STUDIES OF HUMAN AND PORCINE HEPATOCYTE CRYOPRESERVATION AND THEIR SUITABILITY FOR USE IN BIOARTIFICIAL LIVER DEVELOPMENT

Thesis submitted to University of Leicester

MD

Clare Jane Pattenden

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Studies of human and porcine hepatocyte cryopreservation and their suitability for use in bioartificial liver development.

CJ Pattenden

Abstract
The aim of this thesis was to assess feasibility of using chronically injured liver as a source for hepatocyte isolation, to compare inter-laboratory variability, to establish an efficient method for hepatocyte isolation and cryopreservation and to review whether anti-apoptotic compounds have any effect on cryopreservation outcomes.

Method
A two-stage isolation protocol was used, with multiple washes and centrifugation compared with Percoll® purification pre- and post-cryopreservation and pre-incubation. A new cryopreservation media was validated and cells cryopreserved using this with the addition of potential anti-apoptotic agents. Demographic details were collected prospectively and histological samples collected where possible for analysis. Hepatocyte viability, yield per gram, hepatocyte loss, monolayer protein, visual attachment, phase I and II enzyme activity and measures of apoptosis were assessed.

Results
Between October 2003-2005, 83 individuals donated tissue to UKHTB, with an additional 140 donors from elsewhere. 175 hepatocyte isolations were performed yielding more than 50 billion cells. Fresh cell culture was universally successful although purification led to significant cell loss. This was exacerbated during cryopreservation with especially poor porcine hepatocytes. None of the adjuncts to improved isolation or cryopreservation demonstrated reproducible improvement in outcome. There was only a weak negative correlation between histological injury and isolation outcome. Normothermic resuscitation prior to isolation conferred benefit across almost all measurable outcomes.

Conclusion
There is significant inter-donor variability with regard to the outcomes of hepatocyte isolation and all purification methods cause unacceptable hepatocyte loss. A viable cell will attach and function and this is essential for many of the studies that are subsequently performed. Organs which are turned down for transplantation could be used for hepatocyte isolation.

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There is no single animal species that behaves like a human.

Li 1999

Contents

Glossary

AIF	Apoptosis inducing factor
ALF	Acute liver failure
ALP	Alkaline phosphatase
AoC	Acute-on-chronic
ATP	Adenosine triphosphate
BAL	Bioartificial liver
BATB	British Association for Tissue Banking
BCA	Bicinchoninic acid
BLSS	(Excorp Medical) Bioartificial Liver Support System
BNF	β-naphthoflavone
BSA	Bovine serum albumin
BSG	British Society for Gastroenterology
BUF	Bufuralol
CIT	Cold ischaemic time
CLF	Chronic liver failure
CM	Cellulose microspheres
COREC	Central Office for Research Ethics Committees
CYP450	Cytochrome P450
DIC	Diclofenac
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DoH	Department of health
ECM	Extracellular matrix
ECMO	Extracorporeal membrane oxygenation

ECVAM	European Centre for Validation of Alternative Methods
EDTA	Ethylenediaminetetraacetic acid
EGF	Epithelial growth factor
EGTA	Ethylene glycol tetraacetic acid
ELAD	Extracorporeal liver assist device
ER	Endoplasmic reticulum
FBS	Foetal bovine serum
FCS	Foetal calf serum
FHF	Fulminant hepatic failure
HBS	Hepatobiliary scintigraphy
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HD	Haemodialysis
HEPES	(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HGF	Hepatocyte growth factor
(HIF)-1	Hypoxia-inducible factor
HMM	Hepatocyte maintenance medium
HPLC	High performance liquid chromatography
Hrs	Hours
IR	Ischaemia/reperfusion
IVC	Inferior vena cava
LDH	Lactate dehydrogenase
LGF	Liver growth factor
LGH	Leicester General Hospital
LREC	Local Research Ethics Committee

M	Million
MARS	Molecular adsorbent recirculating system
MELS	Modular extracorporeal liver support
MOD	Multi-organ donor
MRC	Medical Research Council
MTT	3-(4,5-dimethylthiazide-2-yl)-2,5-diphenyl tetrazolium bromide
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Reduced form of nicotinamide adenine dinucleotide phosphate
NAFLD	Non-alcoholic fatty liver disease
NF-κB	Nuclear factor-kappa B
NHBD	Non-heart beating donor
NMGH	North Manchester General Hospital
NRES	National Research Ethics Service
NPC	Non-parenchymal cell
OLT	Orthotopic liver transplantation
PARP	Poly(ADP-ribose) polymerase
PB	Phenobarbitol
PC	Parenchymal cell
PEEK-WC	Polyetherether-ketone
PERV	Porcine endogenous retrovirus
PHEN	Phenacetin
PI	Propidium iodide
PTP	Permeability transition pore
PVA	Polyvinyl alcohol
PVDF	Polyvinylidene difluoride

PVP	Polyvinyl pyrrolidone
RCP	Royal College of Physicians
R&D	Research and Development
RIF	Rifampicin
ROS	Reactive oxygen species
SAPK	Stress activated protein kinase
SMA	Superior mesenteric artery
TEST	Testosterone
TNF	Tumor necrosis factor
TUNEL	Tdt-mediated dUTP-digoxigenin nick-end labelling
Tx	Transplant/transplantation
UDCA	Ursodeoxycholic acid
UKHTB	UK Human Tissue Bank
UW	University of Winconsin solution
WIT	Warm ischaemic time
ZVAD-fmk	Benzyloxycarbonyl-Val-Ala-DL-Asp-fluoromethylketone
γ GT	Gamma glutamyl-transferase
2D	Two-dimensional
3D	Three-dimensional
7HC	7-hydroxycoumarin
7HCG	7-hydroxycoumarin glucuronide
7HCS	7-hydroxycoumarin sulfate

Introduction

1.01 Overview

Interest in external liver support including both artificial and bioartificial systems (BAL) began in the 1950s and continues to develop. Originally a concept for the treatment of fulminant hepatic failure it potentially has wider applications. These include the concept of "bridging" patients with hepatic failure until a donor organ is available for liver transplantation which is currently the only long-term treatment (Strong 2001). Donor organ shortages and the length of time patients wait for a transplant organ have made the need for an artificial system capable of supporting patients increasingly acute.

Advances in surgery and anaesthesia have led to dramatic advances in the field of liver surgery. Primary and secondary malignancies can be treated by major resections of up to 75% by volume of functional liver tissue (Clavien *et al.* 2010). Larger resections are not currently feasible as the patients' remaining liver is unable to cope with the metabolic demands placed upon it. It is hoped that a functioning BAL will aid such patients while the liver remnant recovers sufficiently, therefore allowing more extensive resections to take place. A resection of 90% of the liver parenchyma leaving just the caudate lobe would become theoretically possible.

If a human tissue bank were successfully established it might abolish the requirement for a porcine cell BAL where safety concerns have been expressed (Dowling & Mutimer 1999). These include hypersensitivity reactions and the potential for humoral sensitization (Cotterell *et al.* 1995). Additionally there is concern regarding zoonoses, in particular the pig endogenous retrovirus (PERV) that has been transmitted to human cells *in vivo* (Patience *et al.* 2001).

Early attempts at constructing a BAL concentrated on detoxifying the blood with plasma or blood exchange, haemodialysis, haemofiltration, cross-circulation and the use

of charcoal. These measures had little impact in the long-term prognosis of patients and it was realised that the complexities of acute liver failure were not going to be resolved by these methods. Hepatocytes were required to detoxify and maintain metabolic functions, the synthesis of protein and macromolecular structures and to partake in immune and hormonal pathways.

In 1969, Berry and Friend developed a method for isolating fresh hepatocytes that enabled the study of hepatocytes in an extra-corporeal bioreactor. This has lead to the production of a new generation of bioreactors and their application to animal models (Fremond *et al*. 1993, Sheil *et al*. 1996) and human trials (Watanabe *et al.* 2001).

The basic design of most BAL systems consists of a hollow fibre cartridge housing hepatocytes in culture (figure 1). Detoxification is achieved by the flow of plasma or blood across the surface of the structure allowing diffusion across the interface. The different designs under evaluation have been repeatedly reviewed, Strain & Neuberger 2002, Court *et al*. 2003, Park & Lee 2005) and the array of models clearly illustrates the complexity of such a system.

Figure 1 Basic BAL design (www.landesbioscience.com).

One of the difficulties with BAL development is the availability and source of fresh hepatocytes. The methods for isolating and maintaining hepatocytes in culture are well described from various animal species and humans (Loretz *et al*. 1989). Extraction of fresh human hepatocytes relies on the availability of liver tissue which is in short supply. With the limited time that cells function once isolated, there is the need to develop protocols for the long-term storage of functioning hepatocytes. Cryopreservation has long been considered an ideal solution but is challenging in practice and no standard protocol has been internationally accepted although some have been suggested.

There has been significant work undertaken with both human and porcine hepatocytes, reviewing variables that may impact on human hepatocyte yield and viability. These include donor factors, freeze rates and pre-incubation and the storage concentration of hepatocytes. Despite promising results, research should continue to improve cryopreservation procedures.

Whilst hepatocyte viability declines over time after isolation, the extent to which we can achieve the goal of a clinically significant BAL depends on maintaining the function of these cells. There is consequently increasing interest in the use of anti-apoptotic and anti-necrotic agents within the field of cryopreservation. Apoptosis contributes to cell death in banked hepatocytes and therefore strategies are required to increase viability. Agents have been developed which demonstrate anti-apoptotic activity in cultured hepatocytes and include benzyloxycarbonyl-Val-Ala-DL-Asp-fluoromethylketone (ZVAD-fmk) (Nyberg *et al*. 2000), caspase I inhibitor V (CryoStor CS 5N®) (Baust *et al*. 2000) and glucose (Fu *et al*. 2001). Further effort aimed at modulating apoptosis could significantly improve pre-existing methodology.

1.02 Anatomy and Physiology

1.02.01 Gross human anatomy

The liver is the largest organ in the body, weighing approximately 1500g in a healthy adult (figure 2).

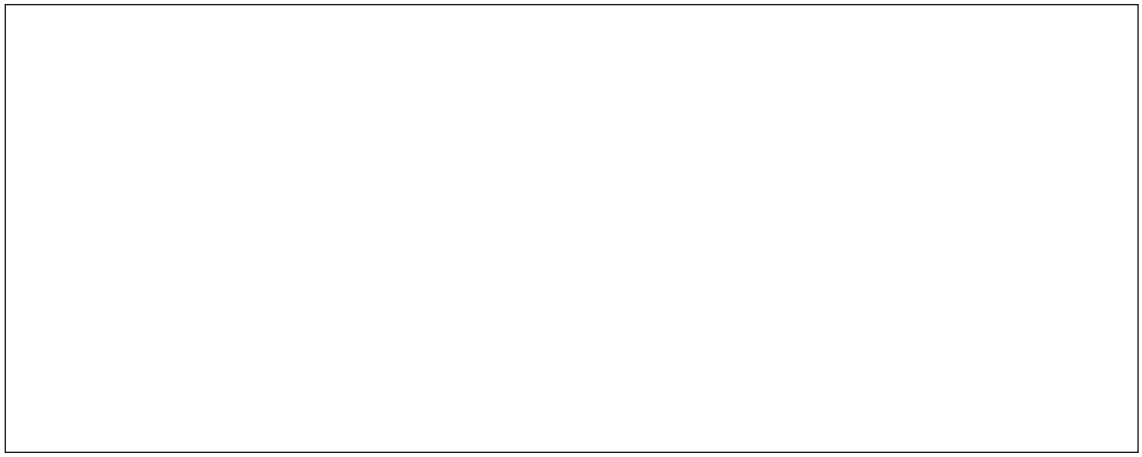


Figure 2 Liver anatomy (Encyclopedia Britannica 2011).

On the basis of circulation and biliary ducts, the liver is divided into eight segments as first described in 1957 by Couinaud (figure 3).

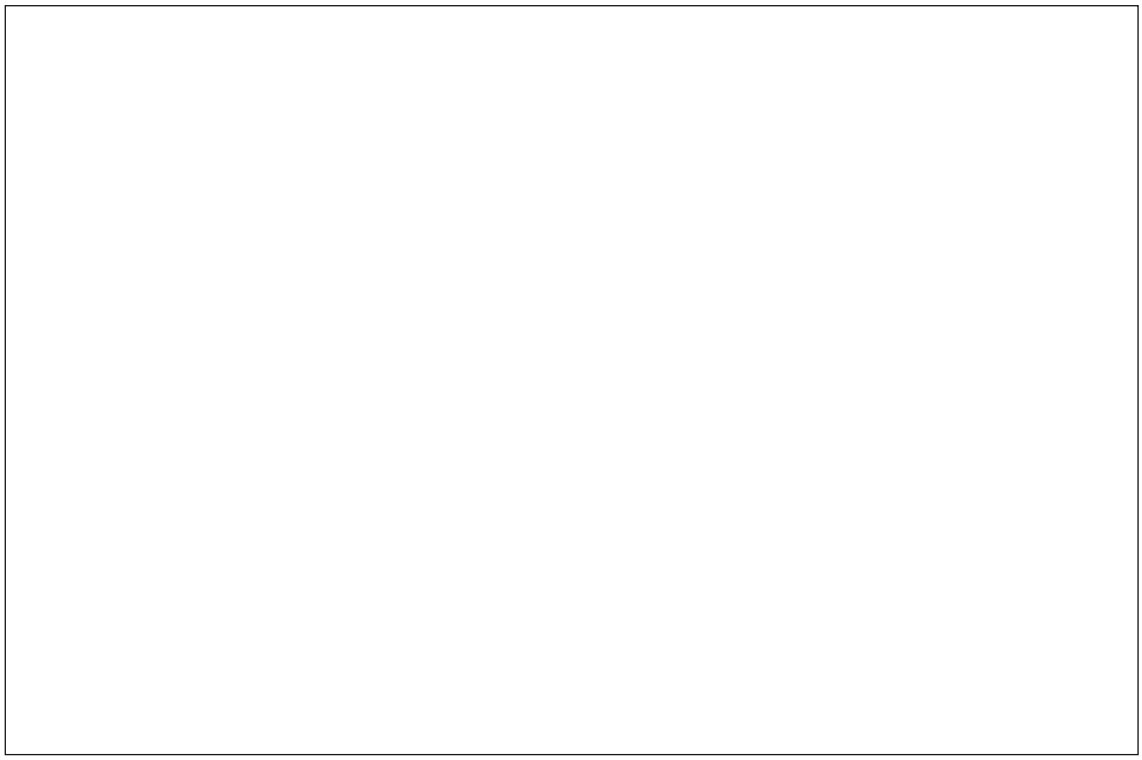


Figure 3 Segmental liver anatomy (About cancer 2011).

The liver receives 1500 ml of blood per minute (25% of cardiac output) through the main vessels positioned on the visceral surface, with hepatic veins emerging posteriorly. Arterial blood is supplied by the common hepatic artery which divides into right and left at the porta hepatis. Venous circulation is via the portal vein, again dividing at the porta hepatic into right and left, with segmental branches thereafter. Portal blood is rich with the products of digestion. Understanding basic anatomy allows for efficient hepatocyte isolation procedures (Sinnatamby 1999).

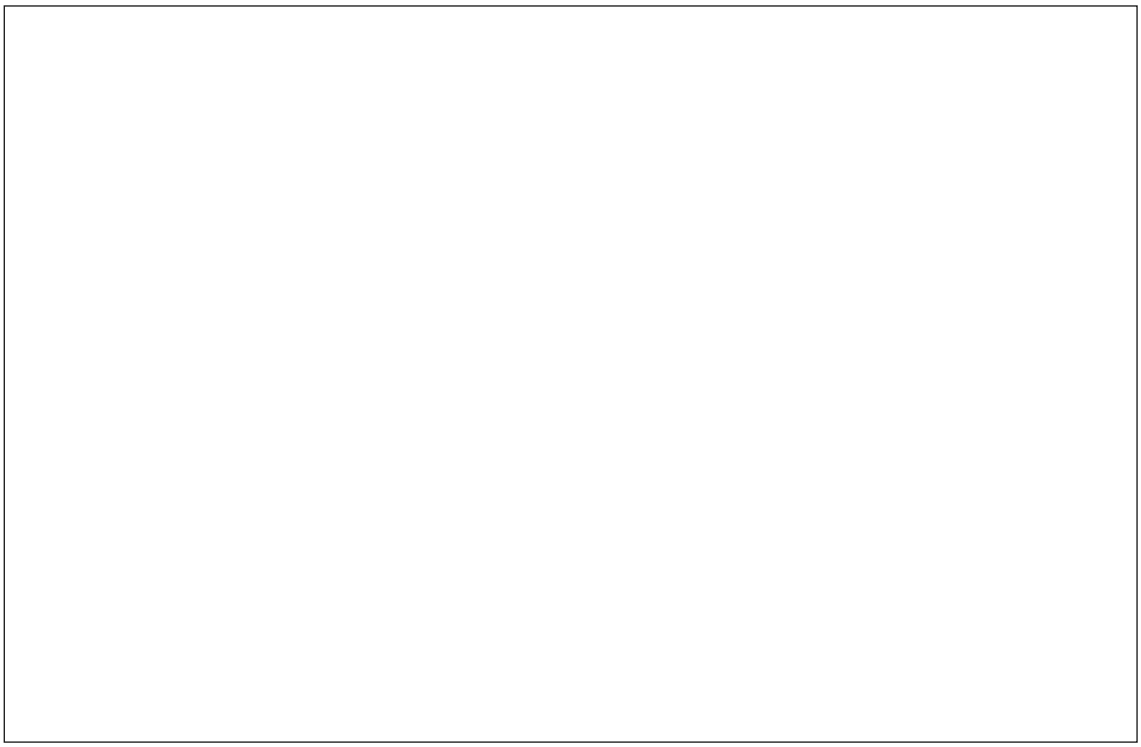


Figure 4 Diagrammatic representation of a liver lobule (Cunningham & van Horn 2003).

On a cellular level (Burkitt *et al*. 1993), (figure 4) the liver parenchyma (made up of hepatocytes) is arranged in polyhedral lobules, bound by a supporting collagenous membrane (collagen type III) around a central terminal hepatic venule fed by sinusoids running around the individual hepatocytes. These are separated by the narrow space of Disse which drains the lymphatics of the portal tracts. Stellate cells encircle the sinusoid with microprojections approaching the hepatocytes, with Kupffer cells (resident macrophages) and Pit cells (natural killer cells) moving through blood and tissue

compartments. Bile is collected by the bile canaliculi, which form a network amongst the hepatocytes draining to a collecting duct that runs with terminal hepatic artery and portal vein branches located at the angle of the lobule boundary in portal tracts/triad. Portal tracts in contrast to the hepatic venules drain more than one lobule. Hepatocytes lay one cell thick, in plates being exposed to blood therefore on at least two surfaces. These then branch and anastomose forming a three dimensional structure that macroscopically resembles sponge.

At the periphery of the liver the collagenous fibres become continuous with the tough Glisson's capsule, which covers its entire external surface and is an aid to hepatocyte isolation.

1.02.02 The hepatocyte

Hepatocytes are large polyhedral cells, with prominent nuclei containing peripherally dispersed chromatin and prominent nucleoli (figure 5). Hepatocyte nuclei are varied in size reflecting their polyploidal complement of chromosomes. More than half the hepatocytes in a normal liver are greater than diploid. On cross section, binucleate cells may be seen, with an estimated 25% of cells actually containing two separate nuclei. There is extensive cytoplasm which represents the storage capacity of healthy tissue, but glycogen and lipid are removed during histological processing and is not represented well with histopathological sections. The cytoplasm is eosinophilic due to the high mitochondria content with basophilic granularity due to free ribosomes and rough endoplasmic reticulum (Burkitt *et al.* 1993).

Figure 5 A hepatocyte.

1.02.03 Porcine anatomy

The pig is an omnivore with digestive and endocrine functions similar to that of the human and as such, it is the animal model of choice in studies of both hepatic function and bioartificial liver development. Morphologically the liver is very different to that of the human. It is flatter and wider, with a negligible falciform ligament. The porcine liver contains four lobes (unlike human liver, with three including the caudate) and is essentially clover shaped (figure 6).

There is a middle or median lobe divided into left and right by the umbilical fissure, a left lateral lobe, a right lateral lobe and a caudate lobe (figure 6).

Figure 6 Porcine liver (Fernandez-Cordero *et al.* 1992).

1. Left lateral lobe 2. left medial lobe 3. right lateral lobe 4. right medial lobe 5. quadrate lobe 6. caudate lobe 7. porta hepatic 8. gallbladder 9. median plane 10. caudal vena cava 11. hepatic artery 12. portal vein and lymph nodes 13. bile duct 14. oesophageal notch.

1.02.04 Function

The liver has a multitude of functions; synthetic, metabolic, immunological, hormonal and detoxifying (table 1).

1.02.05 Phase I and II metabolism

The liver is the major site of enzymatic metabolism (also known as biotransformation). This process is typically two stage or biphasic with oxidation, reduction or hydrolysis preceding subsequent conjugation with an endogenous agent. Phase I biotransformations (functionalisation reactions) can be viewed as the step that 'prepares' a compound for the phase II reaction (detoxication or conjugation reactions) (table 2).

Function	Example
Synthetic	Bile
	Plasma proteins (albumin)
	Plasma lipoproteins
	Amino acids
	Clotting factors: I (fibrinogen), II (prothrombin), V, VII, IX, X and XI, protein C, protein S and antithrombin
	Hormone synthesis: insulin-like growth factor, thrombopoietin, angiotensinogen
	Red cell production (first trimester)
Metabolic	Cholesterol synthesis
	Lipogenesis
	Gluconeogenesis
	Glyconeogenesis
	Glycogenesis
	Hormone degradation
	Deamination of ammonia to produce urea
	Drug and toxin breakdown
	Destruction of spent red blood cells and reclaiming of their constituents
Storage	Vitamins A, D, B12
	Iron
	Copper
Immunology	Reticuloendothelial system carries antigens to the portal system

Table 1 Summary of liver function.

Phase I	Phase II
Oxidation Reduction Hydrolysis Hydration Dethioacetylation Isomerisation	Glucuronidation/glucosidation Sulfation Methylation Acetylation Amino acid conjugation Glutathione conjugation Fatty acid conjugation Condensation

Table 2 Reactions classed as phase I or II metabolism (Gibson & Skett 1994).

Following the phase II reaction, the inactive compound can then be excreted in bile or urine. Phase I metabolism is most commonly performed by the cytochrome p450 isoenzyme family (CYP450) (table 3). Although each may catalyse similar reactions they display selectivity towards substrates (Caldwell *et al*. 1995). They are found within the inner membrane of the mitochondria or outer face of the endoplasmic reticulum (ER) and are most abundant within liver.

