable function at 2-5 days, in both fresh and cryopreserved human and porcine hepatocytes.

For human hepatocyte isolation there is considerable inter individual variation (Li, 1997). Both patient and operative factors have little impact on the yield and viability of the isolated hepatocytes in these studies. As the patient numbers increase some of these factors may become significant, however the results are encouraging in that almost all the livers isolated, regardless of the patient or operation, resulted in a reasonable and consistent viability and yield. Our observations have not identified single significant criteria that would preclude the use of any hepatocytes isolated. Extension of this work with European collaborators (University of Beasançon; Fondation Transplantique, Strasbourg) involving studies with a larger number of patients demonstrated that i) liver tissue no larger than 150g should be isolated for optimal hepatocyte yield ii) the digestion phase should be no longer than 20 minutes (section 10.6). It is encouraging that using similar methods independently has resulted in such a consistent isolation pattern in terms of yield and viability.

The important question of whether these factors have an influence on function have not been conclusively answered. Liver isolations where functional data was obtained as part of the cryopreservation experiments was analysed but no statistical difference was found. As this was a relatively small number of the isolations studied and the analysis performed retrospectively the data was not deemed useful and of limited value. The only other study that has looked at such a large number of human hepatocyte isolations (149) was performed by Alexandre *et al* (Alexandre *et al.*, 2003). In broad terms they too found that the various factors did not alter hepatocyte function significantly.

The analysis of function and comparison with isolation factors is statistically complex as each liver demonstrates such a broad range of metabolic abilities especially cytochrome P450's (Hengstler *et al.*, 2000; Li *et al.*, 1999). In our study the metabolism of lignocaine produced a wide range of MEGX amounts, reflecting this individual variation. Li *et al.* measured a number of cytochrome P450 isoenzymes and demonstrated a large range across each isoenzyme. (Li *et al.*, 1999). This particular property could be advantageous to BAL development. It is probable that the hepatocytes used to construct the BAL will consist of hepatocytes from a number of donor livers. The combined functions should eliminate deficiencies in some donor livers to produce an overall adequate range. Obviously further work is required to characterise this phenomenon fully to ensure the converse does not occur where there is a severe deficiency in function from a number of poor livers.

## 9.4 Discussion of Experimental Methods

### 9.4.1 Viability

The measurement of trypan blue exclusion assesses the ability of the cells to take up and extrude the dye. To evaluate any intervention to the isolation and cryopreservation process a more accurate measurement of cellular viability would be desirable. As discussed in section 3.4.2.1, a number of different methods have been cited in the literature, an indication of how complex a task the development of a standard viability method is. For the development of a store of hepatocytes it could be argued that a trypan blue viability count adds little to the data. It has been shown in our study that it does not correlate with function following culture, and does not have a significant predictive impact on the number of returned hepatocytes following cryopreservation. What is more important is having a standardised method of attachment assessment that reflects function of hepatocytes. The argument for continuing to measure viability by trypan blue is that it is quick to perform and it gives a broad estimate of viable hepatocytes. Concentrations for culture can therefore be adjusted to ensure culturing is performed at a suitable density.

### 9.4.2 Attachment

During the study it was recognised that a measure of how many hepatocytes attached in culture was required to compare the function of hepatocytes between interventions. The protein method developed only gave a crude estimation of attachment and a calculation of function per hepatocyte was still not possible. A possible error within this study methodology was not calculating a value of how much protein correlated to how many

hepatocytes. However it has been published that measuring protein in this way is a method in establishing an attachment value (Loretz *et al.*1989; Lawrence and Benford, 1991; Alexandre *et al.* 2002, and personnel communication with Professor Richert, University of Besançon, France).

Alexandre *et al.* performed the simplest amendment to this method to obtain a percentage attachment figure. They performed the protein assay as outlined in our section 4.4.4. However they also collected the unattached cells in the media, and performed a second protein assay, to derive a percentage of attachment (Alexandre *et al.*, 2002). This method would have been useful in this study and has been adopted for later studies. Other more complex methods have been described that give a more accurate figure to the number of hepatocytes attached. This has included measuring intracellular LDH of attached and non-attached hepatocytes (Pathernik *et al.*, 1996).

Whatever the final acceptable international method this numerical value is important to give a clearer understanding of how hepatocytes attach and would allow comparisons to be made between future studies.