Reaction	Substrate
Aromatic hydroxylation Aliphatic hydroxylation Epoxidation N-Dealkylation O-Dealkylation S-Dealkylation Oxidative deamination N-Oxidation S-Oxidation Phosphothionate oxidation Dehalogenation Alcohol oxidation	Lignocaine Pentobarbitone Benzo[a]pyrene Diazepam Codeine 6-Methylthiopurine Amphetamine 3-Methylpyridine 2-Acetylaminofluorene Chlorpromazine Parathion Halothane Ethanol

Table 3 Reactions performed by the microsomal mixed-function oxidase system (Gibson & Skett 1994).

All of these reactions require molecular oxygen, NADPH, CYP450 and lipid. Initially an oxygen atom is inserted into the molecule. Subsequent rearrangement or decomposition occurs leading to the final product. These CYP450 pathways are involved in reactions such as detoxification and elimination of endogenous and exogenous substances (including drugs), formation of pharmacologically active drugs and generation of toxic metabolites.

The phase II conjugation reactions involve a diverse enzyme group with each reaction requiring a combination of energy-rich intermediaries or 'activated' precursors. They are grouped dependent on the energy source for the process, either from the conjugating agent itself or the prior phase I reaction (table 4, 5). In the vast majority of cases the end product is water soluble. Unlike the phase I reactions, only glucuronosyl transferase is found within the ER with all others found in the cytoplasm.

Reaction	**Enzyme**	**Functional group**
Glucuronidation	UDP-Glucuronyltransferase	-OH -COOH -NH_2 -SH
Glycosidation	UDP-Glycosyltransferase	-OH -COOH -SH
Sulfation	Sulfotransferase	-NH_2 -SO_2NH_2 -OH
Methylation	Methyltransferase	-OH -NH_2
Acetylation	Acetyltransferase	-NH_2 -SO_2NH_2 -OH
Amino acid conjugation		-COOH
Glutathione conjugation	Glutathione-S-Transferase	Epoxide Organic halide
Fatty acid conjugation		-OH
Condensation		Various

Table 4 Phase II conjugation reactions (Gibson & Skett 1994).

Reaction	Substrate
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 5 Phase II metabolism of endogenous compounds (Gibson & Skett 1994).

1.02.06 Cytochrome P450

The CYP450 enzymes are a family of closely related isoenzymes present in the ER and is classified as a haem-containing enzyme with an iron protoporphyrin IX as the prosthetic group (figure 7).

Cytochrome P450

$$NADPH + H^+ + RH \rightarrow NADP^+ + H_2O + ROH$$

RH represents the substrate
ROH represents the hydroxylated substrate

Figure 7 Cytochrome P450 stoichiometry.

These are non-covalently bound and their name is derived from the fact that the cytochrome exhibits spectral absorbance at 450nm when reduced and complexed with carbon monoxide. Full-length CYP450 amino acid sequencing has now been achieved with more than 200 isoenzymes isolated, although just over 30 of these are specific to humans. This has in turn led to a unified nomenclature for these enzymes. Vital to the study of the major human hepatic CYP450 isoenzymes is the understanding that each has both inhibitors and inducers of action.

1.03 Liver disease

1.03.01 Acute liver failure

The increasing need for hepatic replacement therapy stems from the increasing prevalence of liver disease across the globe. Acute liver failure (ALF) is defined as onset of encephalopathy within eight weeks of the onset of jaundice in a patient with no history of liver disease (Trey & Davidson 1970). This was redefined in 1993 (O'Grady *et al*. 1993) describing hyperacute (less than seven days), ALF (8-28 days) and subacute (5-12 weeks). Fulminant hepatic failure (FHF) is the appearance of severe acute liver disease progressing to hepatic encephalopathy in a previously healthy person within a two week period. ALF may be caused by viral infection, drugs, toxins, metabolic disorders, drug overdose or reaction. The mortality rate of ALF is as high as 80% (Lee 1993), treatment is supportive and in some cases culminates in whole organ transplantation. To avoid confusion the term ALF will encompass all of these subdivisions.

1.03.02 Chronic liver disease

Chronic disease (CLD) can be categorised into alcohol induced, non-alcoholic fatty liver disease (NAFLD), viral, cholestatic, autoimmune, metabolic and malignant. Many pathologies are multi-factoral (Ostapowicz & Lee 2000) and all can lead to cirrhosis and ALF.

There is no comprehensive registry of patients affected by ALF either in the UK or the USA and the only definitive treatment is liver transplant. The number of referrals to National Transplant waiting lists are likely to significantly underestimate the prevalence of this condition.

1.03.03 Disease burden

In the USA the incidence of CLD is estimated at 72.3 per 100000 population, with hepatitis C (57%) and alcohol (24%) the most common aetiologies (Bell *et al*. 2001). Liver disease causes 44677 deaths per year in America, equating to nearly 2% of all deaths and the 10th highest killer in total (CDC 2002). Although the data is old, in 1985 direct estimated cost of liver disease totalled $1.5 billion, within indirect costs of $2.4 billion (Everhart 1994), equivalent to 1% of the national health budget.

The United Kingdom is the only developed country in the world where the incidence of liver disease is increasing (Kaner *et al*. 2007). It is now our fifth biggest killer (Office of National Statistics 2007) with no clear governmental strategy to tackle this. Much of the increasing incidence of liver disease is a consequence of lifestyle, with obesity, alcohol abuse and viral hepatitis the main culprits. Although the development of liver disease is silent it often presents as an acute illness with a 25-50% immediate mortality (Office of National Statistics 2007).

In 2005 a total of 13865 people died from liver disease and related conditions (Office of National Statistics 2007). Every year over 100 people die awaiting a liver transplant on the UK Transplant waiting list (NHS Blood and Transplant 2011).

1.03.04 Resectional surgery

Surgical liver resection patients may also be at risk of ALF. The liver has enormous potential for regeneration in response to cell loss by hypertrophy and hyperplasia. The mechanisms for hepatic regeneration are unclear, complex and may be dependent on insult (Court *et al*. 2002). Broadly speaking, as with rats (Rozga 2002), at best two-thirds of the liver can be safely resected in patients with healthy tissue. In humans it is clear that patient selection for hepatectomy must take into account CLD and other co-morbidities (especially

diabetes). The more positive markers, the higher the risk of postoperative liver failure (Shirabe *et al*. 1999). No single risk factor has yet been identified, nor is there an adequate predictive scoring system (Mullin *et al*. 2004).

Surgery is usually for a malignant process and may be primary or secondary, although there are less common benign liver tumours that may be excised. Colorectal cancer is the most common and principally metastasises to regional lymph nodes and the liver. Up to 50% of all patients with colorectal cancer eventually develop liver involvement (Bramhall *et al*. 2003). Primary hepatic malignancies (hepatocellular cancer, cholangiocarcinoma) may also be cured by surgical resection.

Depending on disease distribution and type of lesion, variable amounts of parenchyma are resected. Up to 6 of 8 anatomical liver segments may be removed without causing postoperative liver failure providing the remaining liver parenchyma is normal (Clavien *et al*. 2010). If more than 70-90% of functioning liver is resected, the patient risks liver failure and death. In high risk patients (the elderly or those with severe steatosis or cirrhosis) the functional volume that can be safely resected is less. The remarkable ability of the liver to regenerate, may make it possible to safely resect larger volumes of liver tissue than currently considered acceptable, provided that adequate support could be provided in the postoperative period to aid regeneration. One concern with BAL therapy is that stimuli for regeneration could be lost in the dialysate or filtration process (Mullin *et al*. 2004).

Hepatic surgery has significant morbidity and mortality irrespective of underlying hepatic disease. In the presence of hepatocellular disease these risks are magnified and patients tolerate surgery less well, with more complications. Post-operative hepatic failure is a surgical catastrophe and carries a poor prognosis. In these circumstances residual parenchyma is inadequate, there is a gradual deterioration in the condition of the individual, the tissue is unable to recover and regenerate and the patient progresses to ALF.

Many scoring systems used to evaluate function of the liver have little or no value in determining post-operative risk or assessing functional reserve of the remnant *in situ*. Scoring systems currently used to evaluate function of the liver have little or no value in determining post-operative risk or assessing functional reserve of the remnant *in situ* (Mullin *et al.* 2004). More recently, radioisotope hepatobiliary scintigraphy (HBS) using labelled ^{99m}Tc has been developed, with studies demonstrating improved risk assessment, especially when compared with CT volumetry (de Graaf *et al*. 2010). Patients with underlying hepatic disease are unpredictable safe resectional surgery is difficult. Improvements in statistics are due to a combination of technical innovation, use of tertiary centres for intervention, multi-disciplinary decision making and better patient selection with skilled and experienced medical teams. An effective BAL would allow more radical surgery, giving more patients curative surgery with reduced morbidity in borderline patients. In patients with underlying hepatic disease predicting safe resection volumes for surgery is difficult. Improvements in outcomes represent a combination of technical innovation, use of tertiary centres for intervention, multi-disciplinary decision making and better patient selection with skilled and experienced medical teams. An effective BAL would allow more radical surgery, giving more patients access to curative surgery and with reduced morbidity in borderline patients.

1.03.05 Animal models of ALF

Before hepatic replacement therapy can be put through phase I clinical trials using human subjects, animal models must be developed to test these therapies in terms of efficacy and safety. The complicating factor in this model is that the animal should be conscious as hepatic encephalopathy (an essential component of ALF) can only be measured in the conscious subject.

Large animal models of ALF have been described and subdivided dependent on the surgical procedure involved. The partial hepatectomy model can be used to assess liver regeneration (Kahn *et al.* 1988, Court *et al*. 2004), but the difficulty here is that surgical skill is required to perform the surgery, with reasoned judgement ensuring the production of ALF with enough tissue to regenerate. This would be the ideal candidate for a model to mimic that of a patient following liver resection surgery.

The anhepatic model is a pure model of ALF as there is no remnant to perform any function. Surgery is more straightforward with ligation of hepatic artery and common bile duct with subsequent reconstruction of the retrocaval portal vein. Thereafter the liver is excised and it would be possible in this model to use the liver for hepatocyte isolation (Sosef *et al*. 2002). This model would be the ideal representation of irreversible ALF, such as following primary graft failure after transplantation.

The ischaemic model follows either temporary or permanent clamping of the hepatic artery with a portocaval shunt (van de Kerkhove *et al*. 2004). This is often a less significant insult as collateral circulation may continue to provide blood supply to the organ. When this does not occur there is an additional burden of large volumes of necrotic tissue *in situ*.

Finally, the hepatotoxic model involves exposing the pig to an appropriate toxin such as acetominaphen (Terblanche & Hickman 1991) or galactosamine (Patzer *et al*. 2002). Both agents cause hepatocyte necrosis, with the former having unpredictable dose-responses and the latter carrying potential hazard to personnel. Although representing a post-paracetamol overdose human target, these models are rarely used.

1.04 Logistics

1.04.01 Banking human hepatocytes

Cells culture techniques were first described in 1885 (Roux 1885). Biomedical research continues to be performed using cells of animal origin, although the trend is to use human tissue when possible. Unfortunately, this is expensive and unpredictable in supply. This contrasts with animal tissues that can be kept and killed as required.

It takes approximately 10-15 years and $800000000 to bring a new drug to market (DiMasi *et al*. 2003), reflecting the fact that only 1:10000 new compounds investigated ultimately succeeds as a therapeutic agent. Practical and emotive reasons have led to an increased enthusiasm for using human tissue in drug development. The pharmaceutical industry has historically relied heavily on the use of experimental animals during drug discovery programmes based largely on the assumption that the anatomy and physiology of these animals parallels that of the human (Hawksworth 1994). Fortunately many basic biological pathways are preserved however, there is often a lack of concordance due to variations in hormonal and neuronal influences along with different end-organ receptors.

Despite the value of human tissue research, there are some difficulties. These include ethical issues, optimizing acquisition and supply networks, timing of tissue acquisition and the limitations of an *in vitro* approach. Other concerns include the quality of the specimens; cold/warm ischaemic times, the process of dissection, the type of donor (cadaveric versus living), pathology and pre-operative diagnostic accuracy. Donor heterogeneity must be considered which may be intrinsic (age, gender, past medical history) or extrinsic (lifestyle issues, smoking status, alcohol consumption, drug therapies). The provision of comprehensive donor information may be important in interpreting findings from human tissue studies.

1.04.02 Human hepatocytes

The benefits of using human hepatocytes over xenogenic hepatocytes may be significant, depending on the type of research undertaken. Human hepatocytes are a proven *in vitro* model for the study of metabolism and pharmacotoxicity (Ponsoda *et al*. 2001). It eliminates risk of interspecies transmission of retroviruses in transplantation and BAL development (Patience *et al*. 1997) and removes issues of inter-species variation (Vilei *et al*. 2001). Additionally it is an attractive alternative to use of animals and the associated political, ethical and social concerns involved; however human tissue is not risk-free, with human-human viral transmission a concern.

1.04.03 The UK Human Tissue Bank (UKHTB)

UKHTB was established 10 years ago (Orr & Trafford 2004) and is a cost recovery, non-profit making organisation that accepts and promotes the need for good quality human tissue for research. Donation of both surgically resected tissue surplus to diagnostic requirement and non-transplantable organs from cadaveric donors for this purpose is actively encouraged.

The vast majority of work involves hepatocyte isolation and distribution, although the value of other cell fractions is being recognized.

1.04.04 Leicester General Hospital

Tissue for cellular research is obtained from surgical specimens, removed as part of a liver resection. Excess tissue is not removed and the process of tissue donation is performed under an approved Research and Development (R&D) protocol with Local Research Ethics (LREC) and subsequently Central Office for Research Ethics (COREC) supervision. Due to the changes in the provision of this service across the UK (BSG 2001) workload is

gradually increasing, making this source of tissue increasingly practical (table 6).

Operation	1997	1998	1999	2000	2001	2002	2003
Hemihepatectomy	18	23	28	26	26	25	34
Extended hemihepatectomy	1	3	4	7	9	12	20
Segmental liver resection	10	16	11	13	6	19	22
Wedge metastectomy	5	9	5	9	7	9	20
Total number of liver resections	34	51	48	55	48	65	96

Table 6 Number of potential resections yielding tissue per year at Leicester General Hospital.

1.04.05 The challenge of human hepatocyte tissue banking

There is a perceived international scarcity of human tissue whether for transplantation (Oosterlee & Rahmel 2009) or research (Dickson 2002). Whether this is a true lack of tissue is difficult to elicit. Many hepatocyte banks produce large volumes of cells without distributing them all. Some are used for in-house research and quality control but there is a problem with wastage. Isolations sometimes yield more cells than are needed locally and nationally. Therefore there is a need for long-term preservation techniques so cells can be transported whilst retaining viability and functional capacity.

1.04.06 Donors

In the UK, human hepatocytes are usually obtained as a result of collaboration between NHS departments and tissue banks. Tissue can also be obtained from multi-organ donors whose tissue is unsuitable for transplantation (Baccarani *et al*. 2005) and hepatocytes can

be successfully isolated from tissue removed as part of a graft reduction procedure pre-transplant (Baccarani *et al*. 2005), explanted tissue (from a transplant recipient) (Gerlach *et al*. 1994) and non-heart beating donors (NHBD). Internationally, the use of NHBD organs is an expanding field as a source of tissue for both transplantation and research (Jimenez-Galanes *et al*. 2009, Ferrigno *et al*. 2011). Cadaveric donation in the absence of organ procurement for transplantation is not relevant to the discussion of hepatocyte research as prolonged warm ischaemic times make use of this tissue impossible.

Eurotransplant data from 2009 shows there were 1984 liver donations over that twelve month period. 15.3% (353 organs) were turned down for transplantation for reasons of steatosis or fibrosis (Oosterlee & Rahmel 2009). 1691 patients received cadaveric organs and 98 a living transplant. There were 2641 patients on the waiting list by the end of the year (a need for 852 further transplants/year). Not all were 'high urgency' patients and it is not inconceivable the un-used organs could be used for BAL therapy and might be adequate to bridge the highest risk patients to their transplant.

1.04.07 Tissue banking

Tissue Banks are defined as '*the recovery, processing, sterilisation where appropriate, storage, labelling and distribution of tissues for research and therapeutic use*' (BATB 1999). They are repositories of flash frozen or fixed tissue samples, which may include tissues in both diseased and normal states and constitute a valuable resource for academic and industrial research. It is the duty of the tissue bank to ensure informed consent for use of the donation is obtained and that the research performed has ethical approval. It requires a co-ordinated approach, liaising between transplant and tissue co-ordinators and surgical teams for acquisition of tissue and researchers, pharmaceutical companies, drivers and ethics committees for distribution.

The tissue bank is in an unrivalled position to advance human cell biological research due to their unparalleled access to tissue. With regard to hepatocyte research, tissue banks should be integral to the resolution of international debate regarding all stages of the isolation process, from procurement to storage which include:

Pre-freeze protocols:

- storage solutions
- isolation method
- cell density
- pre-incubation
- Percoll® density gradients

Freezing protocols:

- cryoprotectants and their concentration
- culture configuration
- suspension media
- rates of freezing

Thawing protocols:

- Percoll® gradients
- rates of warming

Additionally the continued development of a bank of cryopreserved hepatocytes is challenged by more esoteric issues that include:

- ethics
- informed consent
- logistics
- legislation
- practicalities

consumer demand

marketing

distribution

funding.

Bureaucracy and evolving administrative guidelines often threaten to engulf tissue banking facilities in the UK (DoH 2003, Human Tissue Act 2004, MRC 2001) as the costs involved in managing such a practice make achieving a financially independent organisation almost impossible. Although many tissue banks tend to focus their efforts on fresh tissue, cells and cellular fractions with their distribution, the need for long-term storage involving the cryopreservation of unused specimens for later dispatch is increasingly recognised as a means of making tissue/cell delivery more practical.

Transporting tissues preserved at low temperature in a dry ice environment means the urgency to use the cells has gone and experiments can be planned. It also means driver and tissue bank technicians are not working late into the evening to dispatch tissues.

Cryopreservation allows for functional assessment in a sample before distribution and at its destination to ensure suitability for the planned work. Tissue banking has the potential to minimise wastage and maximise outcome of each donation.

1.04.08 Ethics

There are a number of complex, inter-related ethical issues facing tissue banking, including the requesting of informed consent, logistics and recent legislation.

Governmental bodies, medical ethics groups and the UK Royal Colleges in respecting the principles of biomedical ethics have stated '*research investigation on human subjects should conform to codes such as the Declaration of Helsinki'* and that '*investigators* (or research scientists) *should not be the sole judges of whether their research does so*

conform' (DoH 2001, RCP 2007). In turn, the declaration of Helsinki explains, '*in medical research on human subjects, considerations related to the well-being of the human subject should take precedence over the interests of science and society*' (World Medical Association 1964). In view of this, ethical committees were developed to review and assess appropriateness of planned research.

1.04.09 Research Ethics Committees

In the UK, research is deemed acceptable by committees of medical, scientific and lay persons. The organisational structure of these committees has evolved over the last ten years although their basic aims remain unchanged. Originally LRECs assessed research projects conducted in the same geographical area where tissue was obtained. Multi-centre research ethics committees became involved when researcher and tissue were in different geographical or multiple areas. Subsequently COREC co-ordinated all UK research projects with a more standardised approach and now the National Research Ethics Service (NRES) performs this role.

These groups have a duty to protect the patient and researcher whilst ensuring a high standard of research is being performed in this country. It is the research ethics committee that decides whether planned research is ethical and therefore whether it may be performed (RCP 2007).

1.04.10 Tissue Co-ordinators

With every donation of organs or tissues there is always a 'co-ordinator'. This individual is the intermediary between tissue bank (or transplant registry) and donor and may be a doctor, specialist nurse or tissue or transplant co-ordinator. In each case they are the individual requesting consent. A large co-ordinator network is required to provide an

adequately significant throughput of tissue. Of note, in the case of transplant or tissue co-ordinators, consent for transplantation is understandably the primary concern and research donation is only requested in the majority of these cases if the explanted organs are found to be unsuitable for transplantation.

1.04.11 Informed consent

Informed consent *is* '*at the heart of ethical research. All studies must have appropriate arrangements for obtaining consent, and the ethical review process pays particular attention to those arrangements*' (Nuffield Council of Bioethics 1995). The law relating to the retention and use of organs and tissues from living and cadaveric sources was reviewed following public inquiries at Bristol Children's Hospital at Bristol Royal Infirmary in 2000 and the Royal Liverpool Children's Hospital; Alder Hey in 2001. These together with the Isaacs Report in 2003 (DoH 2003) demonstrated that organs and tissues from children and adults were commonly stored without proper consent and documentation. These public consultation documents suggested that the current laws required updating and this was undertaken aiming to achieve 'good practice' (The Human Tissue Bill 2003).

The public uproar led by the tabloid press at the time of these inquiries resulted in significantly heightened public awareness of issues surrounding consent and use of human tissue in research. In the Alder Hey inquiry, although the investigation was focused on retained tissues and a lack of consent for storage, many parents found it equally as distressing that that tissue had not been put to use in biomedical research and was essentially wasted. Of course, '*particular care is also needed when research involves tissues or organs of the deceased. The consent of their relatives must always be obtained*' (DoH 2003).

On a practical level, donation of tissue from surgical patients and donors for transplantation is not predictable. Liver resection surgery is scheduled but the actual operation may not be as expected, thus altering the availability of tissue. Procedures may be cancelled at the last minute due to patient health or a simple lack of high dependency beds required postoperatively. Certain patient groups are excluded from donation secondary to infection concerns. Non-English speakers are not asked for consent due to the difficulty of being certain that they have been able to give 'informed' consent as per LREC guidelines. Equally patients only planning to undergo wedge excisions are not consented as this might introduce uncertainty into the individuals' mind about the nature of their surgery. If an appropriately trained co-ordinator is unavailable to request consent, the patient cannot be approached. Despite this, there is significant evidence suggesting that the vast majority of these people are happy to donate their surplus tissue to research (Jack & Womack 2003) and that families do donate multi-organ donor tissue to research (Womack & Jack 2003).

1.04.12 Legislation

New legislation was introduced in 2004 to replace a parliamentary Act more than 40 years old pertaining to the use of tissue samples in the research setting. The Human Tissue Act (2004) is the English legislation produced in response to the organ retention scandals in the UK. The Act focuses on consent as a fundamental principle behind tissue and organ retention with the emphasis on 'appropriate' rather than 'informed' consent.

The removal, storage and use of tissue is regulated and the purposes for which consent is required documented. Regarding tissue from living donors, consent is not needed for tissue analysis from surplus surgical tissue provided the use is clinical audit, education or training relating to human health, performance assessment, public health monitoring and

quality assurance. When this tissue is used for research, ethical approval rather than patient consent is required by law. If data pertaining to the patient is required then both ethical approval and patient consent is required.

1.05 The Contribution of the Donor to isolation outcome

1.05.01 Overview

Every donor is unique, both with regard to demographics and in terms of macroscopic appearance of their tissue including vasculature and steatosis. Equally following hepatocyte isolation there is inconsistency in terms of cell number, viability and quality of the subsequent monolayer. Clearly there is unavoidable variability in terms of the tissue available, but the isolations are performed in an essentially identical manner. The donor therefore appears to dictate outcome rather than other factors.

Comparison of the literature is somewhat challenging, there are few analyses of these factors published and each paper uses different donors, methodologies and outcome measures. Nevertheless this is an attempt at unravelling some of these factors and discussing the potential effects of fibrosis and steatosis on the outcome of donations.

There are few large reviews of hepatocyte isolation and most are reliant on a solitary centres work (Alexandre *et al*. 2002, Lloyd *et al*. 2004, Vondran *et al*. 2007). In the former, yield of viable hepatocytes and percentage digestion of the donor tissue were the measured end points, assessing solitary donor factors such as cholestasis or Percoll® purification against these measured outcomes. Two of these studies (Lloyd *et al*. 2004, Alexandre *et al*. 2002) were later analysed in a collaborative paper (Richert *et al*. 2004). This reviewed a self-selecting group from both papers: those with data obtained prior to Percoll® purification.

1.05.02 Steatosis and fibrosis

In two studies (Alexandre *et al*. 2002, Vondran *et al*. 2007) cholestasis was graded using donor serum bilirubin, alkaline phosphatase and gamma glutamyl-transferase levels and

given a rudimentary rating based upon this. A histopathologist graded the tissue as cholestatic or not (although this is not described, nor graded further and there is no correlation with serum data). Increased cholestasis was statistically insignificantly associated with lower viable hepatocyte yield, although their assessment of cholestasis was clinically inappropriate. With this grading system viable yield from cholestatic human tissue drops from 7.6M +/-1.3 to 5.1M +/-1.2 and with fibrotic tissue from 8.6M +/-1.6 to 5.2M +/-1 (Richert *et al*. 2004).

The 2002 study concluded that although mild steatosis as measured by macrovesicular fat did not affect yield, greater than 10% steatosis did, albeit without statistical significance (Alexandre *et al*. 2002). In the only other study, tissue deemed steatotic at isolation produced hepatocytes of a significantly higher viability than not, with fibrosis not assessed (Lloyd *et al*. 2004). In another large review, steatosis was graded histologically with the percentage of steatotic hepatocytes either less than 10%, 10-20%, 20-30% or greater than 30%. Manifestations of cirrhosis were also commented upon. Worsening steatosis and cholestasis had no effect upon viability, with worsening histology correlating insignificantly with smaller yields (Vondran *et al*. 2007).

Isolation work conducted with organs rejected for transplant also produce high quality hepatocytes, even though these organs are usually morphologically significantly fibrotic, cirrhotic or steatotic (Gerlach *et al*. 2003, Barbich *et al*. 2004, Terry *et al*. 2005). Importantly up to a third of the organs in one study (Gerlach *et al*. 2003) failed at isolation, with viabilities of less than 40%. Comparing steatotic and fibrotic tissues in another human study, again using only organs unsuitable for transplantation, the significant findings were that cirrhotic livers took longer to digest and had a smaller yield of total hepatocytes per gram (Baccarani *et al*. 2001). Total viable hepatocytes were not commented on and cannot be calculated from the paper. Steatotic livers produced the highest yields and viabilities.

Other work has avoided using livers with greater than 50% cirrhosis or steatosis due to perceived poor outcomes (Dorko *et al*. 1994). Hepatocytes isolated from surgical patients have improved viabilities and less signs of cellular injury whether fresh or cryopreserved when compared to organs turned down for transplant (Terry *et al*. 2005). Nevertheless these organs provide hepatocytes with viability and function for practical use.

Using human tissue, yield tends to be low when tissue fatty (n=1) or fibrotic (n=2) (Gerlach *et al*. 1994). Steatotic and cirrhotic tissues faired worse in another human (non-transplantable) tissue study where CIT was long (7-14 hours). Both yield and viability were significantly lowered in organs defined as being cirrhotic by a histopathologist. Over time there was gradual deterioration in CYP450 activity as measured by diazepam metabolism, but equally over time activity became comparable irrespective of histological appearance (Baccarani *et al*. 2003).

1.05.03 Chemotherapy

None of these studies assessed the role of chemotherapy on isolation outcome. Oxaliplatin has activity against colorectal cancer and has been integral to improved downstaging of disease and increased operability.

Although pre-operative chemotherapy is usually well tolerated, these agents are potentially hepatotoxic, with morphological alteration that potentially affects function peri-operatively (Kooby *et al.* 2003). The predominant abnormality is that of steatosis (Rubbia-Brandt *et al.* 2004), with clear links between oxaliplatin use, severe sinusoidal obstruction and perisinusoidal fibrosis. With the use of irinotecan especially in the obese, severe steatohepatitis has been reported (Vauthey *et al.* 2006).

1.05.04 Demographics

The only generic patient factors affecting isolation outcome are age and presence of liver disease: older donors yielded fewer hepatocytes without viability being affected (Dorko *et al*. 1994, Vondran *et al*. 2007), although in other studies viability was reduced (Lloyd *et al*. 2004). Viability and yield from normal and diseased liver did not vary but function as measured by P450 activity was reduced in hepatocytes from diseased tissue with variability determined by donor conditions (Iqbal *et al*. 1991). Few authors describe results by donor (David *et al*. 1998, Mitry *et al*. 2003, Haghighi *et al*. 2004). Reported endpoints differ, therefore data comparison is impossible.

Extremes of tissue weight resulted in worst percentage digestion (Alexandre *et al*. 2002) with biopsy weight not affecting viable hepatocyte yield. Size of tissue failed to give statistical difference in terms of yield/g nor viability and the greater the duration of digestion, the smaller the viable yield (Richert *et al*. 2004). Low weight tissue yields more hepatocytes per gram (viability is not commented on) and use of the Pringle manoeuvre does not affect outcome (Lloyd *et al*. 2004, Vondran *et al*. 2007). In these studies the Pringle manoevre was only used for a maximum of 20 minutes whereas in the French study (Alexandre *et al*. 2002) hepatic vascular clamping was associated with poorer outcomes. Warm ischaemic times of greater than 30 minutes were associated with reduced percentage digestion and yield.

Tissue from patients with benign disease produced more viable hepatocytes with higher yields than those with metastatic disease and those with primary liver cancer faired worst (Serralta *et al*. 2003, Vondran *et al*. 2007).

Finally, using glue to artificially reconstitute Glisson's capsule unsurprisingly led to improved yield and digestion (Alexandre *et al*. 2002, Lloyd *et al*. 2004).

1.06 Hepatocyte isolation

1.06.01 Overview

Hepatocytes have been produced *in vitro* for many years. Initially 0.15% collagenase solution and mechanical degradation was used to isolate rat cells (Howard *et al*. 1967). Subsequently *in situ* perfusion using hepatic vessels (rats) was followed by 0.05% collagenase digestion with *in vitro* 'shaking' and filtration (Berry & Friend 1969). Human studies comparing agitation to *ex vivo* perfusion show clear benefit with the perfusion techniques (Takahashi *et al*. 1993) in terms of yield, without benefit to viability.

Hepatocyte isolation is a time efficient procedure performed using a modified two stage perfusion and collagenase digestion (Seglen 1976). Cells are purified either using density gradients such as Percoll® (Alexandre *et al*. 2002) or repeated wash and centrifugation. They can then be plated, left in suspension, cultured in three-dimensional configurations or cryopreserved.

All protocols remove calcium from the preparation prior to digestion, with the wash step not just the removal of red blood cells (Seglen 1976). The effect of calcium is more significant and not understood.

When basic demographics such as source of tissue, gender, age and hepatic pathology are disclosed, there is no subsequent correlation of findings with any of this data and it is presented for the entire group *en masse*. Study size is often unreported and some papers do not even identify the species from whom the cells are isolated (Evans 1995). Techniques, solutions, assays and endpoints all vary and as such meta-analysis is not possible

1.06.02 Warm and cold ischaemic times

Irrespective of the source of tissue for isolation, liver is exposed to warm- and cold-ischaemic time (WIT/CIT). In practical terms this is represented by the Pringle manoeuvre (clamping of the common hepatic artery and portal vein, intermittently or permanently) during liver surgery and vessel clamping and transection during organ retrieval. Subsequently the tissue undergoes cold perfusion and storage. The Pringle is not the only period of WIT. With larger resections, individual supplying vessels are taken one by one, this further period of WIT commences when the first vessel is taken and ends when the tissue is excised and cold perfusion commenced.

Reduced ischaemic time positively affects yield, viability and maintenance of normal synthetic and metabolic activity (Iqbal *et al.* 1991). After 8 hours CIT with normal tissue, viabilities from human whole organ isolation were low, ranging between 57-72% (Gerlach *et al.* 1994).

1.06.03 Preservation solutions

UW is the internationally accepted liver preservation solution for transplantation, with alternatives and supplementary additives regularly researched. Soltran is a hyperosmolar citrate, licensed and used worldwide as a kidney preservation solution. It is relevant to note that although Soltran may be used to cold perfuse a MOD prior to organ procurement, UW is always immediately circulated thereafter.

There is no literature pertaining to the effects of cold storage solution on subsequent human hepatocyte isolation although pre-isolation preservation media must affect hepatocyte quality. There is some data for rats, with human hepatocyte research focusing on storage solutions post-isolation.

These include simple comparative studies whereby cells were isolated, cultured

overnight, washed and re-cultured, with markers of injury assessed. Here Celsior was a better preservative than HTK, equal to UW (Janssen *et al*. 2003). Locally, UW and ET-Kyoto solutions were compared as media pre- and peri- cryopreservation (Illouz et al. 2008) with ET-Kyoto proving advantageous with viability as a primary endpoint.

1.06.04 Perfusion

Uniform perfusate flow is important, ensuring collagenase reaches all cells in the tissue, with single cannulae in large vessels not allowing for even distribution throughout the tissue specimen (Dorko *et al.* 1994). In tissue there is unequal vascular resistance throughout the tissue with non-uniform vasculature both of which will affect distal perfusion. Older studies used direct injection in an attempt to adequately perfuse the tissue (David *et al.* 1998) but it is difficult to see how the vessels used physiologically cannot be superior.

Hepatocyte isolation is a multi-step process with some authors advocating use of sequential perfusion media during the initial wash step (Lloyd *et al*. 2004). In both studies looking at multi-step perfusion protocols (3-4 solutions) the authors felt there was benefit to porcine cells in terms of viability and yield, statistically significant for yield in one only study (Gerlach *et al*. 1994, Baccarani *et al*. 2003). This has not been repeated in humans.

1.06.05 Isolation

In one of the few papers comparing isolation methodologies, two species (human and porcine) were studied. Viability (as measured by Trypan blue exclusion) and yield (as measured by mass digested) were the primary outcome measures (Gerlach *et al*. 1994). Basic light microscopy was used, without real quantification and the suggestion was that no hepatocytes formed a true monolayer in culture. With whole organ (porcine) isolations

it appears that perfusion and digestion using both venous and arterial circulation is superior to using vein only, with thorough perfusion to remove red blood cells advantageous. Not surprisingly a collagenase free digest media produced the worst results (Gerlach *et al*. 1994). Prolonged digestion or excess exposure to collagenase is toxic (Gomez-Lechon *et al*. 1990), although few studies report digestion time.

1.06.06 Washing

Hepatocyte viability increases with reduced centrifugal force during washing and improves purification (Seglen 1976), but the variety of centrifugal forces used means this cannot be commented upon.

1.06.07 Media

The type of incubation medium used to culture isolated hepatocytes profoundly affects function of hepatocytes, changing their response to stimulus (Elaut *et al*. 2005). Few studies directly compare culture media. One author reports a comparison of four different culture media (Williams E, Chee's, Sigma hepatocyte medium and GIBCO hepatozyme). Culture conditions were both 2D and 3D. Hepatocyte integrity and morphology was assessed by light and electron microscopy, function was measured by albumin secretion. Chee's medium was optimal for monolayer culture but failed to support spheroids unlike Sigma hepatocyte medium. Hepatocytes in spheroids formed bile canaliculi and expressed actin resembling hepatocytes *in vivo*. Medium requirements of hepatocytes differ markedly depending on culture model employed (Hamilton *et al*. 2001).

1.06.08 Hypothermic preservation

When cold preserved over three days, porcine hepatocytes maintained optimum viability

and attachment with UW when compared with Leibowitz-15 (Pahernuk *et al*. 1996). Initial viability was 93%, decreasing to 85% at 24 hours, 74% at 48 hours and 72% at 72 hours. Although these cells did attach in monolayer culture with metabolic function, attachment was poor at 72 hours (72% to 51%). Other authors suggested isolated functional improvement, for example enhanced albumin secretion with periods of hypothermic incubation (Katsuki *et al*. 2004). In hypothermic conditions (maintained at 10°C) hepatocytes maintain function and preserved morphology, but in the longer term (from day nine) they start to die and by day 14 few survive (Griffiths & Evans 2000).

1.06.09 Long-term culture

Hepatocytes in culture repeatedly show a 24 hour adaptation phase with high enzyme activity but low secretory rates with function improving thereafter (Zeilinger *et al*. 2002). Long-term monolayer culture (up to day 34) with regular media changes can produce hepatocytes with synthetic and metabolic activity as measured by albumin and fibrinogen production and CYP450 expression (Pichard-Garcia *et al*. 2002). Other work has looked at even longer culture (up to day 150), with maintained albumin secretion (Chen *et al*. 1998), however there is gradual reduction in CYP450 activity over time (Skett & Bayliss 1996).

Conventional monolayer culture is not the answer to long-term culture as hepatocytes fail to thrive over time (LeCluyse 2001). Optimization of culture conditions will involve assessing effects of culture conditions on gene expression, the influence of the extra-cellular matrix and cell density on CYP450 enzyme regulation, the role of the cytoskeleton in hepatic function, cell-cell interactions and the affect of culture media on outcome (LeCluyse 2001).

1.06.10 Percoll®

Percoll® is a silicon colloid with a PVP covering used as a density gradient for cell purification. It works based on principles that sedimentation of a particle is dependent on size, density in proportion to its media, viscosity and centrifugal force (Amersham Biosciences 2001). In early studies it was suggested Percoll® improved pancreatic islet purification (Buitrago *et al*. 1977). Subsequently it was used with rat hepatocytes in an iso-density preparation which proved advantageous in terms of viability, CYP450 activity and attachment (Kreamer *et al*. 1985).

Percoll® is documented sometimes incompletely, having been was used in 'some' of the six isolations performed (Gerlach *et al*. 1994), with no clear differentiation in terms of results other than reduced contamination (2% to less than 0.5%). Some groups advocate Percoll® both pre- (Alexandre *et al*. 2002, Goll *et al.* 1999) and post-cryopreservation (de Souza *et al*. 1991, 1996, Dou 1992). Percoll® will often produce hepatocytes with greater than 80% viability in both circumstances (David *et al*. 1998, Talameni *et al*. 1997, McGinnity *et al*. 2004). Importantly purification work should be performed at 0-4°C to minimise aggregation and to render the hepatocytes metabolically minimally active (Seglen 1976).

Other authors advocate Percoll® on a 'need' basis, with low viability hepatocytes purified through this density gradient (Ostrowska *et al*. 2000), with 'low' in most studies equating to less than 80%. Equally some studies will not use a gradient to purify the cells further to avoid further loss of non-parenchymal cells (Zeilinger *et al*. 2002). Other authors report particularly poor experiences using Percoll® with fatty preparations (Mitry *et al*. 2003).

It is clear that using a Percoll® gradient causes viable hepatocytes to be lost with yield dropping from 18.7 +/-1.7M/g to 13.2 +/-1.4M/g in one of the few studies to publish this

data (Vondran *et al*. 2007). What is impossible to say is whether these cells were indeed normal and whether losing them is worth the improved purity.

1.06.11 Outcomes

Differences in study objectives, conditions and end points all hinder comparison of hepatocyte research. '*Numerous modifications introduced at various steps during the cell preparation procedure are discussed with no attempt to compare*' (Seglen 1976). Even assessing viability is prone to debate and difference in opinion. Demographics are rarely shared and often have huge discrepancies along with availability of this information. Fresh cell viability is uniformly higher than that of cryopreserved cells (Donini *et al*. 2001, Baccarani *et al*. 2005) and they travel reasonably well whether plated or in suspension (Lloyd *et al*. 2003).

End points vary with one author suggesting efficiency of a method for tissue disaggregation should be evaluated from the initial hepatocyte suspension obtained (Seglen 1976). Others use attachment efficiency, assessed as a percentage of protein content comparing supernatant and monolayer protein (Richert *et al*. 2004). This fails to take into account the protein within the media and protein production.

1.06.12 Cell differentiation

Hepatocytes are difficult to maintain during *in-vitro* culture, with poor proliferative capacities despite *in vivo* ability to regenerate after liver injury or hepatic surgery. In suspension or plated, they progressively lose their cell specific functional activity and their morphological characteristics change. Maintaining these differentiated functions is one of the many 'holy grails' of hepatocyte research. It depends on parameters such as culture media, the extracellular matrix and maintaining cell-cell interaction (Hu *et al*. 1995).

Hepatocyte growth factor induces de-differentiation of hepatocytes in monolayer culture with collagen entrapment matrix abrogating this effect. The mechanisms of de-differentiation are poorly understood. Growth factors and cytokines seem to encourage proliferation but do not alter the loss of differentiated state and hepatocyte-specific function (Block *et al*. 1996).

1.06.13 Co-culture

Tissue engineering with regard to hepatocyte culture and BAL design attempts to create an environment akin to normal liver. One answer to the dilemma of poor long-term function may be co-culture. Here hepatocytes are cultured with at least one other cell type with the aim being to reproduce the hepatocyte single layer plate system.

There is relatively little human work evaluating co-culture, with most studies using rat cells. In one, culture of human hepatocytes with biliary epithelial cells maintains CYP450 function over a significantly protracted time period when compared with monoculture of hepatocytes alone. It impressively restores synthetic and metabolic function in culture as measured by factor VII production, albumin expression and lignocaine metabolism (Auth *et al*. 2005).

Both liver-derived and non-liver derived cells have been used including liver epithelial cells (Gugen-Guillouzo *et al*. 1983), stellate cells (Higashiyama *et al*. 2004), sinusoidal endothelial cells (Morin & Normand 1986), Kupffer cells (Zinchenko *et al*. 2005) and whole non-parenchymal cellular fractions (Shimaoka *et al*. 1986). Of the non-liver derived cells, the most commonly evaluated are embryonic murine cells (the 3T3 murine fibroblast line, a long established and accessible cell line in most incidences) (Bhandari *et al*. 2001), although pancreatic islets (Kaufmann *et al*. 1999) and bone marrow cells (Murakami *et al*. 2004) have been used. Non-parenchymal cells can proliferate vigorously in culture and

predominate where circumstances allow (Funaki *et al*. 2002).

Co-cultured hepatocytes and non-parenchymal liver cells inoculated into a bioreactor and cultured over a five week period enabled the development of parenchymal-like islands which resemble sinusoids (Gerlach *et al*. 2003). Over the study period cells were metabolically active, although over time function did globally deteriorate. As with other studies there is initial injury expressed by high LDH and transaminase release, which later recovers. Together this suggests improved long term survival, retention of hepatocyte-specific function and differentiated state with appropriate co-cultivation.

1.07 Cryopreservation

1.07.01 Overview

'*Cryopreservation is lethal to most living systems, yet it can also preserve cells and their constituents and it may one day permit the long-term storage of whole viable organs*' (Mazur 1970). It is defined as the process by which cells are preserved by cooling to sub-zero temperatures, typically -196°C (boiling point of liquid nitrogen).

Scientists have been fascinated by the prospect of suspending biological animation by cooling for hundreds of years, with the first basic studies reported in 1683 by Robert Boyle, although more detailed studies had to wait for technological advances.

In 1937, Luyt performed pioneering experiments researching the effect of cold on life, using yeast in his experimental model (Luyt 1937). It is he who discovered the phenomenon of vitrification, defined as the transformation of a substance into a glass, with cooling below the water/cryoprotectant glass transition temperature without ice formation.

In 1949 Polge and Smith successfully cryopreserved fowl sperm accidentally in glycerol, thus discovering the first documented cryoprotectant agent, which remains a commonly used compound. Subsequently the cryoprotective properties of dimethyl sulphoxide (DMSO) were discussed in 1959 (Lovelock & Bishop 1959).

It was not until the work of Mazur in 1968 that the mechanism of cryopreservation was better understood. He showed that cells, cooled slowly in the presence of a cryoprotectant, shrink and do not contain intracellular ice. Each cell type has its own optimum cooling rate, which is determined by its water permeability and the role of DMSO is to increase that permeability.Therefore protocols are based on slow freeze and fast thaw of healthy cells in the presence of high protein concentrations and an agent that increases cell membrane permeability.

1.07.02 The effect of freezing on water

Although cryopreservation relies heavily on cryoprotectants, it is the management of water in this technology that is the key to success. Water is the primary solvent in living creatures and it is water's transformation from liquid solvent to solid during the cooling process that provides the ability to preserve or destroy. Rate of cooling determines survival rate and variety of damage to the cell (Bishof *et al*. 1997). When cells are cooled too slowly the extracellular environment freezes forming extracellular ice (Acker *et al*. 1999). The chemical potential difference across the cell membrane causes osmosis of water which dehydrates and shrinks the cell. The slower they are cooled the longer cells are dehydrated. On the other hand, when the cells are cooled quickly they retain water within the cell which expands when frozen, with intracellular ice formation physically destroying the cell (Mazur 1970, Bishof *et al*. 1997).

Formation of ice varies with culture configuration, freezing rate and cryopreservation environment (Acker *et al*. 1999, Hubel *et al*. 1991). The cryoprotectant is a solvent, displacing water and achieving the balance between reducing intracellular water content without dehydration. These compounds are variably toxic and may affect cellular metabolism or chemical potential difference across the membrane. DMSO is the most commonly used cryoprotectant presently, having first been recognised in 1959 (Lovelock & Bishop 1959). Cryoprotectants stabilize proteins during freezing, but destabilize at physiological temperatures in a paradoxical fashion. With DMSO it is hypothesized that at low temperature these compounds are excluded from the proteins' hydration shell, reducing the amount of ice produced during cooling and thus reducing thaw-related damage to the cell. At higher temperatures they interact hydrophobically with the membranes' phospholipid bilayer, denaturing proteins (Anchordoguy *et al*. 1991).

Synthetic agents such as polyvinyl alcohol (PVA) have been used as an adjunct to

cryoprotectants such as DMSO which in turn allow reduced concentrations of these more toxic compounds (Wowk *et al*. 2000).

1.07.03 Hepatocyte cryopreservation

The theoretical advantages of using cryopreserved hepatocytes over fresh hepatocytes are numerous. Work with cryopreserved hepatocytes can be planned at will, they can be characterised before use and it can be ascertained they have adequate metabolic capacity for demand. The same donor can provide repeated hepatocytes for use at different times, cells can easily shipped between destinations or they can be pooled from multiple donors (Li *et al*. 1999). Specific to hepatocyte cryopreservation quality of the cells prior to cryopreservation is particularly important.

1.07.04 Pre-incubation

Basic isolation and culture methodologies may alter ability of cells to survive the stressors of freezing and thawing. A period of recovery prior to the insult of cryopreservation may improve outcomes.