### 9.4.3 LDH release

LDH release is another method to compare the success of the attachment process (Chen *et al.*, 2002; Chesne *et al.* 1991). A high value of LDH measured within the medium correlates with a high amount of leakage from the cell and therefore damage to the membrane. In this study leakage from 4 hours post initial culture to day 2 and from day 3 to day 5 was measured. As expected the first value was considerably higher as

hepatocytes remaining after day 2 are normally considered healthy. Unfortunately, the measurement of LDH in this study was not useful as only two time points were considered. A useful comparison would have been to measure the amount of LDH after 1, 2, 3 and 5 days. This would have given a comparison as to how damaged the hepatocytes were, especially when a value per hepatocyte could be calculated. Just measuring at day 2 negated the fact that most of the release would occur over this time period but when exactly could not be calculated (Chen *et al.*, 2002).

### 9.4.4 Cytochrome P450 activity

The importance of cytochrome P450 function in isolated hepatocytes for drug metabolism or BAL development has been discussed in Chapter 2. There is, however, no agreement in the literature, as to which isoenzyme correlates to good preserved function. The lignocaine metabolism test and the subsequent measurement of MEGX, used in our study, were originally used for the prediction of graft survival in liver transplant. Oellrich *et al.* published a review of 171 transplants undertaken and demonstrated that a good production of MEGX correlated significantly with good graft survival (Oellerich *et al.*, 1991). It has subsequently been used to assess the cytochrome P450 function of a developing bioreactor (Shatford *et al.*, 1992). It gives a comparative marker for the cytochrome P450 function more specifically the isoenzyme CYP3A4. CYP3A4 remains one of the more general P450 isoenzymes and is responsible for the metabolism of many common drugs. In the assessment of hepatocytes for bioartificial livers other isoenymes have been assessed including 1A1, 1A2, 2E, 2E1, 2B, 2E1 (Wu *et al.*, 2000; Donata *et al.*, 1999; Alexandre *et al.*, 2002). Further studies should look at other cytochrome P450's in conjunction with CYP3A4 to evaluate any trends. It is difficult and time consuming to

carry out a large number of assays but an agreed cross section may provide useful data in assessing hepatocyte suitability.

# Chapter 10

# Future Developments

10.1 Hepatocyte survival following cryopreservation.

10.2 Improving Cryopreservation

10.2.1. Pre-incubation

10.3 BAL development

10.4 Stem cells

10.5 Percol purification

10.6 Improving isolation process and pharmacological studies

10.7 Final conclusion

## 10.1 Hepatocyte survival following cryopreservation.

From the studies undertaken and the reviewed literature only small improvements in cellular viability and return, from cryopreservation, can be achieved. To improve this further there is a need to understand the molecular, biophysical and biochemical functions associated with cells death and destruction caused by the isolation and freeze thaw processes. It is not clear if cell death is due to the physical factors of ice formation, or the recently identified triggering of apoptosis. Another form of cell death, anoikis, has been described (Zvibel *et al.*, 2002, Smets *et al.*, 2002) and it has been proposed that this be initiated once the cells have become detached from the extracellular matrix. In summary, attached cells receive "survival signals" mediated by the extracellular matrix and the integrins- especially integrin β1. The exact mechanism has not been fully described, but the activation of Caspase –8 and Caspase –3 is one of the major initiating events that leads to a cascade and the eventual death of the cell (Yagi *et al.*, 2001; Fu *et al.*, 2001).

Yagi *et al.* evaluated the cryoprotective effect of a global caspase inhibitor, benzyloxycarbonyl-Val-Ala-DL-Asp-fluoromethylketone (ZVAD-fmk) on cryopreserved hepatocytes (Yagi *et al.*, 2001). They found an increase in Caspase –3 activity following thawing but on the hepatocytes frozen with ZVAD-fmk a significantly reduced activity was found. This correlated to reduced apoptosis and increased viability in the ZVAD-fmk positive group. The cell function was also improved measured by Temazepam metabolism.