Pre-culture and pre-incubation are interchangeable terms used to describe cell culture in albumin enriched media prior to cryopreservation. There are a number of hepatocyte studies using pre-incubation (Silva *et al*. 1999, Alexandre *et al*. 2002, Terry *et al*. 2005) all demonstrating improved post-thaw viability (table 7). Importantly hepatocytes are metabolically active during pre-incubation (Seglen 1976).

Author	Source	Age of donor (yrs)	M:F	Size of tissue (g)	n	CIT preservation solution	Cannu-lation	Original author	Post-isolation viability (%)	Viability test	Gradient
Silva *et al*. 1999	Transplantable tissue discarded during size reduction/surgical liver specimens			Approx 20		UW		Li *et al*. 1992	>90	Trypan blue	No
Alexandre *et al*. 2002	Surgical liver specimens	33-73	14 male 14 female	30-150	28	25 mM HEPES buffer, pH7.4, 0.8 mM Na^2PO^4, 2.7 mM KCl, 137 mM NaCl	Direct injection or perfusion	David *et al*. 1998, Gugen-Guillouzo *et al*. 1982		Trypan blue	Percoll ®
Terry *et al*. 2005	Multiorgan donors unsuitable for transplant				10	UW		Mitry *et al*. 2003	67 +/-10	Trypan blue	

Author	Viability post-gradient	Yield (viable cells) M/g	Attach-ment (%)	Preincubation media	Duration	Temp-erature (°C)	Density	Cryopre-servation media	Vial size (ml)	Density
Silva *et al*. 1999				Krebs, 15 mM glucose, 12 mM HEPES, pH 7.4	30 mins	37	5M/ml	DMSO 13.3%	5	
Alexandre *et al*. 2002	87.8 +/- 1.4	5.99 +/- 0.78	78.7 +/- 4.5	DMEM, 5% FCS	30 mins	37	3M/ml	DMEM, 2.5% BSA, 10% DMSO, 20% FCS, 2% PVP	1.8	5M/ml
Terry *et al*. 2005			55 +/-15	Glucose OR fructose OR S-adenosyl-L-methionine OR α-lipoic acid OR pentoxifylline	120 mins	4°C and 37°C	3M/ml	10% DMSO in UW	5	

Author	Freezer	Wash	Speed	Cycles	Gradient	Viability	Attachment	Recovery
Silva *et al*. 1999	Minus 20°C for 60 mins, -70°C for 60 mins, liquid nitrogen thereafter	Incubation media	100g	3	No	80 +/- 10% (preincubated 85 +/- 7%)	15 +/- 5% (pre-incubated 66 +/- 11%)	
Alexandre *et al*. 2002	Planer (stepwise cryo-freezer) OR Mr Frosty	HBSS, 10% FCS	50g for 2 mins	1	33% Percoll®	Pre-Percoll® 31.58 +/-4.73 (stepwise freezing) 46.97 +/- 5.72 (progressive freezing). Post-Percoll® 72.4 +/-2.9 (stepwise freezing) 78.8 +/-1.7 (progressive freezing)		Pre-Percoll® 11.8 +/- 1.6 (stepwise freezing) 38.2 +/-2.5 (progressive freezing)
Terry *et al*. 2005	Kryo 10, Planer	Williams E, 10% FCS	50g for 5 mins	1	No	Multiple conditions of pre-incubation. Control 50 +/-6	Control 40 +/- 3	

Table 7 Human hepatocyte pre-incubation experience.

Using a porcine model, whole organs underwent perfusion and digestion with the hepatocytes cultured in a complex media in a spinner flask at 37°C for up to 24 hours (Hubel *et al*. 2000). These were then cryopreserved in 10% DMSO using a Planer cryopreservation (controlled rate) programmable freezer. Thawed hepatocytes were cultured in a hollow fibre bioreactor at 37°C, with circuit perfusion commencing at 24 hours. Numbers were small (n=7 fresh hepatocyte control, n=3 fresh day five control, but n=1 for cryopreserved hepatocytes). Despite this, albumin synthesis was increased in pre-cultured hepatocytes versus immediately cryopreserved hepatocytes. Using similar techniques but monolayer culture, porcine hepatocytes cultured for 24 hours prior to cryopreservation exhibited higher viabilities and albumin synthesis than those without (Darr & Hubel 2001). Improvement correlated with duration of pre-culture (after 24 hours results deteriorate for pre-cultured hepatocytes).

Rat hepatocytes retained metabolic capacity similar to fresh cells when pre-incubated (30 mins at 37°C) prior to cryopreservation (Zaleski *et al*. 1993).

Human hepatocyte studies have demonstrated enhanced long term survival in culture rather than improved initial viability (Silva *et al*. 1999) with 2 day attachment improved from 15 to 60%. CYP450 induction remained similar to that with fresh hepatocytes.

Pre-incubation of rat and human hepatocytes with glucose, fructose or α-lipoic acid at 4°C had a beneficial effect prior to cryopreservation in terms of viability and LDH release, probably conferring an element of membrane protection (Terry *et al*. 2005). At 37°C only fructose and α-lipoic acid conferred benefit.

Other authors have noticed the converse, with no increase in viability or recovery post-cryopreservation with pre-incubation (Alexandre *et al*. 2002).

1.07.05 Cryoprotectants

One of the first steps in cellular cryopreservation is the movement of a cryoprotectant into the hepatocyte (Loretz *et al*. 1989). Complex cryopreservation media often fair better in human hepatocyte studies than 20.5% DMSO alone, possibly representing additional cytoprotectant activity of the combination (Coundouris *et al*. 1993). In other work maximal viability and attachment was seen with 15% DMSO and high serum levels in the cryoprotectant media (Loretz *et al*. 1989). Despite extensive work most human cryopreservation protocols involve 10% DMSO. A similar protocol has been used with monkeys, albeit with lower attachment rates (Sun *et al*. 1990). PVP use appeared to enhance post-thaw viability (de Souza *et al*. 1991) but this was not reflected in improved attachment (Lawrence & Benford 1991).

There is a large body of evidence suggesting 10% DMSO is optimal (Hengstler *et al*. 2000), which must be added slowly as it has a high osmolarity.

1.07.06 Cell density

Concentration of hepatocytes per ml in cryopreservation vials (Madan *et al*. 1999, de Loecker *et al*. 1998, Diener *et al*. 1993) has been well investigated with reduced cell number (low concentration) also conferring a cryoprotectant affect (Hengstler *et al*. 2000) although this is not truly understood. There is evidence suggesting cryopreservation in 3D culture may confer protection (Guyomard *et al*. 1996, Koebe *et al*. 1996), but these are non-human studies. Cryopreservation in monolayer culture configuration may confer benefit (McKay *et al*. 2002), with poor initial viabilities but rapid recovery. Thawing may cause plasma membrane injury, thus high take up of trypan blue with subsequent recovery (Watts & Grant 1996). These hepatocytes retain CYP450 activity and albumin synthesis.

1.07.07 Freezing rates

Intracellular ice formation affects hepatocyte outcome following cryopreservation with large amounts of work undertaken to understand this further (Diener *et al*. 1993, De Loecker *et al*. 1993, Lawrence & Benford 1991, Chesne *et al*. 1993, Koebe *et al*. 1990). Freezing protocols play a major role in cell recovery with progressive, steady rates clearly improving outcome (Alexandre *et al*. 2002, Lloyd *et al*. 2003). Reproducible results are seen with DMSO/FCS cryopreservation media and a 1-2°C/minute cooling rate is preferred (Guillouzo *et al*. 1999).

1.07.08 Longevity

Duration of storage is a contentious issue, but there is little good quality evidence suggesting it significantly affects outcome (Guillouzo *et al*. 1999). Outcome as measured by hepatocyte viability and yield remain unchanged when cells are stored for as long as one year (Li *et al*. 1999).

1.07.09 Outcomes

As with fresh hepatocytes, when cryopreserved they have synthetic and detoxification capacity, with prolonged culture conferring benefit in terms of function and membrane integrity (Dou *et al*. 1992). CYP450 activity is preserved in cryopreserved (rat) hepatocytes, but this activity in monolayer culture is not sustained and is significantly reduced when compared with fresh cultured hepatocytes. Only 46-56% of cryopreserved hepatocytes were recovered with low viabilities (67-75%) (Jackson *et al*. 1985). Similar findings were reported with human, monkey and canine hepatocytes (de Souza *et al*. 1996). In other studies with different end points cryopreserved rat hepatocytes exhibited near normal morphology when compared with fresh hepatocytes. Their induction capacity and

CYP450 activity were similar, suggesting cryopreserved hepatocytes would be a suitable *in vitro* system for evaluating xenobiotics (Madan *et al*. 1999, McKay *et al*. 2002). So although post-cryopreservation hepatocytes may not exhibit some normal metabolic behaviour (de Souza *et al*. 1991), many studies suggest minimally altered metabolic capacity post-thaw (Donini *et al*. 2001, Baccarani *et al*. 2005). For example CYP450 activity in cryopreserved hepatocytes is 70% of fresh hepatocytes (Coundouris *et al*. 1993).

Returning to rat hepatocytes with which most studies are conducted, using a 10% DMSO cryoprotectant a significant viability drop is seen post-cryopreservation from 82 +/-7% to 69+/-6%, although in plated hepatocytes, metabolic function is identical (Utesch *et al*. 1992). Regarding albumin secretion and P450 activity, no major differences were seen between fresh, frozen and 24 hour UW cold preserved rat hepatocytes, using a cryopreservation media comprising of Leibowitz buffer, 10% FCS and 16% DMSO. Cryopreservation did however reduce viability from 82+/-2% to 60+/-4% and attachment from 69+/-4% to 38+/-9% (Fautrel *et al*. 1997).

There are a number of freeze-thaw protocol reviews written, with little pertaining solely to human hepatocytes. It is accepted that these cells are more sensitive to cryopreservation than rat cells (Lawrence & Benford 1991, Adams *et al*. 1995) with canine hepatocytes more sensitive (Guillouzo 1998) therefore applying data from other species to human hepatocytes is not straightforward. Researchers cannot always agree on this with other groups demonstrating human hepatocytes being more resistant than rat to cryopreservation with viabilities of 67% at 24 hours and 49% at two weeks (Coundouris *et al*. 1993). Nevertheless, all agree hepatocytes are particularly sensitive to the rigors of the freeze/thaw process, with poor recovery (Dou *et al*. 1992) that manifests as reduced viability and attachment (Loretz *et al*. 1989, de Sousa *et al*. 1999).

Metabolic activity of cryopreserved hepatocytes is uniformly less than that of fresh

hepatocytes (de Loecker *et al*. 1990) but given a period of culture over 48 hours, activity improves and perhaps allows recovery from their cryopreservation injury thus regenerating synthetic and metabolic capacity (de Loecker *et al*. 1993). CYP450 activity is preserved most of the time for a short period at least. Recovery is uniformly poor along with attachment when compared with fresh hepatocytes, although recovery or percentage lost is often impossible to calculate as the number of hepatocytes thawed is rarely identified. In rat studies, recovery ranges from 33% (Powis *et al*. 1987) to 78% (Diener *et al*. 1993). In one of the few clear human hepatocyte studies, recovery is only greater than 50% in 20% of preparations although hepatocytes that survived did plate with good efficiency and function (Alexandre *et al*. 2002). After cryopreservation, viability is reduced typically by 10-25%, but lower viability is reported frequently (Guillouzo *et al*. 1999) and recovery may be as low as 22%.

Hepatocytes can be returned with a good range of functions including preservation of CYP450 isoenzymes (Li *et al*. 1999, Dou *et al*. 1992) and they have been used in some of the BAL trials (Rozga *et al*. 1993). Nevertheless cryopreserved hepatocytes are viewed with a degree of suspicion as to their value (de Souza *et al*. 1991).

The optimal cryopreservation protocol despite over 25 years of work is yet to be defined and increased percentage recovery of cells is desperately needed with research continuing across the world.

1.07.10 Post-cryopreservation Percoll® gradient

Cryopreservation leads to significant cell death and as such purification may be appropriate. Although study numbers are relatively low, Percoll® does increase hepatocyte viability at the expense of seemingly viable cells whether measured by recovery or relative decrease in viability (de Souza *et al*. 1996, Dou *et al*. 1992, Vondran *et al*. 2007).

As Percoll® concentration increases during purification, gain in viability reduces. Maximal purification is seen at 25% concentrations, above this hepatocyte loss becomes unacceptable although figures are not described (McGinnity *et al*. 2004).

Some cryopreservation protocols progress directly to Percoll® gradient without making any assessment of hepatocyte outcome without purification (Chesne *et al*. 1993). Again there is overall loss of viable hepatocytes, with percentage impossible to calculate from the data. Outcomes in terms of CYP450 function were that fresh and cryopreserved hepatocytes had similar activity, although in animal species there was a 48 hour lag time until equivalent figures. Importantly it was clearly identified that hepatocytes derived from dog, monkey, mouse, rat, rabbit, hamster and human all behave differently when treated equally, whether in terms of response to isolation, cryopreservation, DMSO concentration, CYP450 activity, attachment or viability.

1.07.11 Animal studies

Just as human hepatocyte work is difficult to cross compare, animal work is possibly even worse, although the volume of work is approximately 1:10. Disparate species are used, models of injury are different, there is variation in dosing schedules, variation between animals, nuances in laboratory technique, selection of outcome measures that may be irrelevant in the human population and variable duration of follow-up (Bracken 2008).

Slaughterhouse organs are a huge resource for hepatocyte researchers with porcine cells easily produced from this tissue (Koebe *et al*. 1995). Viable hepatocyte yields of 10M/g from lobar perfusion can be obtained with a total viable hepatocyte number of 1920M per isolation with viabilities of 93%. Importantly no bacterial or fungal contamination was seen either. Equine hepatocytes can also be isolated from abbatoir animals even with 30 minutes WIT (Bakala *et al*. 2003).

The only paper attempting to identify relevant variables in porcine isolation concluded that ionic composition and pH of the collagenase perfusant affected viability along with time taken to perfuse the tissue with EDTA, although delay conferred inexplicable benefit (Wang *et al*. 2000). The only porcine paper that presented data from each donor suggested 19500M cells could be retrieved per organ with a viability of 94.6% (Sielaff *et al*. 1995).

With cryopreserved porcine hepatocytes morphology is altered post-thaw. Viability drops from 88+/-2% to 36-63% with only 60% metabolic activity post-thaw. Controlled freezing rates are most successful at low hepatocyte concentration (3M/ml) (Smrzova *et al*. 2001). Other porcine studies report extraordinary success of cryopreservation. Without Percoll® purification viability post-thaw can be reported as high as 96% (Chen *et al*. 2001).

Variable results are seen with rat hepatocytes. In one study L15 media with 16% DMSO cooled at a rate of 3°C/min adding 0.8M glucose to the thaw solution produced optimal hepatocytes (Chesne and Guillouzo 1988). More than 50% hepatocytes survived cryopreservation, although only 40% attached to media in culture (Chesne & Guillouzo 1988) however, morphology was similar and protein secretion reduced by only 40%. Viability pre-cryopreservation ranged from 80-86%, dropping to 60-68% on thawing. Calculating recovery based on viability rather than hepatocyte number lost is inaccurate. Recovery is measured comparing hepatocyte number thawed with that cryopreserved. Without this data one cannot assess recovery or hepatocyte loss (Chesne & Guillouzo 1988). In the same paper it is claimed that dead hepatocytes could be eliminated by 36% Percoll® centrifugation giving a viability of 90-95%.

Canine hepatocytes can be successfully cryopreserved with viabilities of up to 78% and recovery rates of approximately 50%. These were metabolically active with 40% of fresh function persisting (Kasai & Mito 1993).

1.08 3D culture

1.08.01 Overview

One of the requirements for short- and long-term culture of functional hepatocytes is cell attachment to the culture vessel. Biocompatibility involves the search for structural and surface compatibility. Applied to tissue engineering and 3D hepatocyte culture 'scaffolds' should mimic tissue architecture and extracellular matrix (ECM) of the target tissue, attaching cells whilst providing nutritional support (Wintermantel *et al*. 1996) and enabling normal cellular function. This is especially important as hepatocytes are anchorage dependent cells (Strain & Neuberger 2002).

The culture vessel should be pre-coated with a suitable attachment matrix such as type I collagen (Chen *et al*. 1998) or Matrigel (Hong & Glauert 2000) which corresponds to *in vivo* ECM. Collagen or fibronectin coating on substratum supports high cell adhesion and induces differentiation of hepatocytes (Cima *et al*. 1991) whilst collagen or Matrigel in the form of hydrogel used to encapsulate hepatocytes can better maintain their albumin synthesis and ammonia metabolism (Hu *et al.* 1993).

For long-term *in vitro* maintenance of differentiated hepatocytes and in order to promote intracellular metabolism, culture media is often supplemented with FCS, and/or non-nutritional growth factors such as human growth factor (HGF), epidermal growth factor (EGF), insulin and glucocorticoids including dexamethasone (Kono *et al*. 1997, Strom *et al*. 1998). Many studies allude to BAL technology but actually are simply evaluating 3D culture conditions.

1.08.02 Monolayer (two-dimensional) culture

Hepatocytes are traditionally cultured directly on dishes pre-coated with collagen

(Waxman *et al*. 1990) or Matrigel (LeCluyse *et al*. 1996) with FCS, dexamethasone, insulin, transferrin and selenium. These cells have a shorter life span compared to those cultured in a 3D configuration (LeCluyse *et al*. 1996, Qiao & Farrel 1999) and lose differentiated function within a few days, whereas those cultured in 3D systems maintain differentiated function for up to 8 weeks (Bissell *et al*. 1973). Monolayer cells require a large surface area to attain the cell density required for a functional BAL and there is less scope for up-scaling.

In one study, porcine hepatocytes were cultured in monolayer on stacked, gas permeable membranes forming a flat membrane bioreactor (Bader *et al*. 2000). This reactor demonstrated CYP450 activity and synthetic function but has never been trialled.

1.08.03 Membrane networks

Polymeric semi-permeable membranes are widely used in biomedical devices for detoxification and oxygenation of blood. In BAL membranes can act as immunoprotective barriers whilst providing a large exchange area to supply hepatocytes with nutrients and oxygen necessary for metabolism and acting as substrata for hepatocyte adhesion. The capacity of the membrane to provide support for cell structure depends on surface properties and it has been demonstrated that roughness and pore size of polymeric membranes affects viability and metabolic rate of isolated hepatocytes (de Bartolo *et al*. 2004). It is also reported that rat hepatocytes interact better with hydrophilic as compared to hydrophobic membranes (Catapano *et al*. 1996).

One study investigated the physico-chemical aspects of interaction between membrane and rat hepatocytes to provide guidelines for selection of cytocompatible membranes (de Bartolo & Bader 2001). Six flat sheet microporous membranes with different properties were used (cellulose acetate, polysulfone, polycarbonate, polypropylene and

perfluoropolymer), collagen was the control. These substances were coated onto Petri dishes and the ability of the membrane to absorb water measured. Rat hepatocytes were then isolated and seeded into these dishes. Morphology was observed and ammonia metabolism measured. Cell adhesion increased in the presence of serum proteins adsorbed by the membrane. Metabolic function, measured by urea synthesis is improved on hydrophilic membranes. The membranes with the favoured qualities were polycarbonate and cellulose acetate (de Bartolo *et al*. 2001).

Polymeric membranes composed of modified polyetheretherketone (PEEK-WC) were then investigated as they exhibit chemical, mechanical and thermal stability and have been used in other biomedical devices (Kimmerle & Strathmann 1990). Rat hepatocytes were cultured on Petri dishes coated with collagen or PEEK-WC. The cells formed 3D aggregates larger than those formed on the control membranes with increased ammonia elimination and protein synthesis. These membranes were more hydrophilic than the control correlating with the finding that these membranes improve global function (de Bartolo *et al*. 2004).

1.08.04 Collagen Sandwiches

Common culture matrices are sandwich configuration of collagen-collagen or collagen-matrigel. Hepatocytes are plated onto collagen coated plates, allowing attachment for 2-3 hours, then overlaid with collagen/matrigel to create a sandwich. This morphology is reminiscent of the liver plate and increases hepatocyte longevity in culture compared with monolayer culture (LeCluyse *et al*. 1996, Qiao & Farrell 1999).

Two- and three-dimensional rat hepatocyte cultures using sandwich configuration were compared and microsomal CYP450 and phase II activities were assessed. Oxidative stress of reperfusion was increased in 2D culture measured by reduced catalase activity described

previously during reperfusion (Duval *et al*. 1995). 2D sandwich cultures and 3D Matrigel culture allow longevity of rat hepatocyte cultures and induction of CYP450 but imbalance in detoxification processes in cultured rat hepatocytes occurs, whatever the culture configuration (Richert *et al*. 2002).

1.08.05 Non–woven fabrics

Hyaluronic acid is a natural glycosaminoglycan present in connective tissues that influences cell motility, adhesion, proliferation and cell-cell recognition by interacting with membrane receptors on the cell surface (Abatangelo *et al*. 1994). It is biocompatible, biodegradable and water soluble. Polymers are available in the form of films, non-woven fabrics, gauzes, sponges, tubes and microspheres.

These fabrics can be used as substratum for culture with the effect of the chemical nature and geometry of polymers on hepatocyte morphology and metabolism investigated. Rat hepatocytes were cultured in Petri dishes on films and non-woven fabrics made of polymers of ethyl- and benzyl- esters. Function was evaluated by ammonia elimination. Hepatocytes adhered on substrata formed larger spreading aggregates. Those cultured on collagen had intermediate sized aggregates, spread more and had more protrusions than those cultured on basic non-woven materials. Hepatocytes cultured in non-woven fabrics retained ammonia elimination equal to or better than those adherent to collagen (Catapano *et al*. 2001).

1.08.06 Foams/sponge

Experimental evidence has demonstrated hepatocyte culture in collagen foams or sponges promotes long-term maintenance of liver specific function (ammonia elimination) in rat hepatocytes (Matshushita *et al*. 1991). Rat small hepatocytes and hepatocyte progenitor cells can differentiate into mature hepatocytes and reconstruct sinusoids by interacting with non-parenchymal cells. These cells have the capacity of proliferating into a large colony with one cell potentially multiplying to form more than 30 cells at day 10 (Mitaka *et al*. 1995).

Colonies of small hepatocytes were collected and re-plated on collagen sponge with albumin, transferrin, haptoglobin and fibrinogen secretion examined. Production of proteins by cells in the sponge increased with time and was significantly higher than from cells grown as a monolayer. Morphologically and functionally differentiated hepatocytes and excretion of fluorescein in bile canaliculi were also observed. Sinusoidal endothelial cells were sometimes found inside the sponge with long processes extending from cell body to neighbouring cells (Harada *et al.* 2003).

1.08.07 Spheroids

Hepatocytes cultured as aggregates (spheroids) have enhanced liver function compared with those in monolayer (Landry *et al*. 1985). Urea synthesis in a BAL (Wu *et al*. 1996) improved 1.8-fold using collagen entrapped porcine hepatocyte spheroids rather than dispersed cells. Similarly self-assembling (rat) hepatocyte spheroids exhibit increased CYP450 activity when compared with monolayer culture (Wu *et al*. 1999). Conventional preparation of spheroids depends on culture substratum and surface area for initial hepatocyte attachment. A large surface area is the limiting factor in the preparation of spheroids for a BAL (Koide *et al*. 1990).

Hepatocytes co-cultured with non-parenchymal cells (heterospheroids) or liver derived epithelial cells have longer survival and enhanced liver function. When cultured in equal parts they had higher rates of albumin secretion and ammonia elimination (Yamada *et al*. 2001). This is probably because cell-cell interactions play important roles in organogenesis, regeneration, expression and maintenance of liver function.

1.08.08 Encapsulated hepatocytes

Bioencapsulation uses a drop method of encapsulation in which cells are retained in a semi-permeable membrane. This method involves isolating the hepatocytes and suspending them in solution of the substance which would constitute capsule membrane. This is then extruded through a needle in a solution of calcium chloride in 2.2% sodium chloride. The beads can then be washed and used with a BAL or transplanted via intraperitoneal, intrasplenic or intraportal injection (Riordan & Williams 1999, Selden *et al*. 2004). Intraperitoneal injection of bioencapsulated hepatocytes was effective in increasing survival time of rats with galactosamine induced ALF and lowered bilirubin levels in rats with Crigler-Najjar (Wong & Chang 1986, Bruni & Chang 1991). They had longer viability than those not bioencapsulated and hepatotrophic factors secreted by hepatocytes are retained inside microcapsules. Unfortunately, a large proportion of biocapsules are coated with fibrin and fibroblasts resulting in adhesion between capsules and subsequent death (Wong & Chang 1988).

Alginate is obtained from brown seaweed and has been extensively used as gelling agent in biotechnology and medical applications. It has been used as 3D synthetic ECM for immobilisation of cells in tissue engineering (Skjaek-Break 1992). Encapsulation of hepatocytes into alginate capsules could produce a high-density culture system. This could offer cellular mechanical support and immunoprotection in the event of implantation.

These hepatocytes had increased secretion of albumin, fibrinogen, prothrombin and -1-antitrypsin and improved CYP450 activity as compared to those in monolayer culture (Selden *et al*. 1999). Urea synthesis was undetectable in monolayer culture but approached *in vivo* levels in bioencapsulated culture. Electron microscopy revealed ultrastructure reminiscent of normal human hepatocytes with abundant ER, Golgi and mitochondria with canaliculi and a network of microvilli, desmosomes and junctional complexes.

Some groups report no loss of viability during encapsulation, with microcapsules cryopreserved for up to 4 months. Permeability is adjustable, allowing for albumin to be secreted but immunoglobulins and leucocytes not to gain access. Encapsulation in bioreactors or transplantation, might immuno-isolate hepatocytes from rejection and provide mechanical protection during cryopreservation. Problems include lack of mechanical strength, long-term capsule degeneration and frequent induction of inflammatory response (Canaple *et al.* 2001).

A BAL connected to a plasmapharesis system inoculated with alginate gel bead-embedded hepatocytes directly in contact with plasma has been developed. This was shown to be able to partially correct specific defects and have the capacity to detoxify xenobiotics (Desille *et al*. 2001). This system was then used in pigs with ALF with reduced encephalopathy and cerebral oedema without survival benefit (Desille *et al*. 2002).

A packed bed or fluidized bed bioreactor filled with microencapsulated hepatocytes has potential advantages with high mass transport rate and optimal micro-environment for hepatocyte culture.

1.08.09 Extracellular Matrix (ECM)

Scaffolds are designed for cell attachment and to facilitate nutrient provision. They should mimic tissue architecture and ECM. Hepatocytes in an implant will work poorly or die if

they are further than 500 microns away from blood vessels or other sources of nourishment. Therefore an ideal BAL matrix is one in which the blood can circulate though the vessel that houses metabolically active hepatocytes (Lysaght & Aebischer 1999).

Collagens are a family of closely related proteins, mainly produced by fibroblasts, which can aggregate to produce filaments, fibrils or mesh-works which interact with other proteins to provide support in the ECM. It is the most suitable material for constructing artificial substitutes for diseased and damaged tissues but is highly thrombogenic, inducing platelet adhesion and aggregation as well as activation of the intrinsic blood coagulation cascade.

Chitosan, an amino-polysaccharide derived from chitin by deacetylation is used in wound dressing and drug delivery systems on account of its non toxic biocompatible nature. It causes formation of granulation tissue and angiogenesis in wound healing by inducing fibroblasts to release interleukin-8, which is involved in migration and proliferation of fibroblasts and endothelial cells (Mori *et al.* 1997).

To overcome thrombogenecity, collagen and chitosan were cross-linked by casting a solution of type I collagen and 1% chitosan then air drying the films, thus allowing use as ECM. Murine hepatocytes were then cultured on these films and viability and morphology measured. The addition of chitosan to a collagen matrix can improve adhesion and division of hepatocytes. Additionally, cross-linked collagen and chitosan matrix (CCM) compared to collagen matrix has excellent anticoagulant properties (Wang *et al.* 2003).

ECM proteins such as collagen and fibronectin are popular choices for coating of substrates for hepatocyte culture, but they promote adhesion and differentiation and collagen or Matrigel in the form of a hydrogel can better maintain cellular synthetic functions. In one study, hepatocytes embedded in collagen gel within a hollow fibre BAL impeded oxygen and nutrient transport thus potentially compromise performance of such

a design (Nyberg *et al.* 1993).

Rat hepatocyte attachment efficiency on galactosylated substrate was similar to that of collagen coated surface. The attached hepatocytes self-assembled into spheroids within a day. These spheroids exhibited higher cell functions (albumin synthesis and CYP450 activity) compared to unmodified polyvinylidene difluoride and simple collagen coated surfaces (Lu *et al*. 2003).

Cellulose microspheres (CM) containing cell adhesive peptides were designed to aid 3D culture with NKNT-3 (immortalised human hepatocyte) cultured with these CMs. Cells were seen to attach after six hours of stirring the culture and within 48 hours there were immobilized cells on CMs with high density, the cells gradually forming large aggregates. Scanning electromicroscopy showed cells on CMs with well developed microvillous protrusions (Kobayashi *et al*. 2000, 2002).

No single study has compared the different 3D cultures with identical conditions and hepatocytes. This makes comparison between methodologies difficult. Most studies are rat or porcine, with some using immortalised human hepatocyte cell lines. Very few use normal human hepatocytes creating concern regarding reproducibility of the results in a human model.

1.09 ECVAM

1.09.01 Overview

ECVAM (the European centre for the validation of alternative methods) was established in 1992 as a result of a Communication from the Commission to the Council and the Parliament in October 1991 (SEC(91)1794), pointing to a requirement in Directive 86/609/EEC on protection of animals used for experimental and other scientific purposes. The Commission and Member States should support development, validation and acceptance of methods which could reduce, refine or replace the use of laboratory animals. It is a unit of the Environmental Institute based in Italy. ECVAM answers to the European Commission and is an independent scientific body.

ECVAM co-ordinates independent evaluation of the relevance and reliability of tests for specific purposes, so that chemicals and products can be manufactured, transported and used economically and more safely, whilst the current reliance on animal test procedures is progressively reduced (ECVAM 2009). Research activities are undertaken in collaboration with organisations in the EU Member States and worldwide.

1.09.02 Harmonised protocols

Attempting to compare studies is essentially impossible as no two groups appear to share the same method for hepatocyte preparation let alone buffers, anaesthesia, species, oxygenation, temperature, perfusion, washing or digestion media or culture conditions. These issues led to the first ECVAM workshop (Blaauboer *et al*. 1994) and the desire to harmonise isolation methodologies. ECVAM were prompted to grant a pre-validation study on the use of human hepatocytes for systematic evaluation of prediction of CYP450 induction (ECVAM contract number 19471-2002-05 F1ED ISP FR).

The first stage defined criteria for hepatocyte isolation using results generated by the participating laboratories (Richert *et al*. 2004, LeCluyse *et al*. 2005).

The second stage involved harmonised protocols for human hepatocyte isolation and culture, with assessment of response to treatment with various inducers of CYP450 activity.

1.10 Liver support

1.10.01 Organ Replacement Therapy: Hepatic transplantation

Orthotopic liver transplantation (OLT) was first performed in humans in 1963 (Starzl *et al*. 1963) and is the gold standard for irreversible ALF. With increasing success of cadaveric OLT, there is a widening gap between the shortage of organ donors and potential recipients. This has been addressed by non-heart beating donation (NHBD), split liver transplant, live donors and domino transplant. With the potential exception of auxillary transplantation patients undergoing OLT are committed to a lifetime of immunosuppression and its associated complications with only 66% of organs functioning at five years (Strong 2001).

Although liver transplantation has provided an alternative to patients with ALF (Strong 2001) as with all transplant programs a shortage of donors has led to the deaths of many patients before transplant can be undertaken (Riordan & Williams 2000). An alternative liver replacement therapy would be advantageous and potentially utilised in many clinical scenarios. The principle of maintaining a patient using artificial support and thus 'bridging' them to transplantation is attractive (Riordan & Williams 1999, 2000). Equally it is anticipated that in some patients this support may allow endogenous liver regeneration and subsequent recovery without transplantation thus 'bridging to recovery'.

1.10.02 Hepatocyte transplantation

Cell transplantation is a more recent concept with the first human hepatocyte transplant performed in 1992 (Mito *et al*. 1992). Results are promising where this is used as a bridge to OLT (Strom *et al*. 1997, Mitry *et al*. 2003, 2004) but realisation of its potential has not yet been achieved (Soltys *et al*. 2010).

1.10.03 Artificial liver support (ALS) and bioartificial liver (BAL) systems

Prior to successful whole organ transplantation, initial research concentrated on the removal of toxic metabolites (Kiley *et al*. 1951, 1957). The focus was on dialysis or haemoperfusion using cartridges and membranes that could remove low molecular weight toxins. Charcoal perfusion (Silk *et al*. 1978) gained popularity, but demonstrated no impact on survival.

Advances in cell biology and in-vitro cell culture methods, led to the possibility of incorporating biological systems (Dixit 1994). This new generation of BAL devices offered potential detoxifying and synthetic hepatic function whilst avoiding indiscriminate removal of useful stimulants to hepatic repair such as hepatocyte growth factor (Seldon *et al*. 1986).

The ideal hepatocyte would be human, of normal (non-malignant) phenotype, readily available, rapidly and easily grown in culture to high density, stable, remaining differentiated and capable of the synthetic and detoxifying features of mature hepatocytes. Hepatic replacement therapy is both filtration and bioartificial, although most systems use a combination of both and are hybrid. The need to develop a bioartificial support system was first proposed by Sorrentino in 1956 and the first clinical BAL described by Matsumara in 1987.

1.10.04 ALS: haemodialysis

More than 50 years ago the first technically successful use of haemodialysis for treatment of animals with ammonia intoxication was described and applied to cirrhotic human patients (Kiley *et al*. 1957). It was postulated these results could have implications for the treatment of hepatic coma. Later, a polyacrylonitrile membrane was used to treat 24 patients suffering from viral ALF (Opolon *et al*. 1976). Improvement in coma without survival benefit was achieved.

1.10.05 ALS: charcoal

Charcoal haemoperfusion uses charcoal to bind water-soluble molecules thought to contribute to metabolic encephalopathy. Clinical improvement in biochemical parameters and consciousness in an alcoholic with ALF was seen following treatment with albumin encapsulated charcoal (Chang 1972). Two years later a larger study of patients with ALF was conducted in London. Of the 22 patients treated, eleven regained consciousness and ten were discharged home, potentially demonstrating a survival advantage (Gazzard *et al*. 1974). Clinical complications arose with hypotension linked to platelet activation and aggregation as they passed through the column (Silk & Williams 1978). This was successfully overcome with concurrent prostacyclin infusion but the documented improvement in biochemistry and encephalopathy (Gimson *et al*. 1982, Hughes *et al*. 2002) had no correlation with survival.

1.10.06 ALS: plasma exchange

This involves exchanging blood for plasma and was investigated as a means of removing toxins associated with ALF (Lee & Tink 1958). Results from early trials demonstrated improvement in hepatic coma and biochemical parameters without survival benefit (Lepore *et al*. 1972, Freeman & Matthewsson 1986). There were significant side-effects including toxicity, viral infection and death (Brunner & Losgen 1987).

1.10.07 ALS: Molecular Adsorbent Recirculating System (MARS)

MARS is albumin dialysis (figure 8), removing water-soluble albumin bound toxins through a semi-permeable membrane. Proteins such as hormones, clotting factors, antithrombin III and albumin remain in the circulation whilst albumin is recycled. Other properties allow the retention of electrolytes and glucose, management of acid/base and

fluid balance (Stange *et al*. 2002).

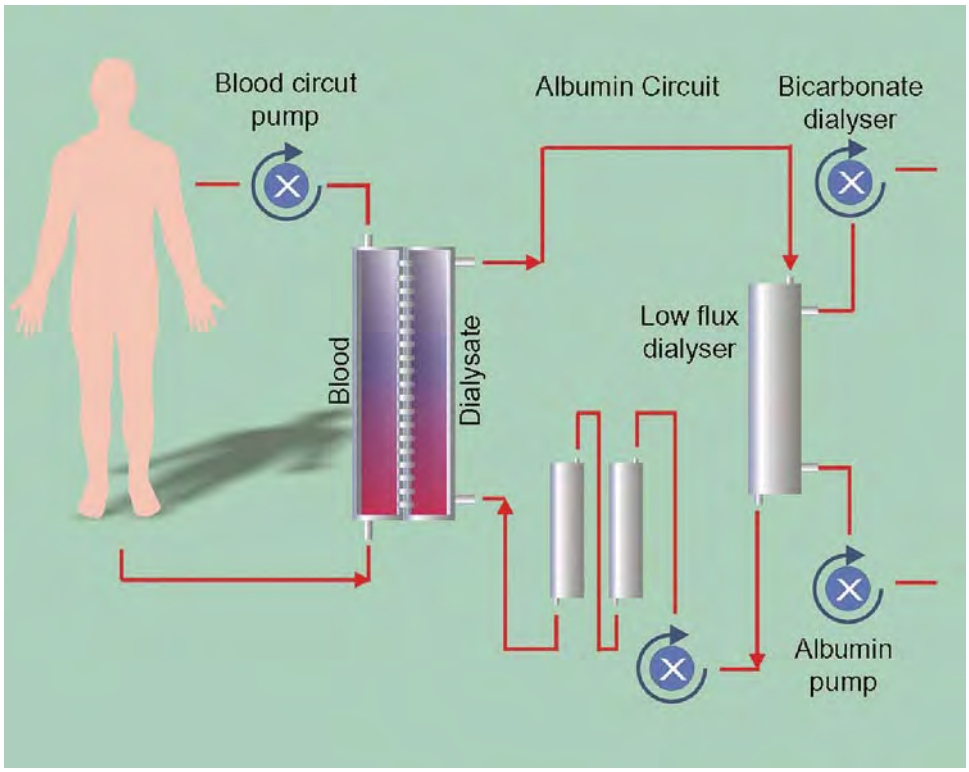


Figure 8 MARS (adapted from Stange 2002).

The system is safe (Mitzner *et al*. 2000) but recently it has been demonstrated in early figures for the RELIEF study (n=189), although MARS confers benefit in terms of dialysis, it does not improve survival (Banares *et al*. 2010).

1.10.08 ALS: Prometheus

The Prometheus system combines dialysis equipment with an adsorber treatment (Rifai *et al*. 2003). Plasma, albumin and small proteins pass through two adsorbers that separate and bind toxins from albumin. Following adsorption, blood is dialyzed to remove remaining water-soluble toxins. The system is similar to MARS with the recent HELIOS trial demonstrating clinical benefit without survival improvement (Rifai *et al*. 2010).

1.10.09 Biological support: whole organ perfusion

Cross-circulation was used in 1967 (Burnell *et al*. 1967) with suboptimal results and complications in the volunteers. The ethical viability of this procedure is questionable with this side effect profile and thus it was abandoned.

Porcine whole organ perfusion was first described in 1965 in an animal model (Eiseman *et al*. 1965). Subsequently the livers of many species (monkey, rat, porcine) have been used as extracorporeal units, with varying results. Early work consisted of *ex vivo* perfusion of multiple livers from 5 animal species over a period of 10 weeks on a single patient (Abouna *et al*. 1970). Improvement in neurological status was seen without survival benefit. In another study five of eight dogs with surgically induced ALF were bridged to recovery (as measured by eight day survival) using a calf liver. The liver only showed early xenograft rejection with native liver regeneration sufficient to correct blood parameters (Abouna *et al*. 2001).

1.11 BAL Technology

1.11.01 Design

Innovations in hepatocyte isolation have directly resulted in BAL development. The complex requirements of BAL have resulted in a multitude of designs with vital considerations being exposure of adequate cell mass to plasma or blood, cells receiving sufficient nutrition and oxygenation, configuration, cell source and scale-up technology.

1.11.02 Hollow fibre reactors

The simplest bioreactor design, the hollow fibre consists of cartridges through which there is perfusion of blood or plasma though a hollow fibre lumen, with the hepatocyte component entrapped in gel and inoculated into the fibres of the extra-fibre space. This is the most commonly used vehicle for BAL (Sussman *et al*. 1992, Watanabe *et al*. 1997). Plasma or blood is circulated through the extracapillary space allowing diffusion of across the interface.

1.11.03 Perfused bed BAL

The perfused bed or scaffold BAL is where a synthetic framework is inserted within the housing cartridge to which hepatocytes attach. Plasma or blood is perfused through the cartridge with direct cell contact. The limitation of this design is the ability of the cells to attach and novel frameworks are often used (Yang *et al*. 2001). Potential immunological consequences of two different individuals (or species) cross-circulating were overcome by incorporating an immunosorbent column. Evaluating this system using porcine hepatocytes with a dog with ALF, it was demonstrated that the system reversed hepatic encephalopathy, increased survival and improved biochemistry. The immunosorbent

column worked sufficiently well to allow xenogenic perfusion (Sosef *et al*. 2002).

1.11.04 Fluidised bed BAL

This consists of a column of encapsulated hepatocytes, through which blood is introduced thus ‘fluidisation’ (Hwang *et al*. 2000).

1.12 Clinically assessed BAL

In 1987 a patient with liver failure was treated with cryopreserved rabbit hepatocytes in the first clinical BAL (Matsumara *et al*. 1987). Although no clinical benefit was confirmed, biochemical improvement offered some support for the potential application of a BAL. The most used systems are described, with their configurations differing (table 8).

System	Configuration	Author
AMC-BAL	Spiral wound, non-woven, polyester matrix, no membrane	van de Kerkhove *et al*. 2002
BLSS	Hollow fibre, cellulose acetate, 100 kDa cut off	Patzer *et al*. 2002
ELAD	Hollow fibre, cellulose acetate, 120 kDa cut off	Watanabe *et al*. 1997
Hepat-Assist	Hollow fibre, polysulphone, 0.15-0.2 μm pores	Demetriou *et al*. 2004
Hybrid BAL	Hollow fibre, polysulphone, 100 kDa cut off, adsorption column	Ding *et al*. 2003
Liver haemodialysis unit	Haemodiabsorption across 5 kDa cut off cellulose membranes	Ash *et al*. 2002
LiverX-2000	Cells imbedded in collagen matrix	Sielaff *et al*. 1997
MARS	Albumin loaded filter, 60 kDa cut off	Stange *et al*. 2002
MELS	Hollow fibre, interwoven, multicompartment	Sauer *et al*. 2003
Prometheus	Haemofilter, 250 kDa cut off, two absorber cartridges, in series with conventional dialsyser	Laleman *et al*. 2006

Table 8 BAL configurations.

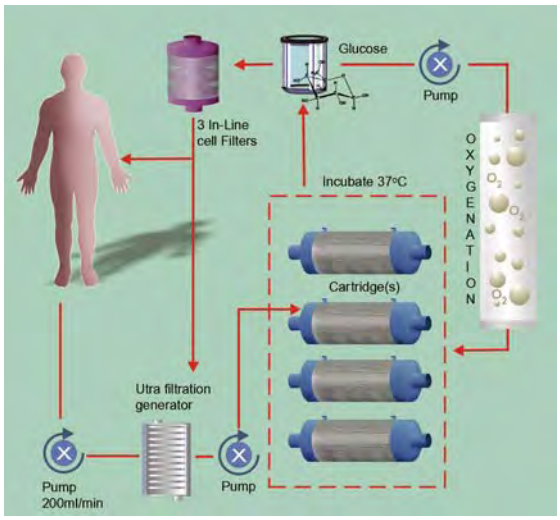


Figure 9 ELAD™
(Adapted from Sussman *et al.* 1992).

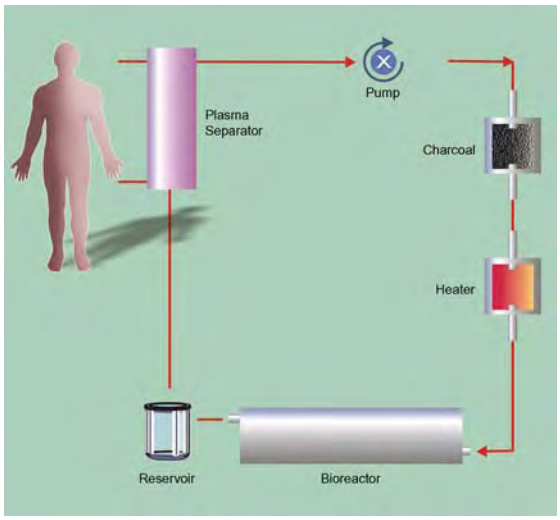


Figure 10 HepatAssist
(Adapted from Demetriou *et al.* 2004).

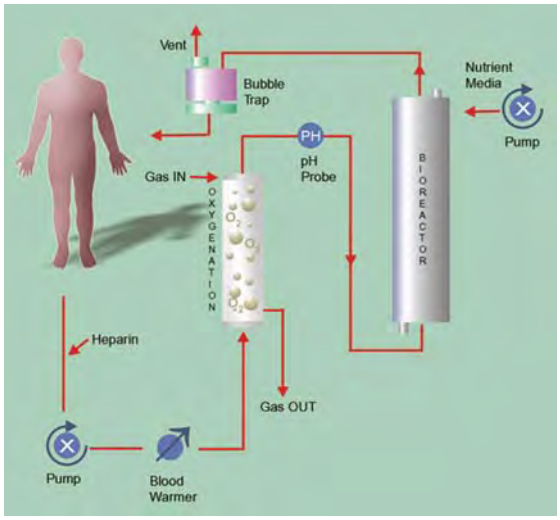


Figure 11 BLSS.
(Adapted from Patzer *et al.* 2002).