An anti-apoptotic pathway also exists in balance with the apoptotic. The anti-apoptotic factor, nuclear factor NFκB has been identified as one of the main pathways that can induce inhibitors of apoptosis proteins (IAPs) thereby blocking caspase –3 and –7. (Black and Behrns, 2002). Manipulation of either of these mechanisms could produce benefits to the hepatocyte. It is unclear where on the isolation-thawing pathway such an intervention should take place. Possible options include pre-treating animals (or humans) with an inhibitor or inducer of either pathway, including such compounds in any of the isolation or storage solutions, or including them in the culture media or cryopreservation solution. Tilles *et al.* noted that the improvement in one parameter maybe to the detriment of another and that possibly several parameters will need to be simultaneously altered to yield improvements (Tilles *et al.*, 2002). To improve cell viability and possibly subsequent function, research into this area may provide important answers to improve hepatocyte cell return.

## 10.2 Improving Cryopreservation

### 10.2.1 Pre-incubation

Positive effects have been observed with pre-incubation in terms of attachment and function (Darr and Hubel, 2001; Chen *et al.*, 2001; Alexandre *et al.*, 2002). In our study no significant improvement was demonstrated. The method and equipment studied are different to that published. Therefore there are a number of areas, which could be studied to allow the development of an optimised method. It is possible that the cell loss experienced during pre-incubation, may represent a population of cells that would not normally attach in culture and should therefore not be considered a true reduction. Further work into this and the dynamics of the cell spinner with a reduction in the

revolution speed may provide an improvement in cellular or cryopreservation return. It is possible that the speed used in the experiment was too fast and was causing unnecessary shear forces on the already damaged hepatocytes.

The length of time of pre-incubation may also be critical. This may depend very much on the technology and methods used, but as a result of current studies a shorter time would prove beneficial (Silva *et al.*, 1999; Alexandre *et al.*, 2002).

## 10.3 BAL development

In parallel with increasing the number of cells following cryopreservation, improving attachment of hepatocytes following isolation or cryopreservation remains a significant challenge to be overcome. Although cell function depends on this occurring, as the study has demonstrated this can be severely reduced following cryopreservation (Guillouzo *et al.*, 1999). Further manipulation that would encourage attachment of cells could improve the hepatocyte numbers. It has been demonstrated that pre-incubation has provided some improvement but the type of surface that the cells attach to may also be critical. Alexandre *et al.* and Silva *et al.* both concluded, that pre-incubation prior to freezing could be sufficient to improve attachment on to Matrigel coated plates but not on collagen-coated plates (Silva *et al.*, 1999; Alexandre *et al.*, 2002). A number of different materials have been used for the attachment surface of the hepatocytes within a BAL. Linti *et al.* used polyurethane fibres because of its "biocompatability", while Demetriou *et al.* attached the hepatocytes to collagen-coated dextran microcarriers (Linti *et al.*, 2002). Further work looking for the most appropriate surface for attachment and perhaps

trying to emulate the normal basement membrane may actually improve the attachment and function and reduce the number of hepatocytes detaching as the culture increases.

## 10.4 Stem cells

Stem cells are considered the "Holy Grail" for functional replacement therapies for all organs of the body. A stem cell can be defined as a cell that has the capacity both to produce daughter cells ("self renewal") and to produce daughters that are fated to differentiate ("committed cells") (Stockmann and Ijzermans, 2002). Its advantage in BAL development is clear and would theoretically negate the debate on human cell shortages and functional deficiencies (Table 18).

| |
|---|
| • Full lineage potential |
| • Superior lifespan |
| • Maximum proliferate capacity |
| • Resistance to ischaemia |
| • Can be obtained from cadaveric livers |
| • Ability to be cryopreserved with high viability and function |
| • Small cell volume for transplant |

**Table 18. Advantages of progenitor cells versus mature**

If these cells could also be induced to divide then cryopreserved hepatocytes would also not be required. Unfortunately, much of stem cell research is limited due to the ethical debates in many counties concerning the use of pre-implantation human blastocytes (Stockmann and Ijzermans, 2002). Recently it has been accepted that the liver contains cells with stem-like properties which, when activated, could proliferate and mature into all types of hepatic cells (Terai *et al.*, 2002). These cells can now be separated by flow cytometry from livers discarded from transplantation. Susick *et al.* have established a procurement programme for livers discarded for transplant and the result of their work is

awaited (Susick *et al.*, 2001). Masson and Currie *et al.* in Edinburgh, have recently presented work on the isolation, characterisation and culture of stem cells from foetal livers. They have maintained the hepatocytes in culture for 1 week where specific functions were demonstrated, although cytochrome P450 activity has not been examined (personal communication) (Masson *et al.*, 2003; Currie *et al.*, 2003).