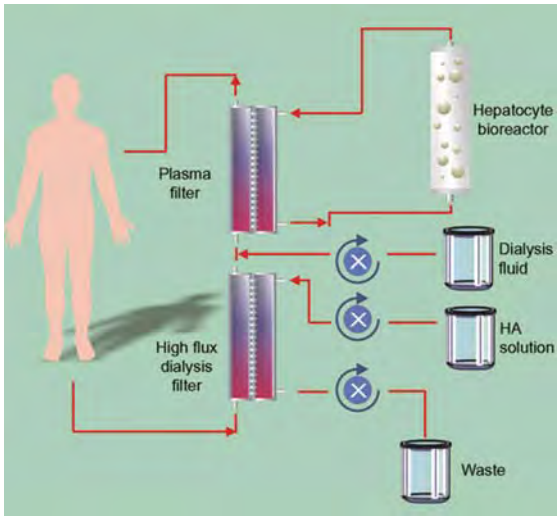


Figure 12 MELS
(Adapted from Sauer & Gerlach *et al.* 2002).

1.12.01 ELAD™

This BAL is a hollow fibre system containing six billion dextran microcarrier attached human hepatoblastoma cell line hepatocytes (C3A cells) (Sussman *et al*. 1992, 1993), (figure 9).

1.12.02 HepatAssist

The most studied system is HepatAssist, which consists of a plasma separator, a high flow

plasma recirculation system, hollow fibre bioreactor and charcoal column to protect hepatocytes from plasma (figure 10). Initially evaluated on animal models (Rozga *et al*. 1994), later it was used in clinical studies (Demetriou *et al*. 1995, 2004, Detry *et al*. 1999).

In 1997 a phase I trial was completed (Watanabe *et al*. 1997) with a single centre trial with encouraging early results in 2002 (Samuel *et al*. 2002). Little has been published since.

1.12.03 Excorp Medical Bioartificial Liver Support System (BLSS)

This is a hollow fibre design utilising porcine hepatocytes infused into the extra-capillary space of the reactor (figure 11) (Patzer *et al*. 1999, Mazariegos *et al*. 2002) and is perfused by whole blood rather plasma. There are phase II clinical trials (Patzer *et al*. 2002) but no randomised trials.

1.12.04 Modular Extracorporeal Liver Support (MELS)

A multi-compartment hollow fibre design (Sauer & Gerlach 2002) incorporating human or porcine hepatocytes (Gerlach *et al*. 1994) contained within a collagen matrix (figure 12). This sits within hollow fibres and is connected to a MARS device for further detoxification. There are no randomised control trials.

Cochrane reviews are independent systematic reviews assessing the effects of healthcare interventions and assist in global healthcare decision-making. Their review of liver support systems suggested a mortality reduction in AoC liver failure, with no improvement in ALF. The strength of evidence was poor and additional clinical trials would be essential before any support system could be recommended (Liu *et al*. 2002). A subsequent meta-analysis contradicts this conclusion, describing improved survival in ALF with no benefit with AoC liver failure (Stutchfield *et al*. 2011). Few clinical trials exist for either ALS or BAL, summarized in table 9. It is fairly clear that little real gain is achieved with BAL therapy.

Table 9 Liver support system outcomes [part 1 of 3]

Authors	Patient population	n	Suitable for Tx	System	Cell type	State	Study design	End-point	Duration of dialysis (hrs)	Outcome
van de Kerkhove *et al*. 2002	ALF	7	Yes	AMC-BAL	Porcine	Fresh	First 7 patients	Tx	18 x1-2	6 of 7 bridged to Tx, 1 recovered
van de Kerkhove *et al*. 2003	ALF secondary to HBV	1	Yes	AMC-BAL	Porcine	Fresh	First patient	Tx	35hrs (2 sessions)	Successfully bridged to Tx
Mazariegos *et al*. 2002	ALF	1	Yes	BLSS	Porcine	Fresh	Case study		12 x2	Died day 6
Patzer *et al*. 2002	Acute on chronic 2, ALF 2	4	Some	BLSS	Porcine	Fresh	I/II	Tx	12 x1-2	3 died pre-transplant (days 5-10), 1 survived to transplant at 16 days
Irgang *et al*. 2003	ALF (2 drug overdose, 2 unknown, 3 hepatitis B)	8	All	CellModule	Porcine	Fresh	Single centre	Tx	27.3 (8-48)	100% survival to Tx, 1 excluded as declined F/U
Burnell *et al*. 1967	ALF	4	No	Cross-circulation	None		Case study		Approx. 4 sessions	3 recovered from coma, 1 completely. Significant volunteer complications
Ellis *et al*. 1996	ALF not fulfilling Tx criteria (I), ALF fulfilling Tx criteria (II)	(I) 17 (II) 7	Some	ELAD	Immortalised human hepatocytes	Fresh	Single centre randomised control trial	In hospital mortality or Tx	Mean 72	Survival group I 78%, controls 75%, group II 33%, controls 25%, no difference, survival in control patients much higher than expected.
Millis *et al*. 2001	ALF not fulfilling Tx criteria (I), ALF fulfilling Tx criteria (II)	(I) 5 (II) 19	Some	ELAD	Immortalised human hepatocytes	Fresh	Randomised controlled phase I trial	30-day mortality		Inadequately powered for significance. Listed for Tx, 30-day survival ELAD 83%, controls 43%. Tx received 92%, controls 43%
Millis *et al*. 2002	ALF (3 idiopathic, 1 drug induced, 1 ischaemic)	5	All	ELAD	Immortalised human hepatocytes	Fresh	First five patients' experience	Tx	51.4 (12-107)	100% survival to Tx, 1 immediate post-Tx death, 4/5 30-day survival
Sussman *et al*. 1993	ALF	11	Yes	ELAD	Immortalised human hepatocytes	Fresh	Phase I	Tx		1 recovered, 4 bridged to Tx 6 died
Demetriou *et al*. 2004	ALF (I), Primary non-function (II)	(I) 147 (II) 24	Yes	Hepat-Assist	Porcine	Cryo.	Randomised controlled phase II trial	30-day survival	6 x1-14	Improved 30-day survival (71% vs 62%)
Pazzi *et al*. 2002	ALF, AoC	13 + 5	Unknown	Hepat-Assist	Porcine	Cryo.	II/III	Bilirubin level	6 x1-14	Treatment was associted with improved biochemistry
Samuel *et al*. 2002	ALF	10	Yes	Hepat-Assist	Porcine	Cryo.	II/III	Tx	6 x1-14	10% 30-day mortality, 9 of 10 bridged to Tx

Table 9 Liver support system outcomes [part 2 of 3]

Authors	Patient population	n	Suitable for Tx	System	Cell type	State	Study design	End-point	Duration of dialysis (hrs)	Outcome
Stevens *et al*. 2001	ALF (I), primary non-graft function (II)	(I) 147 (II) 24	All	Hepat-Assist	Porcine	Fresh	Multi-centre randomised control trial	30-day mortality		Substantial impact of transplant (total of 54% of all patients). 30-day survival: all patients with BAL 71%, controls 62%. All ALF with BAL 73%, controls 59% (p=0.1). ALF due to paracetamol OD with BAL 70%, without 37% (p=0.05)
Watanabe *et al.* 1997	ALF	18	Yes (all ALF)	Hepat-Assist	Porcine	Cryo.	Phase I	Tx	6 +/-2	16 of 18 were bridged to Tx
Ding *et al*. 2003	ALF	12	Unknown	Hybrid BAL	Porcine	Fresh	I	On dialysis death	6 x1-2	25% died
Ash *et al*. 2000	AoC or ALF		Unknown	Liver HD	None		Randomised, controlled trial		6 x1-5	Improved neurological status
Ash *et al*. 2002	ALF (paracetamol overdose)	10	Unknown	Liver HD	None		Randomised, controlled trial	Discharge or Tx	6 x1-5	Some increased bleeding risk, all recovered
Sielaff *et al*. 1997	Induced ALF	0	N/A	LiverX-2000	Porcine	Fresh	Porcine study			
El Banayosy *et al*. 2004	ALF	27	Unknown	MARS	None		Randomised controlled trial	30-day mortality	3 sessions	Improved survival(half vs third of controls survived)
Hassanein *et al*. 2007	Hepatic encephalopathy	70	Unknown	MARS	None		Randomised controlled trial		6 x5	Improvement of encephalopathy
Heeman *et al*. 2002	AoC liver failure	24	Unknown	MARS	None		Randomised controlled trial	Bilirubin level	7.7 sessions	Improved 30-day survival, improvement in renal function and bilirubin
Laleman *et al*. 2006	AoC liver failure, alcoholic hepatitis	18	Unknown	MARS	None		Randomised controlled trial		6 x3	Improved haemodynamic stability
Mitzner *et al*. 2000	ALF	13	Unknown	MARS	None		Randomised controlled trial	30-day survival	x5 mean	Improved 30-day survival compared to 100% in the control group
Sen *et al*. 2004	Alcoholic acute on chronic liver failure	18	No	MARS	None		Randomised controlled trial	Cytokine profile	8 x4	Improvement of encephalopathy, no survival benefit
Stange *et al*. 2002	Acute on chronic liver failure	103	Unknown	MARS	None		Multi-centre report	In hospital mortality or Tx	12-132	Some survival benefit
Sauer *et al.* 2003	Primary non-function	1	Yes	MELS	Human	Fresh	Single patient	Tx	79	Survived to Tx

Table 9 Liver support system outcomes [part 3 of 3]

Authors	Patient population	n	Suitable for Tx	System	Cell type	State	Study design	End-point	Duration of dialysis (hrs)	Outcome
Sauer *et al.* 2003	ALF	8	Unknown	MELS	Human	Fresh	II/III	Safety	7-144	36 bioreactors were prepared, only 10 used to treat 8. No adverse events
Rozga *et al.* 1994	ALF	7	Yes	Hepat-Assist	Porcine	Fresh	Phase I	Tx	x1-3 sessions	All bridged to Tx
Baccarani *et al.* 2005	ALF (1 viral, 1 crytogenic)	2	All	Performer BAL	Human	Cryo.	First two patients	Tx or recovery	1.5 treatments	1 Tx, 1 recovered spontaneously
Dethloff *et al.* 2008	ALF	24	Unknown	Prometheus	None		Randomised controlled trial		6	Nil significant
Kramer *et al.* 2003	Drug induced ALF	1	No	Prometheus	None		I	30-day survival	12 x1-2	Patient recovered
Laleman *et al.* 2006	AoC liver failure, alcoholic hepatitis	18	Unknown	Prometheus	None		Randomised controlled trial		6 x3	Improved bilirubin
Rifai *et al.* 2003	AoC liver failure with renal failure	11	Unknown	Prometheus	None		I		5+/1	8 of 11 died in hospital. None were transplanted
Morsiani *et al.* 2002	ALF	7	Yes	Radial flow bioreactor	Porcine	Fresh	I/II		6 to 24	6 were bridged to Tx
Pascher *et al.* 2002	ALF	14	Yes	Whole liver perfusion	Porcine	Fresh	Review		5 x1-2	No benefit
Pascher *et al.* 2002	ALF	198	Unknown	Whole liver perfusion	Variable	Fresh	Review		5 x1-2	Benefit in those receiving allogenic therapy

1.13 Cell selection

1.13.01 Human hepatocytes

Human cells are the ideal tissue source for a human BAL but use is hindered by low availability (Hoofnagle *et al*. 1995, Strain & Neuberger 2002). The use of this tissue is subject to complex legal conditions that vary between countries but more importantly there is concern over possible transmission of carcinoma or infection (Tsiaoussis *et al*. 2001). This could be overcome by plasma filtering along with efficient screening and quality control. Further challenges include the sharp decrease in cellular function after culture and inability of the cells to proliferate (Nagamori *et al*. 2000).

1.13.02 Immortalised cell lines (tumour and foetal)

One technique used to preserve cellular function and allow proliferation is to use immortalised cells from primary hepatocellular carcinoma or hepatoblastoma cells (HepG2) or foetal material. This could produce an unlimited supply of hepatocytes as required but a number of key CYP450 functions may not be preserved (Morsiani *et al*. 2002). There is also the theoretical risk of transmitting immortalised cells or tumorogenic products to the patients' circulation (Tsiaoussis *et al*. 2001) with unknown consequences. Foetal hepatocytes have been immortalized by introducing a plasmid (SV3neo) expressing a simian virus 40 large T-antigen (SV40Tag) gene (Kobayashi *et al*. 2000, 2001). They demonstrated no tumourigenesis after transplantation, with some metabolic function in a rat model and later this group were able to clone and maintain this population (Kobayashi *et al*. 2000).

Immortalised hepatocyte lines (HHY41) have been sandwich cultured whereby at four weeks the cells were dividing and continued subcultures were possible (Kono *et al*. 1995).

Reduced hepatocyte function has been documented when immortalised cells are exposed to human serum (Wang *et al*. 1998) and there is debate as to whether they perform better than xenogenic cells (Nyberg *et al*. 1994, Wang *et al*. 1998).

1.13.03 Progenitor or Stem cells

Stem cell research is an interesting recent development (Laurson *et al*. 2005, Muraca 2011). The capacity to regenerate is thought to be secondary to hepatocyte's ability to enter the cell cycle (Muraca 2011). However, liver also contains pluripotent cells with the potential to proliferate and mature into all types of hepatic cell (Susick *et al*. 2001, Alison 2002).

Bone marrow stem cells can differentiate into hepatocytes *in vivo* and *in vitro* with some synthetic and metabolic function (Shi *et al*. 2005). Equally umbilical cord blood derived mesenchymal stem cells have a versatile potential for differentiation and show hepatogenic potential which may enable them to be used as a source of hepatocytes (Hong *et al*. 2005).

The perceived advantages of stem cells over mature cells are that pluripotent cells can differentiate into all cell types, express superior cryopreservation tolerance and have minimal immunogenicity (Susick *et al*. 2001, Ilan 2002).

1.13.04 Xenogenic hepatocytes

The problems with using xenogenic hepatocytes are summarised into four categories: ethics, biocompatability, immunogenicity and zoonoses (Morsiani *et al*. 2002, Sen & Jalan 2005). Their use is attractive in practical terms but acceptance of such a practice by society is unknown. Xenogenic hepatocytes are typically porcine although murine, rat, canine, primate and goat have been used (Vijayalakshmi *et al*. 2004). They could potentially be

harvested from pathogen-free animals with relative ease, cryopreserved and shipped for use. Porcine hepatocytes have been used extensively in BALs with 15000M cells isolated per organ, with initial viability of 92+/-5%, which can improve over 24 hours of culture to 95+/-3%, with a purity of 98% (Li *et al*. 2005). Porcine hepatocytes in culture can maintain phase I and II biotransformation reactions with similarities to human cells, therefore with the paucity of availability of human cells, many conclude the pig is the most appropriate species to use in a BAL (Donato *et al*. 1999). Others report marked and important interspecies variability in hepatocyte metabolic activity (de Souza *et al*. 1991). Another concern is that porcine hepatocyte function and viability can deteriorate on exposure to serum, possibly by activating the complement cascade (Wang *et al*. 1998) with similar findings with rat hepatocytes exposed to sera of human liver cancer patients (Grant *et al*. 2001).

An estimated 7% of the population have pre-formed IgM antibodies against porcine hepatocytes (Takahashi *et al*. 1993), so risk of immunological rejection is a concern. No significant adverse effects have been discovered so far, although this could be due to the relative immunosuppression of the patients suffering from ALF (Tsiaoussis *et al*. 2002). Repeated exposure to a BAL is probably a greater risk; patients receiving two or more treatments with a porcine BAL demonstrate a two- to three-fold increase in anti-pig antibodies (Baquerizo *et al*. 1997). The risk of hypersensitivity reactions and humoral sensitisation with the production of antibodies that would cross-react with human antigens is also possible but not shown experimentally (Cotterell *et al*. 1995). Graft versus host has also been seen with whole organ work which could compromise porcine BAL development (Rees *et al*. 2002). Potential immunological implications of repeated BAL use to the patient and bioreactor need evaluation.

1.13.05 Zoonoses

There is potential that animal cells may transmit infection to human hosts and the discovery of PERV has cast doubt on the suitability of using porcine tissue. PERV has been introduced to human cells *in vitro* with successful replication (Patience *et al*. 1997, Paradis *et al*. 1999). This is so common in the porcine population, to breed virus-free pigs may not be possible (Le Tissier *et al*. 1997). Despite these concerns there has been no demonstration that clinically applicable infection has been seen in people exposed to porcine BALs or xenografts (Patience *et al*. 1997), despite PERV being seen in BAL media (Liu *et al*. 2005). Many studies only test for PERV immediately after exposure (within 24 hours) and it is difficult to believe this can truly be representative of risk (Falasca *et al*. 2001, Kuddus *et al*. 2002). Few studies have long-term follow-up (Heneine *et al.* 1998, Irgang *et al*. 2003) and as such transmission is unknown.

Viruses are adept at changing to suit their environmental conditions (Ludwig *et al*. 2003) and theoretically PERV could lead to new oncogenic or immunodeficient infections in humans.

1.13.06 Cell mass

Hepatic encephalopathy occurs when liver function falls below 25-30% of normal therefore, it might be assumed that this volume of hepatocytes are needed to provide effective liver support.

There is no consensus regarding the ideal number of hepatocytes required to support a patient with liver failure and mass cannot be equated directly with function (Sauer *et al*. 2001). Hepatocytes within a BAL may have reduced functional ability when compared with a normal whole organ. Liver resection data suggests the body requires at least 25% (Clavien *et al*. 2010) of normal functional liver mass to support life. This equates to 100-

400g (Rosenthal 2000) of cells, assuming there is no endogenous liver function. There is still some functioning liver in most patients with liver failure (Hui *et al*. 2001), so sufficient cell mass to provide support rather than complete replacement may suffice.

In BAL developed to date there is no consensus as to cell number (Tsiaoussis *et al*. 2001). HepatAssist uses approximately 6 billion porcine cells, corresponding to approximately two percent of normal cell number (Rozga *et al*. 1993). ELAD uses 40 billion C3A cells (Sussman *et al*. 1992), 15% of normal hepatocyte mass. MELS uses 100 billion porcine or human hepatocytes, equivalent to 400-600 g of tissue and 33% of normal hepatocyte mass (Gerlach *et al*. 1994), whilst BLSS (Patzer *et al*. 2002, Mazariegos *et al*. 2002) utilised 70 g of porcine hepatocytes (5% of normal mass). In experimental models (de Bartolo *et al*. 2000) only 10 billion porcine cells (3.3% of normal mass) have been used although it is difficult to see this small number of cells providing adequate support.

1.14 Apoptosis

1.14.01 Historical perspective

Between 1962 and 1964 two types of cell death were histologically distinguished: classical necrosis and 'shrinkage' necrosis (Kerr 1971). It is this latter group that were later described as undergoing the process of apoptosis (Kerr 1972). Kerr correlated this cellular process with descriptions given in 1890 by Councilman who identified hyaline bodies in the livers of patients with Yellow fever. Subsequently DNA fragmentation was described (Wyllie 1980). Later the Nobel Prize in Physiology or Medicine (2002) was awarded for discoveries concerning genetic regulation of programmed cell death.

1.14.02 Morphology of apoptosis and necrosis

Apoptosis is an energy consuming mechanism of cellular death characterised by distinct morphological change and biochemical abnormality. These standardised changes suggest a common pattern to this process. It is characterised by condensation and fragmentation of nuclear chromatin, compaction of cytoplasmic organelles, dilatation of the endoplasmic reticulum, reduced cell volume and alterations to the plasma membrane. The overall shrinkage and nuclear compaction is the easiest to identify. These changes allow rapid recognition and destruction of the shrunken cell by phagocytes and neighbouring cells *in vivo* (Studinski 1999).

The pattern with necrosis, a passive cell death are that changes in the plasma membrane stimulate loss of sodium and calcium homeostasis, with acidosis, osmotic shock, chromatin clumping and nuclear pyknosis (shrinking and condensation) (Sandritter *et al.* 1975). Intracellular sodium is reduced with subsequent water loss to maintain osmotic pressure across the plasma membrane. Potassium ions are lost into the interstitium thereafter

(Chaudary *et al*. 1981). Intracellular calcium increases, leading to mitochondrial dysfunction (Fitzpatrick *et al*. 1984) and a reduction in adenosine triphosphate (ATP) production. Membrane integrity is destroyed, there is cellular and organelle swelling and the cell dies by necrosis (Halliwell 1989). The toxic cellular content is expelled into the extracellular fluid with subsequent inflammation (Rosser & Gores 1995).

Apoptosis is difficult to measure *in vivo* as phagocytes engulf these cells thus preventing further tissue injury (Raffray & Cohen 1997). *In vitro*, the absence of phagocytes makes measuring apoptosis more straightforward. It is a cell driven event fundamental to normal development with necrosis always being pathological.

1.14.03 Mechanisms of cell death

Apoptosis is a complex process involving activation of a hierarchy of events leading to cell death. It is a single cell process, although many cells may be undergoing an identical process concurrently, whereas necrosis is a more global phenomenon. It is not programmed cell death nor physiological cell death.

Triggers are classically intracellular although extracellular influences play their role. Mitochondria are vital, possibly integrating and co-ordinating the process and finally the pro-apoptosis machinery interacts with cell survival mechanisms and executioner caspases. Although the exact mechanisms of apoptosis remains inadequately understood there are two hypothesised pathways, ‘surface death receptor mediated’ and ‘mitochondrial’ pathways.

1.14.04 Death receptor mediated pathway (Fas)

This pathway can be initiated by activation of death receptors on the cell membrane that belong to the tumour necrosis factor (TNF) superfamily. Hepatocytes express Fas (CD95)

and TNF-R1 (TNF receptor-1) (Yoon & Goves 2002). It is the intracellular component of these receptors that contains the death domain signalling apoptosis. Others have used the term ‘induced proximity’ whereby fas receptors aggregate into complexes which recruit multiple pro-caspase-8 molecules. Under these conditions low protease activity inherent to pro-caspases is sufficient to drive activation of receptor-associated pro-caspase-8. Reports have suggested this latter theory is also associated with the activation of caspase-9 in humans (Hengartner 1998). Thus fas recruits effector caspase-8 and mediates transduction of the death signal in cells (Maeda 2002).

1.14.05 Mitochondrial (or caspase) pathway

Inactive pro-enzymes are herded together to increase local concentration and allow conformations that promote activation of the caspase cascade (figure 13) with sequential activation of downstream effector caspases or pro-caspases (Hengartner 1998).