The perceived advantages over mature cells are that they can be expanded, differentiate into all cell types, express superior cryopreservation tolerance and have minimal immunogenicity (Susick *et al.*, 2001; Ilan, 2002). It is too early to fully appreciate what role this type of cell will have on hepatic support therapies, but its development has considerable potential, and may negate many of the difficulties associated with bioartificial liver development.

## 10.5 Percol Purification

An area that has not been investigated in this study is the role of Percol purification. As previously presented (Section 3.4.1.) a Percol density gradient can be created following the isolation of hepatocytes and the dead or nonviable cells separated from the liver (Sun *et al.*, 1990). The reports on subsequent cell culture have indicated that this step aids the function and attachment of hepatocytes (Lawrence and Benford, 1991; Dou *et al.*, 1992; Donata *et al.*, 1999). The arguments against performing this are that a different population of hepatocytes with specialised functions may be selected out, and that there is a significant overall loss of the total hepatocyte population including viable hepatocytes.

In the context of the bioartificial liver project in Leicester the role of Percol following isolation and even cryopreservation needs to be identified. The potential loss of hepatocytes would be a concern, however, it is not known whether the remaining cells would be in a stronger position to survive cryopreservation. Following cryopreservation approximately 50% (own data) are deemed non-viable by trypan blue exclusion. If these cells were introduced to the proposed mesh how would the many dead cells affect the viable cells in attaching? It is possible that they could interfere with the clumping of the viable hepatocytes. It would also be important to identify whether a Percol density gradient would be useful to remove these dead hepatocytes prior to inoculation into the mesh. A method has been recently agreed by two prolific centres (Fondation Transplantique, Strasbourg, France and the University of North Carolina, USA) as part of the ECVAM validation study (personal communication, see section 11.6). This method requires evaluating within the present project to determine its position in hepatocyte acquisition for bioartificial liver usage.

## 10.6 Improving isolation process and pharmacological studies

The liver is the major organ involved in the metabolism of xenobiotics. In particular, microsomal cytochrome P450 dependent oxidation pathways are involved in reactions such as detoxification and elimination of endogenous and exogenous substances and the formation of pharmacologically active compounds from pro-drugs. (Parkinson, 1996). Due to their inducibility (increases in enzymatically active microsomal protein content after exposure to specific chemical), these enzymatic systems have been shown to be involved in various side effects such as profound endogenous hormonal disturbances,

drug-drug interactions and exacerbated toxic effects. Therefore, routine evaluation of the inducing potential of a given chemical on these pathways would be invaluable for human safety assessment (Madan *et al.*, 2003).

Due to major species differences both in the catalytic activities and regulation of this group of enzymes, such an evaluation can be accurately performed only with human tissues (Parkinson, 1996; Kocarek *et al.*, 1995, Silva *et al.*, 1998). During the past decade, primary cultures of isolated human hepatocytes have proved to be a valuable model in which to study the inducing potential of drugs on human cytochrome P450 isoenzymes (Kostrubsky *et al.*, 1999). Major families of inducers have been identified and recently nuclear receptors involved in specific induction pathways have been discovered (Waxman, 1999). Many different matrix conditions have been tested and found to be appropriate given that the proper cell density of high quality of viable hepatocytes is utilised (Maurel, 1996; Silva *et al.*, 1998; LeCluyse *et al.*, 2000). In particular, variability in the rifampacin-induced testosterone 6β-hydroxylase activities is most likely due to subtle differences in seeding density due to differences in cell attachment efficiency (LeCluyse *et al.*, 20001a, b).

This prompted the European Centre for the Validation of Alternative Methods (ECVAM) to fund a collaborative study to harmonise protocols for the isolation and use of human hepatocytes. It was intended that a systematic evaluation of cultured human hepatocytes should be performed to attempt to predict cytochrome P450 enzyme induction. This was to be conducted in several laboratories utilising a large number of donor livers. A pre-

validation study was thus initiated between three laboratories, two in Europe (France and Leicester General Hospital, UK) and one in the USA.