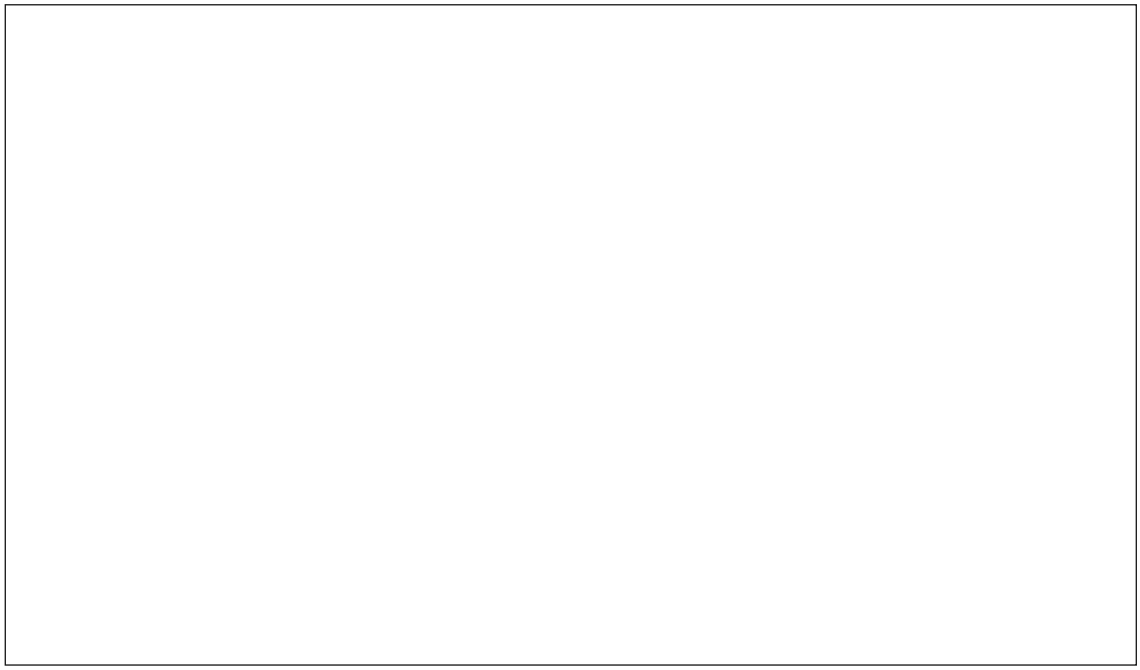


Figure 13 Caspase cascade (from Holcik & Korneluk 2001).

Mitochondria are essential for energy metabolism, production of membrane lipids and cell

growth, but they are also stress sensors and executioners. Mitochondrial membrane abnormalities lead to release of apoptotic mediators (Kroemer *et al*. 1995). They can provide critical antioxidant source (ROS) as a by product of oxidative phosphorylation and although important, apoptosis can be independent of this process. Electron transport disruption disorganises oxidative phosphorylation with resultant altered ATP and redox potential with release of proteins that trigger caspase activation (Green & Reed 1998). Along with ROS, pro-caspases and cytochrome c can be released with subsequent calcium liberation from the ER (Lui *et al.* 1996). This precedes nuclear apoptosis and is inhibited by the anti-apoptotic proteins of the Bcl-2 family (Yang *et al.* 1997). However, without rupture the large outer membrane channel is theoretically capable of liberating cytochrome C. This is an attractive hypothesis as it obviates a need for mitochondrial swelling which is a feature absent in apoptotic cells *in vivo*.

Hepatocytes are rich in mitochondria and thus these cells demonstrate a propensity for mitochondrially-induced apoptosis activated via inappropriate exposure to reactive oxygen species. Release of mitochondrial proteins leads to membrane or permeability transition pore (PTP) opening. Active effector caspases also induce opening of these PTP (Marzo *et al*. 1998) thus feedback loops are inevitable.

If PTP is induced there is mitochondrial dysfunction, lipid peroxidation, altered cell signalling, DNA damage and death. In the presence of widespread PTP, ATP concentration drops significantly and results in necrosis (Lemasters *et al*. 1998).

Cytochrome c is an essential component of the electron transport chain in mitochondria and a pro-apoptotic protein which complexes with pro-caspase 9. This subsequently cleaves and actives the caspase cascade (Schoemaker & Moshage 2004). Progression of this cascade ends with the activation of caspase-3 which is the execution phase of apoptosis (Yang *et al*. 1997). Cytochrome c kills the cell by fast apoptosis or slow necrosis secondary

to the collapse of the electron transport system. Release is not prevented by caspase inhibitors thus it may also contribute to Fas-mediated apoptosis.

In summary, metabolic alterations in mitochondria lead to disruption of PTP, changes in the cellular redox state and apoposis.

1.14.06 Apoptosis and cryopreservation

The mechanism of cell death during cryopreservation is not fully understood. During cryopreservation ATP stores are depleted and intracellular acidosis increases. This prevents or reduces the amount of calcium that can be extruded from the cell and intracellular calcium increases although the exact role of calcium has not been elucidated. Storage and thawing of cryopreserved cells is associated with severe cellular damage and existing methods for cryopreserving hepatocytes do not allow for adequate cell recovery. Careful control of cryopreservation media incorporating anti-apoptotic agents could improve outcome in terms of viability and reduced cell loss by permitting reduced concentration of cryoprotectants by regulating apoptosis (Baust *et al*. 2000).

Cryoinjury induced apoptosis is mediated by a death receptor independent, mitochondrial pathway that does not involve non-caspase proteases (Stroh *et al*. 2002). The freeze-thaw process induces caspase-3, with Bcl-2 conferring protection against cryoinjury.

Hepatocytes entrapped in cylindrical collagen gels located within the hollow fibres of a bioartificial liver undergo apoptosis at a rate of 40-45% at 24 hours (Rivera *et al*. 1999). Similar findings were reported with murine hepatocytes (Fu *et al*. 2001), 7% of fresh cells contrasting with 45% of cryopreserved cells were apoptotic. Addition of glucose improved findings in both groups.

When adherent hepatocytes are detached and maintained in suspension, their cell-cell

bonds are disrupted and a proportion of the population will die in a process termed anoikis. With endothelial detachment there is a rise in intracellular ROS thought to be from mitochondria which in turn increases caspase activity (Li *et al*. 1999).

Whether in static culture or 3D culture fresh and cryopreserved porcine hepatocytes showed reduced caspase-3 activity, apoptosis, cytochrome C and slower mitochondrial membrane potential abnormalities when cultured with a global caspase inhibitor (Matsushita *et al*. 2003). Treated cells had improved synthetic and metabolic function. In fresh culture 34% of cell death at 24 hours in the fresh group were apoptotic, rising to 74% with cryopreserved cells.

01.14.07 Curcumin

The exact mechanism of action of curcumin [1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadien-3,5-dione] (figure 14) derived from the spice turmeric has not been clearly elucidated.

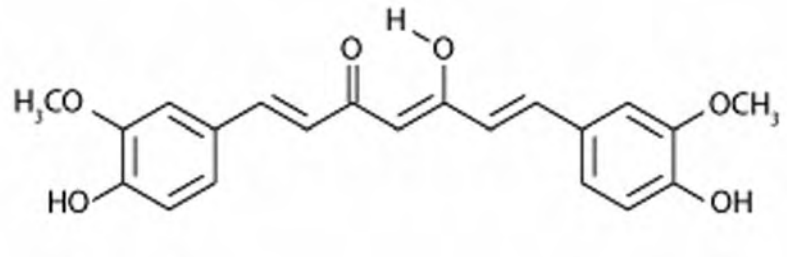


Figure 14 Chemical structure of curcumin.

It has chemopreventative effects (Rao *et al*. 1995) with additional hepatoprotective effects against alcohol-induced liver disease (in rats) by inhibiting lipid peroxidation and expression of NF-κB dependent genes (Nanji *et al*. 2003). Curcumin is an antioxidant, able to inhibit Ca^{2+} induction of increased mitochondrial membrane potential by inhibiting superoxide production and lipid peroxidation (Morin *et al*. 2001).

Injection of curcumin inhibits rapid transcriptional suppression of CYP2E1, CYP3A2

and CYP2C11 (Cheng *et al*. 2003) as seen with systemic insult (Morgan 1997). In contrast, in a rat liver microsomal study pre-treatment with oral curcumin was associated with dose dependent inhibition of CYP1A1 and CYP1A2 (Thapliyal & Maru 2001).

At lower dose there is cell swelling, which reduces as dose increases: it has both pro- and anti-apoptotic properties in a biphasic profile (Nanji *et al.* 2003). The combination of these effects might indicate curcumin could be a useful compound *in vitro*.

1.14.08 Ethanol

Solid chemicals need a solvent for dissolution for *in vitro* studies and many are water insoluble. Ethanol (figure 15) is an ideally positioned solvent with potential toxicity. Many studies demonstrate a dose-dependent increase in apoptosis in hepatocytes exposed to ethanol.

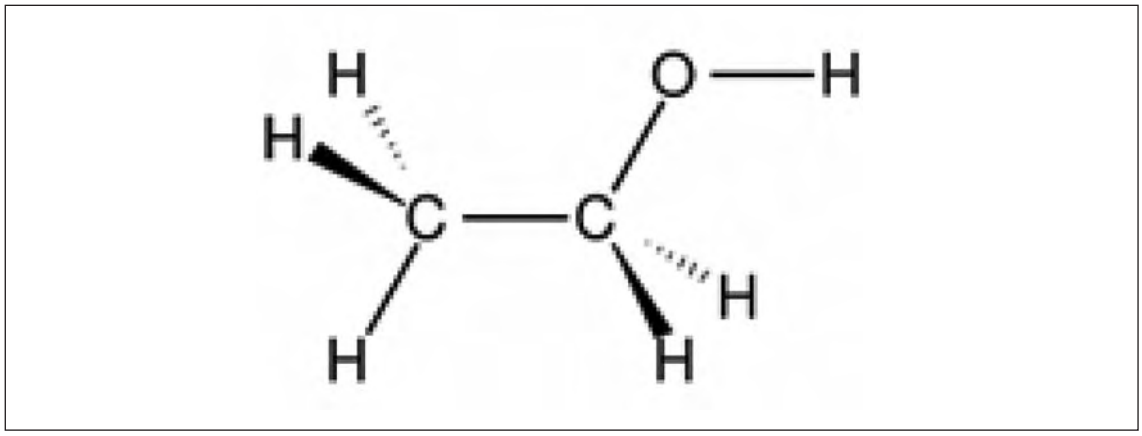


Figure 15 Chemical structure of ethanol.

Using a HepG2 population 100 mmol/l ethanol resulted in 22% increase in apoptosis *in vitro* (Katz *et al*. 2001). In a similar study, apoptotic cells were observed ranging from 6-26% with dose ranging from 40-80 mmol/l ethanol (Neuman *et al*. 1999). When ethanol was given more frequently apoptosis increased to 55%. Fresh human hepatocytes were more sensitive than HepG2 cells. Human hepatoma cells incubated with 100 and 200 mM ethanol had in increased cell membrane permeability, decreased metabolic activity and

viability (Henzel *et al*. 2004). Smaller concentrations (1 mmol/l) caused inhibition of cell proliferation in rat hepatocytes and HepG2 cells (Castaneda *et al*. 2000). In contrast, similar concentrations (1-2 mmol/l) reduced necrosis measured by LDH release from human and rat hepatocytes, with 10 mmol/l inducing a reproducible increase (Castilla *et al*. 2004). Presence of ethanol in culture has been seen to increase CYP450 activity in HepG2 cells (Alexandre *et al*. 1999).

1.14.9 Ursodeoxycholic Acid (UDCA)

UDCA (figure 16) is a hydrophilic dihydroxylated bile salt used to treat cholestatic liver disease although the evidence that it causes clinical improvement is questionable (Poropat *et al*. 2011).

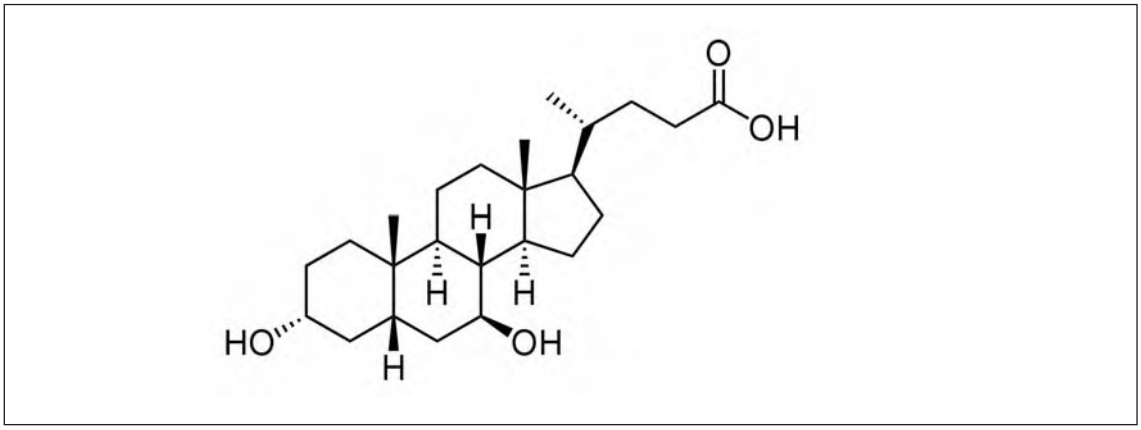


Figure 16 Chemical structure of UDCA.

Despite extensive investigation it remains unclear how UDCA affects hepatocytes at a cellular level. Co-administration in culture with known pro-apoptotic agents including ethanol resulted in significantly reduced apoptosis and PTP (Rodrigues *et al*. 1998). In a rat study, culture with UDCA inferred mitochondrial change with reduced cytochrome c release and an 80% reduction in nuclear fragmentation when cells were stimulated by pro-apoptotic compounds (Rodrigues *et al*. 1999). Co-incubation of astrocytes with UDCA

inhibited mitochondrial swelling and cell death (Rodrigues *et al*. 2000) and it may have some capacity to protect against Fas-ligand induced apoptosis (Castro *et al*. 2005).

1.14.10 ZVAD-fmk

ZVAD-fmk (figure 17) is a cell permeable irreversible tripeptide caspase inhibitor [benzyloxycarbonyl-Val-Ala-Asp(OMe) fluoromethyl ketone]. It inhibits apoptosis early affecting the cascade at the apex (Nyberg *et al*. 2000). Mitochondria contain procaspase-3 and apoptosis inducing factor (AIF), which processes pro-caspase-3 *in vitro* and is blocked by ZVAD-fmk (Zheng *et al.* 2000).

Use of ZVAD-fmk in porcine hepatocyte culture is associated with reduced caspase-3, increased viability and reduced apoptotic cell death (Yagi *et al*. 2001). Using a rat model of gel entrapment there was dose-dependent cytoprotection in cells treated with ZVAD-fmk (Nyberg *et al*. 2000), reducing apoptotic cells from 35% to 23%. In another rat hepatocyte study however, ZVAD-fmk had no effect on ischaemia-reperfusion injury as measured by caspase-3 activity and morphological findings (Gulraj *et al*. 2001).

ZVAD-fmk does not appear to influence cellular physiological processes other than cell death via the caspase pathway. Short-term use is superior to prolonged exposure where ZVAD-fmk appears to exert an inhibitory and possibly toxic effect (Stroh *et al*. 2002).

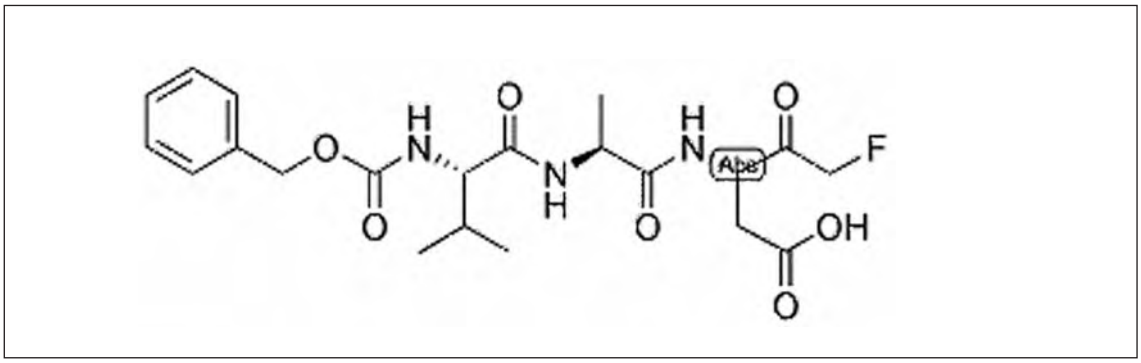


Figure 17 Chemical structure of ZVAD-fmk.

1.14.11 Apoptame™

Apoptame Q™ is a pan-caspase inhibitor that theoretically inhibits apoptosis in fresh culture and cryopreservation. Few studies have looked at this inhibitor and none using hepatocytes. Apoptame Q™ is no longer available, marketed only between 2004 and 2006.

1.15 Ischaemia-reperfusion injury

1.15.01 Overview

Ischaemia is defined as the interruption of circulation to a tissue, thus reperfusion is the re-establishment of blood flow. An ischaemia-reperfusion (IR) injury is the consequence of these events and is an unavoidable cause of morbidity and mortality in liver surgery and transplantation (Howard *et al*. 1990).

1.15.02 Cell death

In the presence of ischaemia cells are deprived of oxygen, resulting in anaerobic metabolism and increased local concentrations of lactic acid that affects enzyme kinetics (Rhodes *et al.* 1980). It is the loss of ATP that is the critical factor in this process and reoxygenation worsens injury by increased ROS formation.

Cold ischaemia causes sinusoidal endothelial cell injury, with detachment, loss of cytoplasmic processes, altered morphology and sloughing into the sinusoid lumen (Otto *et al*. 1984).

There is debate as to whether hepatocyte death is a result of necrosis or apoptosis with contradicting evidence. Authors postulate predominantly necrotic (Gujral *et al*. 2001, Jaeschke 2002) or apoptotic (Kohli *et al*. 1999) cell death. Experimental results are contradictory potentially due to reliance on TdT-mediated dUTP-digoxigenin nick-end labelling (TUNEL) (Gavrieli *et al*. 1992). TUNEL will stain any DNA demonstrating a strand break irrespective of injury which is seen with all cell death, potentially giving false positive results. Equally, if apoptosis is the primary mode of death, one would expect caspase inhibition to have a beneficial tissue effect not seen in practice (Gujral *et al*. 2001). The apoptosis proponent's views are shifting towards a dominant fraction of cells being

necrotic (Selzner *et al*. 2006) based on transplantation models rather than cultured cells.

1.15.03 Warm ischaemic injury

A basic understanding of the mechanisms of cellular injury can guide its management with each component contributing to the overall injury to a greater or lesser extent with overlap (Grace 1994). All tissue has a WIT of often unclear duration.

Kupffer cells play an initiator role during warm IR injury (Jaeschke 1998) producing tumour necrosis factor (TNF), interleukin-1 (IL)-1, nitric oxide (NO) and leukotrienes (Teoh & Farrell 2003) all of which exert pro-oxidant stress thus increasing the imbalance between pro- and anti-oxidants. Some authors suggest hepatocytes are the primary source of ROS in the IR model (Kumamoto *et al*. 1999). Oxidative stress is driven by production and release of ROS from CYP450 oxidases, mitochondria, phagocytic cells and neutrophils (Teoh & Farrell 2003). Complement is activated (Jaeschke & Farhood 1991) and later there is an inflammatory response mediated by neutrophils (Teoh & Farrell 2003). Mitochondria become permeable due to the presence of high intracellular calcium and ROS (Fujii *et al*. 1994) which in turn relates to reduction in hepatocyte viability.

1.15.04 Reperfusion injury

Reperfusion may be later compromised by a 'no-reflow phenomenon' or microcirculatory collapse secondary to microvascular obstruction: another poorly understood concept (Grace 1994). Sludging of red cells and platelet aggregation are likely to be important factors (Quinones-Baldrich *et al*. 1991), the presence of cellular oedema may cause extrinsic capillary occlusion (Leaf 1973) and the vasodilatory and vasoconstricting properties of released mediators (Clemens *et al*. 1997) may be predominant factors. This may be relevant when discussing hepatocyte isolation, which is reliant on good

microcirculatory perfusion of collagenase.

1.15.05 IR injury and hepatocyte isolation

Sinusoidal injury is clearly evident following IR injury (Natori *et al*. 1999, Sindram *et al*. 2000) but it is the global affect this has on the hepatocyte that is relevant. Kupffer cell and sinusoidal injury in the absence of subsequent hepatocyte injury is unlikely (Takeda *et al*. 2003). Once the liver has been excised, the isolation process although initially having a perfusion step, involves a closed circuit. The chemical mediators present in the tissue will not be removed from the process and could continue to drive cell injury.

1.15.06 Injury prevention

Protective mechanisms against IR injury include surgical intervention, pharmaceutical agents and gene therapy. There is growing thought that ischaemic preconditioning (IPC) may allow endogenous adaptive mechanisms to be activated thus reducing ischaemic related injury (Koti *et al*. 2003, Schauer *et al*. 2003). The mechanism of IPC is not understood and is largely postulated. The Pringle manoeuvre is the practical way of achieving this whilst also potentially improving the safety of surgical procedures in the context of surplus tissue donation.

Reducing the IR insult may improve tissue quality, thus creating better tissue for transplant or cell isolation. One way of resuscitating tissue might be to consider the use of whole organ perfusion, the principles of which would permit use in MOD organs and possibly in smaller specimens such as surgical resected specimens. This might allow daylight isolations whilst also improving yield, viability and function.

1.16 Whole organ liver perfusion

The biomass needed to support an adult human requires advanced tissue culture techniques that commence with procurement of the donor tissue and ends with cells in configuration maintained and supported in a BAL. These techniques are expensive, difficult and not easily available. A further potential solution is based around the principles of whole organ perfusion where a healthy organ *ex vivo* is used to maintain physiological stability. First experimentally described in a canine model in 1958 (Otto *et al*. 1958), the principle of normothermic perfusion is to recreate the physiological environment by maintaining normal temperature and providing the essential substrates for cellular metabolism, oxygen and nutrition (Reddy *et al*. 2009).

Clinically an Extracorporeal Liver Perfusion system (ECLP) was first used in humans with ALF in 1964-65 (Eiseman *et al*. 1965). At this time, the organ was porcine (ECPLP) and survival rates were poor (20-40%). Advancements in extracorporeal membrane oxygenation and cardiopulmonary bypass technology when applied to the ECPLP proved encouraging (Horslen *et al*. 2000).

Many studies use porcine tissue as people feel human tissue is impractical. As with BAL development, clinical application of ECPLP for liver support received a setback with the reports of PERV infecting human cells (Patience *et al*. 1997). Direct exposure of patients' blood to porcine tissue will always pose a theoretical threat for transmission of xenogeneic viruses into the human circulation and risks must be eliminated to drive this technology forwards in humans. Application of dialysis membrane technology in ECLP models would allow diffusion of toxins and metabolites across the membrane whilst providing protection against PERV (Nyberg *et al*. 1999). The membrane must be permeable to albumin (66 kDa) for clearance of albumin-bound toxins and hepatocyte

inhibitory factor (25 kDa). Equally, it should inhibit free movement of the smallest porcine endogenous retrovirus (120 kDa) and hepatocyte growth factor (84 kDa). If human organs could be used then there is no concern regarding zoonoses although appropriate viral testing would be necessary.

Whole organ human liver support is not an unreasonable aim dependent on the duration of time an organ can be maintained on such a system. All livers undergo injury during the process of donation so being able to extend the preservation time of livers may minimise wastage and increase organ availability. Equally if not transplanted, the organ is suitable for hepatocyte isolation.