The first step to harmonise this process was to define the conditions required to isolate hepatocytes from human liver tissue. An analysis of data from the Fondation Transplantique, Strasbourg and University of Besançon, was used to identify trends (Alexandre *et al.*, 2003). They found that age, sex, previous chemotherapy, alcohol and tobacco consumption had no influence on yield or viability. They did however find that livers with a high fat content and a long warm ischaemic time had a negative influence on cell yield.

Approximate comparison was made with the study outlined in Chapter 5, although there were some methodological differences between the two studies (Table 19).

| | French study | UK study |
|---|---|---|
| Source of human livers | Surgical liver biopsies from patients with benign livers diseases or liver cancer | Surgical liver biopsies from patients with benign livers diseases or liver cancer (MOD) |
| Flushing at the theatre | No | 1. Soltran<br>2. Viaspan for MOD |
| Transport time | Less than 1 hour | 1. Less than 1 hour<br>2. Up to 5 hours |
| Hepatocyte isolation<br>Glue, number of cannulae, flow rate/cannula | 1. Glue +, 2-4 cannulae, 20-40 ml/min<br>2. Glue -, 2-4 cannulae, 20-40 ml/min | 1. Glue +, 4 cannulae, 25 ml/min<br>2. Glue -, 4 cannulae, 25 ml/min |
| Collagenase | Powder collagenase for hepatocyte (InVitrogen) | Hepatocyte digestion medium (Life technologies) |
| Digestion time | 20–30 minutes | 15–45 minutes |

**Table. 19 Comparison of isolation conditions of the human hepatocytes used in the 2 independent laboratories**

These differences precluded accurate statistical assessment, however a protocol was agreed to start the ECVAM study (Table 20). The combined experiences and result,s showed that good quality hepatocytes could be isolated from whole livers by these methods. The identification of significant factors that influenced the quality of the cells was variable between the studies (a possible reflection of methodology) but did not show any major preclusion.

| | Criteria |
|---|---|
| Source of human livers | Surgical liver biopsies from patients with benign livers diseases or liver cancer |
| Flushing at the theatre | 1. No if transport time is not longer than 1 hour<br>2. Yes with Soltran or Viaspan if transport time is longer than 1 hour |
| Transport time | 0 - 5 hours |
| Hepatocyte isolation | |
| - Glue | + |
| - Number of cannulae | 2-4 cannulae |
| - Flow rate/cannula | 20-40 ml/min/cannula |
| - Collagenase | Supplied as powder |
| - Digestion time | Not longer than 20 minutes |

**Table 20. Criteria retained for tissue origin, collection, transport and hepatocyte isolation.**

This study is now ongoing to evaluate the isolation method between the two centres and on the detection of CYP450 induction potency of xenobiotics in human hepatocytes. Further work is also anticipated on the use and isolation of hepatocytes from MOD livers. This is currently an under utilised resource, with the potential to isolate large numbers of hepatocytes.

## 10.7 Final Conclusion

The work presented in this thesis illustrates that the functions of cryopreserved hepatocytes are broadly similar to freshly isolated hepatocytes. Both human and porcine

hepatocytes can be considered for the future evaluation within a bioartificial liver. Further optimisation of the cryopreservation protocol should aim at increasing the number of viable hepatocytes recovered following thawing, and developing further functional assays to assess success of functional preservation.

# Publications and Presentations

## Publications

1. **Lloyd T**, Orr S, Dennison AR.

   The supply and use of human hepatocytes a consumers perspective.

   ATLA 2003: 4 (1) 3-15.

2. **Lloyd T**, Orr S, Skett P, Berry DP, Dennison AR.

   Cryopreservation of hepatocytes: a review of current methods and their applications.

   Cell and Tissue Banking 2003:4 (1) 3-15.

3. **Lloyd T**, Orr S, Patel R, Crees G, Chavda S, Vadeyar H, Berry DP, Sherlock D, Dennison AR

   Effect of patient, operative and isolation factors on subsequent yield and viability of human hepatocytes for research use

   Cell and Tissue Banking 2004:5 (2) 81-87.

4. **Lloyd T**, Orr S, Berry DP, Dennison AR

   The development of a protocol for the cryopreservation of porcine hepatocytes

   Annals of Clinical and Laboratory Science 2004: 34: 165-174.

5. Richert L, Alexandre E, **Lloyd T**, Orr S, Abadie-Viollon C, Patel R, Kingston S, Berry D, Dennison A, Heyd B, Mantion G, Jaeck D.

   Tissue collection, transport and isolation procedures required to optimise human hepatocyte isolation from waste liver surgical resections.

   Liver International 2004: 24: 371-378.

6. **Lloyd T**, Orr S, Berry DP, Dennison AR

The development of a protocol for the cryopreservation of human hepatocytes

Submitted to Cryobiology.

# Published Abstracts

1. **Lloyd T**, Wilkinson J, Orr S, Berry D, Dennison A.

   Studies into the cryopreservation of human and hepatocytes.

   Journal of Pharmacy and Pharmacology 2003;Sept s75

   (Presented to the British Pharmaceutical conference, Harrogate, September 2003)

2. **Lloyd T**, Orr S, Trafford J., Berry D, Dennison A.

   Human hepatocyte provision for the pharmaceutical industry- consumer views and future challenges

   Journal of Pharmacy and Pharmacology 2003; Sept S74

   (Presented to the British Pharmaceutical conference, Harrogate, September 2003)

3. **Lloyd T**, Patel R, Crees G., Chavda, S., Orr S, Berry D, Dennison A.

   Patient, operative and processing variables: the effect on the quality of isolated human hepatocytes and subsequent function

   Journal of Pharmacy and Pharmacology 2003; Sept S74

   (Presented to the British Pharmaceutical conference, Harrogate, September 2003)

4. **Lloyd T**, Orr S, Sheen A, Garcea G, Kingston S, Vadeyar H, Berry D, Sherlock D, Dennison AR.

   Large scale human hepatocyte isolation from surgically resected liver tissue: a review of 1 year of the UK Human Tissue Bank.

   British Journal of Surgery 2003;90:S124

   (Presented to the Association of surgeons of Great Britain and Ireland, May 2003)

5. **Lloyd T**, Orr S, Sheen A, Garcea G, Kingston S, Vadeyar H, Berry D, Sherlock D, Dennison AR.

Human hepatocyte supply by the UK Human Tissue Bank.

Drug Metabolism Reviews 2003;35:Supp 1.

(Presented to the International Society for the Study of Xenobiotics, Dijon, April 2003))

# Presentations

1. **Lloyd T,** Orr S, Dennison AR.

   The supply and use of human hepatocytes - a consumer's perspective.

   -British Association of Tissue Banking. Liverpool, April 2003.

   -Hepatocyte Users Group. Aberdeen, March 2003.

2. Richert L, LeCluyse E, **Lloyd T.**

   An ECVAM sponsored pre validation study on the response of primary human hepatocyte cultures to model CYP inducers - criteria retained for setting up the inter laboratory harmonised protocol.

   -Hepatocyte Users Group. Aberdeen, March 2003.

3. **Lloyd T.** , Orr S, Berry DP, Dennison AR

   Cryopreservation of hepatocytes and the bioartificial liver.

   -Lincoln County Hospital, December 2001.

   -Midlands Hepatobiliary meeting, April 2002.

   -Renal and Clinical Sciences, Research Seminar, January 2002.

   -Cancer Institute meeting, Department of Oncology, Leicester Royal Infirmary, March 2002.

   -Hepatocyte Users Group. Aberdeen, March 2003.

   -Leicester Research meeting, September 2003

   -Hepatocyte Users Group. Aberdeen, March 2003.

4. **Lloyd T**, Orr S, Sheen A, Garcea G, Kingston S, Vadeyar H, Berry D, Sherlock D, Dennison AR.

Large-scale human hepatocyte isolation - the work of the UK Human Tissue Bank.

-British Transplant Society. London, April 2003.

5. **Lloyd T**, Wilkinson J, Patel R, Crees G, Chavda S, Orr S, Berry DP, Dennison AR.

Patient and processing variables: their effect on the quality of isolated human hepatocytes for banking.

-British Association of Tissue Banking. Liverpool, April 2003.

-Hepatocyte Users Group. Aberdeen, March 2003.

# Appendix

## 1. Donor Information Sheet

### Donor Information from October 2001

Age Hospital Sex Date

**Patient Factors**

BMI Race Blood Group

Alcohol units/wk Smoking pack years

Chemotherapy Y N What/When

Past Medical History

Medication

Social History

Allergies

**Pre op Bloods**

| | | |
|---|---|---|
| Bilirubin | HB | Na |
| AST | WCC | K |
| AP | Plat | Ur |
| ALB | | Cr |

Previous Liver Surgery

**Surgery**

Resection Performed Y N

Procedure

Pringle time in total

**Isolation** **Performed by** **at**

Canulation type

Size of tissue

No. of cells isolated Viability %

Transfer time in cold storage

Digestion time required

Digestion Poor Moderate Good

Digestion type used

Time for isolation (once in lab)

**Pathology**

Cirrhotic Y N

Fatty Y N

Underlying pathology for surgery

## 2. Hepatocyte Wash Buffer

| Chemical | Conc. |
|---|---|
| NaCl | 120mM |
| KCl | 6.2mM |
| $CaCl_2$ | 0.9mM |
| Hepes | 10mM |
| Albumin | 0.2% |
| Distilled water | 5 litres |

The solution requires adjusted to pH 7.4, and filtration under vacuum suction in a Biosafety Class II cabinet using a 0.22µl filter to sterilise.

## 3. Porcine perfusion and digestion medium

**Stock buffer** **to 5 litres of ionised water**

| | |
|---|---|
| Sodium Chloride (NaCl) | 154mM |
| Potassium Chloride (KCL) | 5.6mM |
| Glucose | 5mM |
| Sodium Bicarbonate ($NaHCO_3$) | 25mM |
| Hepes | 20mM |

The solution was then adjusted to a pH of 7.4

**Porcine perfusion Buffer I**

To 500ml of stock buffer 1mM EGTA and 40mg/l dexamethasone dissolved first in a small volume of methanol, were added.

**Porcine perfusion Buffer I I**

1mM EGTA added to stock buffer.

**Porcine perfusion Buffer I I I**

100ml of stock buffer

**Porcine digestion Buffer IV**

To 800ml of stock buffer add:

5mM $CaCL_2$ (1.08g)

500mg of collagenase (Sigma-Aldrich, Gillingham, U.K., cat. no. C-5138) suspended in 100mls of buffer IV. Sterilised using 0.22 μm filters and a vacuum pump. This was then added to the remaining 700mls of buffer IV before being circulated through the liver as described in section 4.3.3.

Note: Each solution was filter sterilised using bottle top filters.

## 4. Mobile Phase for HPLC analysis of lignocaine metabolism

To 2 litres of purified water the following were added

- 3.2mls of phosphoric acid (Sigma, cat. no. P6560)
- 400 μl of Triethylamine (TEA) (Sigma, cat. no. T0886)

The phosphoric acid/ TEA solution was mixed at a 85:15 proportion with Acetonitrile 190 FAR UV (Rathburn cat. no. 1016PF).

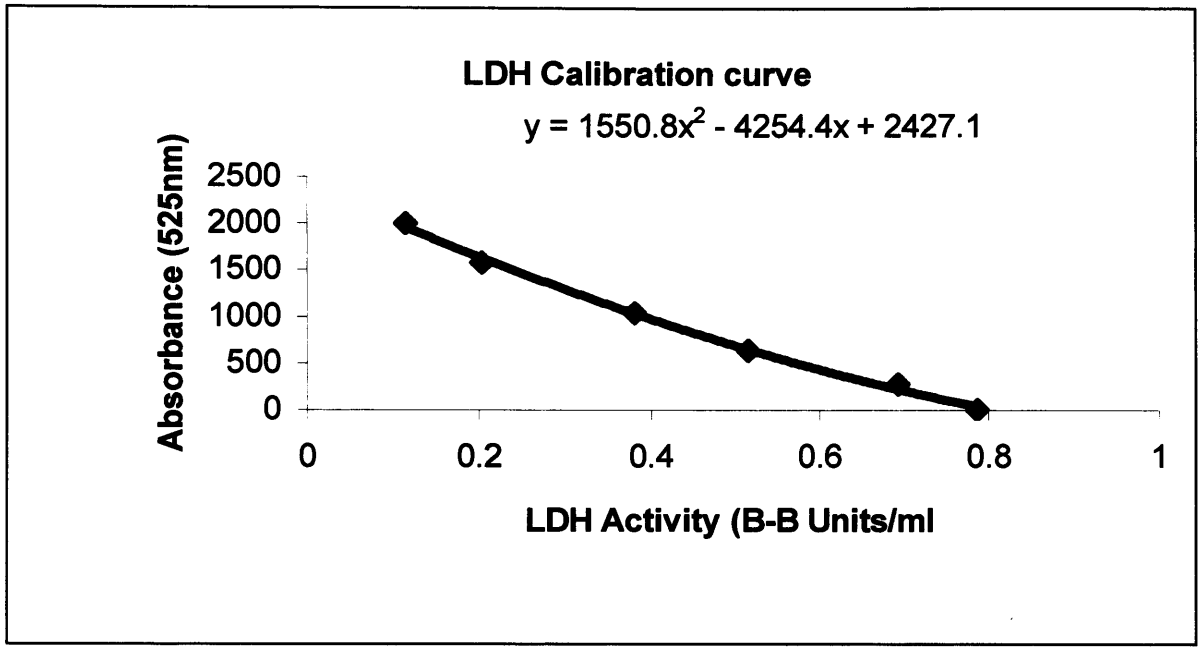
## 5. LDH Calibration

Six eppendorfs were labelled 1-6 and the mixtures in columns 2 and 3 were prepared (table 2).

| Tube No. | Pyruvate Substrate Cat.no. 500L-1 | Water | LDH Activity(B-B Units/ml) |
|---|---|---|---|
| 1 | 100µl | 10 µl | 0 |
| 2 | 80 µl | 30 µl | 280 |
| 3 | 60 µl | 50 µl | 640 |
| 4 | 40 µl | 70 µl | 1040 |
| 5 | 20 µl | 90 µl | 1580 |
| 6 | 10 µl | 100 µl | 2000 |

**Table 2. Calibration Preparation**

To each Eppendorf 100µl of sigma colour reagent (Sigma, cat. no. 505-2) were added, mixed and allowed to remain at room temperature (18-26°C) for 20 minutes. One ml of 0.40N Sodium Hydroxide was then added and mixed by inversion. After at least 5 minutes but no more than 30 minutes the solutions are placed in cuvettes (Starstadt cat. no. 67.742) and read using the spectrophotometer at a wavelength of 460nm. A standard curve was plotted using the absorbance against the corresponding LDH generated.




Using the equation of the line the absorbance results are entered into the equation and the LDH activity calculated

## 6. Determination of cell viability and yield by trypan blue staining

i. To 20µls of suspended cells the following are added,

- 160 µls Hank's Balanced Salt Solution (Sigma cat no. 24020-133).
- 20µl of trypan blue solution 0.4% (Sigma cat no. T8154)

ii. Ten µls of this solution were pipetted on to a Haemocytometer. All 4 squares labelled A-D were counted.

iii. To calculate the viability of the hepatocytes all the cells were initially counted and then all the cells that had taken up the dye were counted. The following calculation was then performed.

$$\frac{\text{Cells taking up dye}}{\text{Total number of cells}} \times 100 = \% \text{ viability of the hepatocytes}$$

iv. Cell yield is calculated by counting the total number of cells (A) and performing the following calculation;

$A/4 \times 1x10^4 \times 10$ = Number of cells /ml of suspension.

The total number of viable hepatocytes calculated by multiplying the number of cells/ml by the volume of the original cell suspension.

## 7. Standards for the Protein Assay

Protein stock solution was prepared by dissolving human albumin in 6M urea solution to a concentration of 0.5mg/ml. The calibration standards were then prepared from the stock solution using the volumes shown in the table below

| Vol. of 0.5mg/ml protein stock (µl) | Volume of 6 M urea (µl) | Final protein concentration (mg/ml) |
|---|---|---|
| 1000 | 0 | 0.5 |
| 800 | 200 | 0.4 |
| 600 | 400 | 0.3 |
| 400 | 600 | 0.2 |
| 200 | 800 | 0.1 |
| 100 | 900 | 0.05 |
| 0 | 1000 | BLANK |

## 8. Sodium Bicarbonate buffer

1 M solution

8g of Sodium Bicarbonate added to 95mls of purified water.

PH made up to 10.5 using 5M Sodium Hydroxide

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