The greatest interest in normothermic perfusion technology is coming from the transplantation groups who hope this will improve donor liver quality, possibly rendering marginal organs transplantable (St Peter *et al*. 2002, Steen *et al*. 2007). Isolated perfused livers overcome the limitations of BAL therapies by providing hepatocytes in their physiological micro-environment: good quality cells, in co-culture, maintained on a fibrous extra-cellular matrix with easy perfusion and drainage systems already in place.

Aims and Hypotheses

2.01 Aims

To assess the feasibility of using chronically injured liver as a source for hepatocyte isolation, to compare inter-laboratory variability, to establish an efficient method for hepatocyte isolation and cryopreservation and to review whether anti-apoptotic compounds could have a beneficial effect on cryopreservation outcomes.

2.02 Experimental hypotheses

ECVAM prevalidation study

With harmonised protocols CYP450 induction will be similar across all three laboratories, without inter-laboratory variation.

Fibrosis and steatosis

Pre-existing histological abnormalities are associated with worse isolation outcomes in terms of viability and viable cell yield.

Curcumin 'dual isolation'

Curcumin will confer benefit post-isolation as measured by monolayer protein and attachment.

Apoptosis studies

The addition of anti-oxidant and anti-apoptotic compounds will improve post-thaw viability and reduce cell loss. ZVAD-fmk will be the superior cryopreservant.

ECPLP and hepatocyte isolation

Allowing a period of recovery following cold storage will improve outcome from isolation.

Materials and Methods

3.01 Health and safety

All experimental work was conducted within a containment level 2 laboratory according to guidelines produced by the Committee on Dangerous Pathogens (ACDP, 1995). Isolation and culturing techniques were undertaken within a Class 2 Microbiological Safety Cabinet (MSC II). To reduce microbiological contamination the surfaces of the MSC II cabinet were routinely decontaminated with 70% ethanol.

Patients considered high risk for blood-borne infection are excluded from the potential tissue donor pool.

Tissue culture waste was inactivated in Trigene for twelve hours prior to drain disposal with excess of water. Contaminated pipettes were placed in a “sharps bin” before disposal by incineration. Solid waste (flasks, centrifuge tubes, contaminated gloves and residual tissue) was placed inside appropriate waste sacks and autoclaved prior to incineration.

3.02 Generic methods

3.02.01 Collection of human samples

Donors were consented for donation pre-operatively in accordance with LREC and Hospital Research & Development guidance (information and consent sheets, appendix 1). Thereafter, under sterile conditions in theatre the donation was dissected from the histological specimen and perfused with 1 l, 4°C Soltran. It was then stored in a sterile container containing Soltran on ice to maintain a temperature of 4°C. A diagram was drawn for the histopathologist, demonstrating the tissue removed and the donated liver transported to the laboratory. Cold and warm ischaemic times and intra-operative Pringle time was recorded. Transport to the laboratory was immediate.

Multi-organ donor liver usually arrived in UW solution in an ice box on ice.

3.02.02 Porcine liver procurement

Porcine livers were obtained from the abattoir at the University of Nottingham campus at Sutton Bonnington. The pigs were stunned by electric shock and exsanguinated. Standard abattoir procedures were followed. The pigs weighed between 50-60 kg.

Once the pig had been stunned and exsanguinated the intestines were removed. The liver was then removed *en bloc* with the kidneys under clean conditions. Either the whole organ was returned to the laboratory or a solitary lobe of liver was excised. Both were perfused *in situ* using all available tissue surface vessels in sequence with ice-cold Soltran and 1 µl/ml of Gentamicin through an arthroscopy set. The tissue was transported to the laboratory as rapidly as possible.

3.02.03 Preparation

Equipment and consumables were sterile. Frozen solutions were defrosted in a 37°C waterbath. Refrigerated equipment was allowed to equilibrate. (Reagents and equipment appendix 2, solutions appendix 3).

When necessary a suitable lobe/segment of tissue was dissected. The size of this tissue varied but isolations were not performed using less than 20 g of tissue. Ideally tissue was encapsulated with one cut surface.

The liver was weighed (50-150 g ideally) and digestion medium (500 ml/isolation) warmed to 37°C. The perfusion and digestion equipment was arranged with the apparatus circulated with water warmed to 37°C. Culture materials were organised, the class II cabinet turned on and surfaces disinfected. Attachment media was prepared and Percoll® diluted.

3.02.04 Cannulation

The surface vessels were cannulated, with the largest vessels used. Smaller vessels typically only perfused small areas of tissue and were only used if all four cannulae had not been deployed. The catheters or cannulae inserted were then secured with a histoacryl glue collar to secure these in place (1-4 per resection). All cut surfaces are sealed. If any part of the native Glisson's capsule was damaged this was repaired by applying more glue (figure 18). All glue was dry before the commencement of perfusion.

3.02.05 Washing step

Liver tissue was placed into a suitable perfusion bowl and tubing attached to ensure circulation of buffer. The tissue floated within the perfusion media and expanded (500 ml at 4°C). The buffer warmed to 37°C during recirculation. Any leaks were repaired so initial

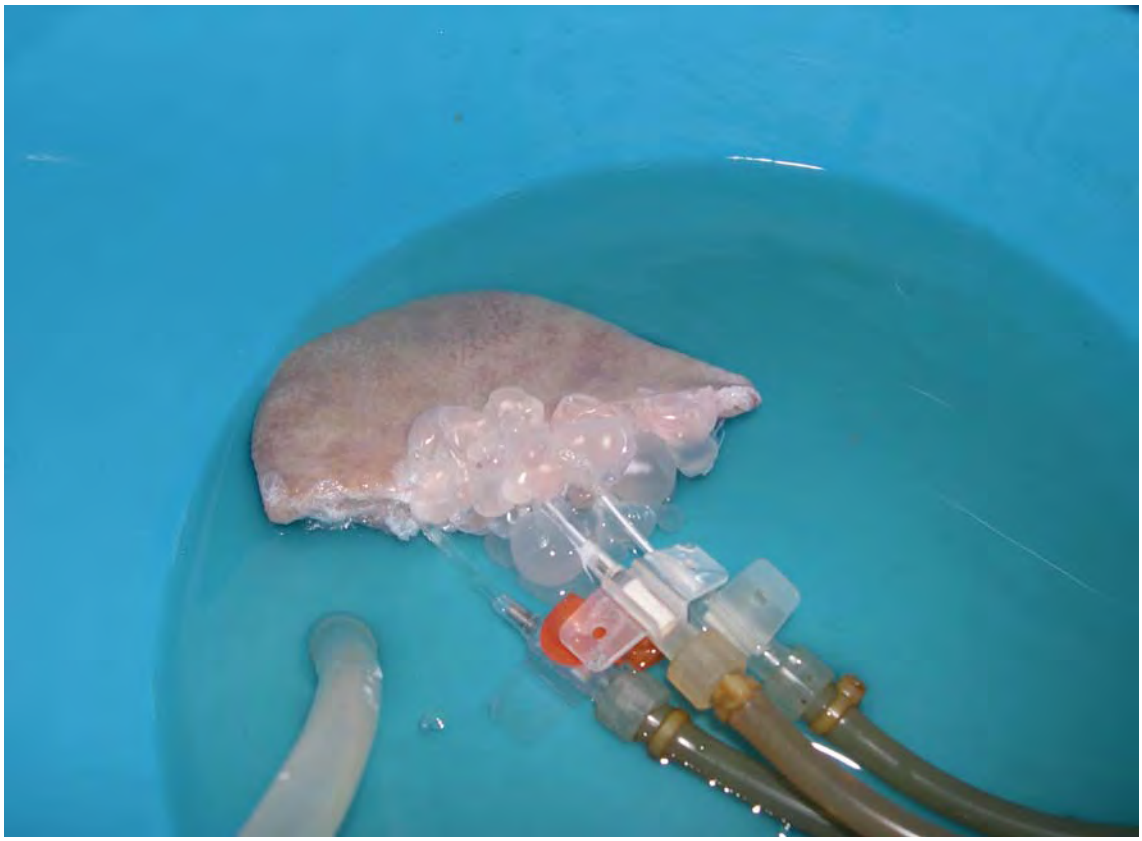


Figure 18 Liver perfusion.

Figure 19 Perfusion and digestion circuit.

pump speed should be low (10 ml/min). Perfusion medium was re-circulated for 10-30 minutes at a flow rate of 40 ml/min (figure 19). Engorgement of the liver and clearing of blood was seen. The more heavily blood stained the discharging buffer, the longer the perfusion was required to run.

With porcine tissue the perfusion and digestion steps were undertaken in the first experiments using porcine perfusion buffer I through III in sequence, with digestion using pre-warmed porcine digestion buffer.

3.02.06 Digestion step

Perfusion buffer was run to waste and replaced with pre-warmed digestion medium (37°C). The flow rate was identical as for the perfusion stage. Digestion took approximately 20 minutes, but no more than 25 minutes with normal human tissue. Digestion was halted when the liver was soft to touch.

3.02.07 Disaggregation of liver tissue

After digestion was complete the perfusion lines were disconnected, the liver placed in a clean bowl and the glue removed. Manual manipulation using scissors and blunt dissection was performed. Undigested liver tissue was weighed and discarded. Cell suspension buffer was added to create a cell suspension that was passed through a series of sieves (850, 400-500, 100 μm) using cold (4°C) suspension media to aid this process. The filtered cell suspension was subsequently transferred to centrifuge tubes (200 ml tubes) and centrifuged (75 G for 5 minutes at 4°C) to ensure cell sedimentation.

3.02.08 Cell counting

Subject to pellet size, cells were re-suspended in suspension media on ice. Hepatocytes

were counted using Trypan blue exclusion and a haemocytometer. Suspensions were observed to be uniform, total yield and viability were determined and recorded.

3.02.09 Further purification/x3 technique

Hepatocytes were plated or cryopreserved. Alternatively, depending on the experiment either two further washing phases were used or a Percoll® gradient. With the three wash (x3 technique) the hepatocytes were re-suspended in cold suspension media and centrifuged twice further, with re-suspension and discarding of supernatant between wash cycles.

Subsequently a further Trypan Blue exclusion test was performed with hepatocyte count and a viability assessment calculated.

3.02.10 Percoll® gradient

50 ml tubes were used containing 3 parts hepatocyte suspension to 0.8 parts Percoll® 90% (39.5 ml cell suspension and 10.5 ml of Percoll® 90%, final Percoll® concentration of 18.9%). Less than 400M total hepatocytes were contained in a tube prior to centrifugation.

3.02.11 Washing

Samples were re-centrifuged at 100 G for 5 minutes at 4°C and supernatant containing dead hepatocytes carefully removed. The pellet was re-suspended in suspension media and centrifuged again at 75 G for 5 minutes at 4°C.

The final pellet was re-suspended in attachment media, pre-incubation (where appropriate) commenced immediately and the hepatocytes re-counted.

3.02.12 Pre-incubation

Hepatocytes were suspended in attachment media and pre-incubated at 37°C in a shaker for 30 minutes with the hepatocytes re-counted prior to plating and cryopreservation.

3.02.13 Hepatocyte plating

Immediately following each count during the isolation process, the hepatocytes were cultured in 12 well plates. Using a concentration of 0.7M hepatocytes/ml HMM, 1 ml of hepatocyte suspension was added to each well. Six wells were required for each step and condition. This provided a confluent layer across the base of the wells (figure 20). Hepatocytes were cultured in the incubator for four hours and then examined for attachment, flattening and spreading. If hepatocytes were attached (figure 21), then the medium was changed and the cells incubated for 18-24 hours.

If hepatocytes were not attached they were incubated for a further 12 hours without changing the medium.

3.02.14 Cryopreservation

Following each count, hepatocytes were cryopreserved. At each step and for each condition cells at a concentration of 5M viable hepatocytes/ml were cryopreserved in 1.8 ml aliquots in 2 ml sterile cryovials.

Hepatocytes were initially cryopreserved in ice-cold DMEM, 20% foetal bovine serum (FBS) and 10% DMSO, added over one minute to the solution. Subsequently, hepatocytes were cryopreserved in ‘media B’ containing 10% DMSO, 2% PVP (polyvinylpyrrolidone) and 20% foetal calf serum (FCS). This is made clear in the experimental chapters. All cryovials were frozen in a ‘Mr Frosty’ within a -80°C freezer overnight (eight hours minimum) before suspension in liquid nitrogen.

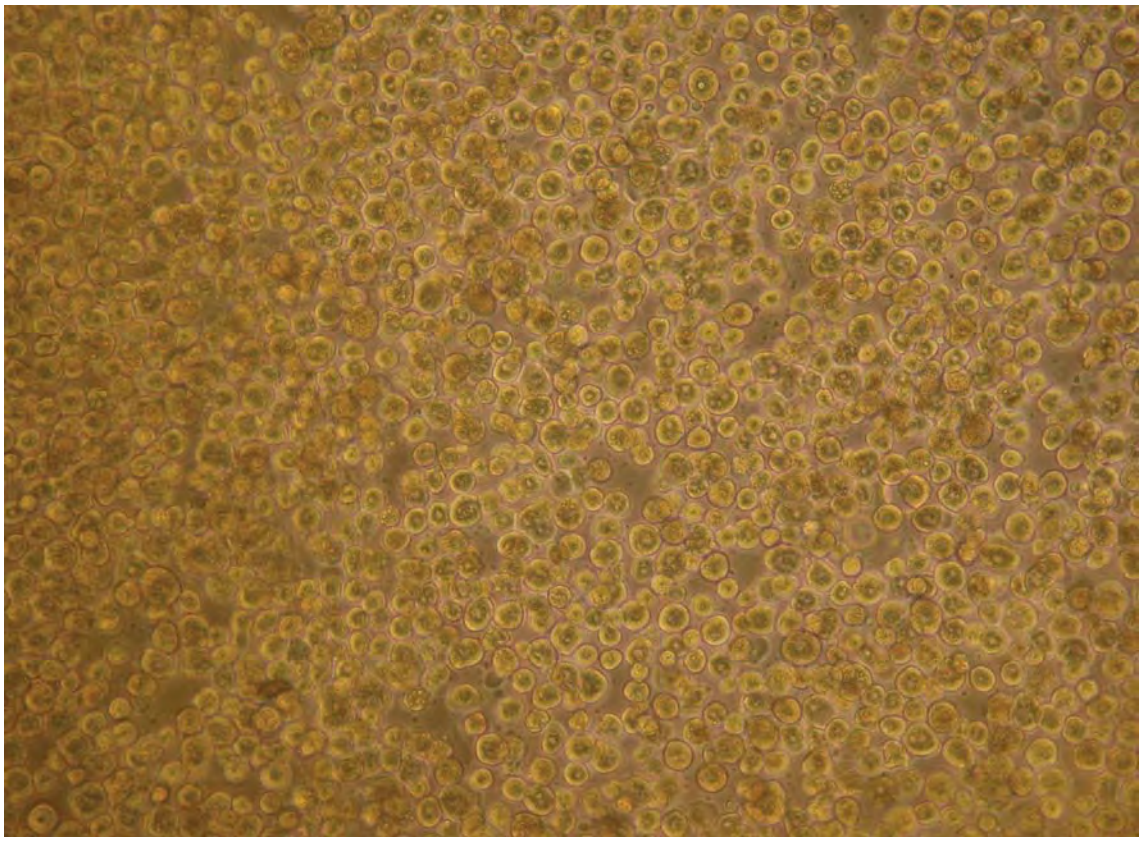


Figure 20 Fresh plated hepatocytes.

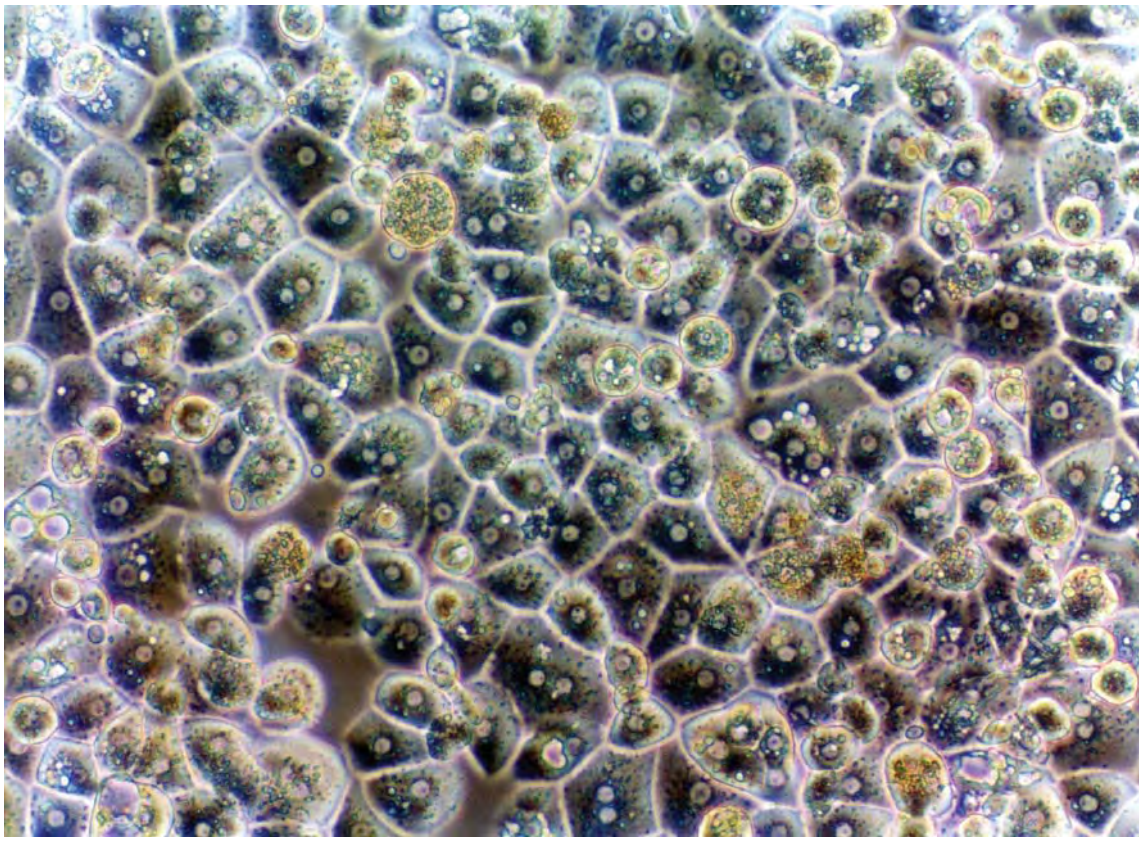


Figure 21 Attached hepatocytes.

3.02.15 Thawing post-cryopreservation

Vials were taken from liquid nitrogen and placed into a 37°C water bath. After approximately one minute the solution become liquid. When the content of the vial moved when inverted, they were emptied into a 50 ml centrifuge tube.

The centrifuge tube was filled with DMEM to at least double the volume and centrifuged at 50 G for 4 minutes. The supernatant was discarded, leaving a hepatocyte pellet which was then resuspended in DMEM. Centrifugation was repeated.

The supernatant was discarded, with the pellet finally re-suspended in HMM and hepatocytes were counted. If viability was poor but hepatocyte number allowed it, a Percoll® gradient was used as with fresh cells. Hepatocytes were plated as with fresh cells.

3.02.16 Hepatocyte culture

24 hours after seeding percentage monolayer coverage was estimated using a microscope, protein assays and all five P450 functional assays were performed for each condition. The media was then aspirated from any remaining wells using a pump suction device and replaced with 1 ml/well HMM.

Day 3

Where number allowed, all assays were repeated.

Day 5

Where number allowed, all assays were repeated and culture was discontinued.

3.03 Specific assays

3.03.01 Trypan Blue exclusion test

Aim:

To determine hepatocyte viability and yield.

Principles:

Trypan Blue is a high molecular weight dye that is does not penetrate the membrane of viable cells. If damaged dye is taken up and they become visibly blue.

Method:

Hepatocyte s were re-suspended in solution and 20 µl cells were added to 180 µl Trypan blue.

A coverslip was placed over the counting chamber 20 µl of this solution positioned under the coverslip. Using a tally counter cells were counted across four haemocytometer grids and viability and yield calculated.

3.03.02 Phase I and II metabolic function

Aim:

To determine CYP450 function of-

CYP3A4	Testosterone 6-beta hydroxylation
CYP2C9	Diclofenac 4'-hydroxylase
CYP1A2	Phenacetin O-Deethylase
CYP2D6	Bufuralol hydroxylation
Phase II metabolism	7-hydroxycoumarin sulfation and glucuronidation

Method:

Following overnight incubation CYP450 activities from each condition were

determined by measuring the metabolism of probe substrates by HPLC. Culture medium was removed from the hepatocytes and replaced with 2 ml of medium containing the final assay concentration of the following compounds. Working solutions at the appropriate concentrations were prepared and 1 µl stock solution was added per 1 ml pre-warmed Krebs solution (table 10).

Compound	Stock concentration	mg/ml in DMSO	Working solution	Final assay concentration (µM)
Testosterone	200 mM	57.6	200 µM	100
Diclofenac	200 mM	63.6	200 µM	100
Phenacetin	200 mM	35.8	200 µM	100
Bufuralol	100 mM	59	100 µM	50
7hydroxycoumarin	200 mM	32	200 µM	100

Table 10 Preparation of probe substrates for estimation p450 activity.

After adding the probe substrates the plates were returned to the incubator and 200 µl samples were removed from the wells at 0, 30, 60 and 120 minutes. The 200 µl aliquots were added to eppendorfs with stop solution (table 11).

Assay	Stop Solution	Volume of stop solution/ 200µl sample
Testosterone	Methanol	100 µl
Diclofenac	Acetonitrile 94% acetic acid 6%	50 µl
Bufuralol	65% Perchloric acid	25 µl
Phenacetin	65% Perchloric acid	25 µl
7HC	1:1 zinc sulphate / barium sulphate	25 µl

Table 11 Stop solutions.

Following collection of the final samples, eppendorfs were centrifuged in a microfuge at

16000 G for 10 minutes and the supernatant transferred to labeled HPLC vials.

Analysis of samples was performed using HPLC conditions (appendix 4). To ensure minimum wastage specimens were kept at -20°C and subsequently centrifuged and assayed *en masse*. No samples were frozen for longer than six months. Results of analysis were expressed as µmoles of substrate production/min/million hepatocytes.

3.03.03 Bicinchoninic acid protein assay (BCA)

Aim:

To measure the concentration of the protein sample in mg/ml.

Principles:

This assay relies on the formation of a Cu^{2+}-protein complex, followed by reduction of Cu^{2+} to Cu^{1+}. The amount of reduction is proportional to the amount of protein present. BCA forms a purple-blue complex with Cu^{1+} in alkaline environments.

Method:

The supernatant was taken from one well of a twelve well plate into an eppendorf and centrifuged at 75 G for five minutes. The supernatant was discarded and the pellet re-suspended in 400 µl 0.1 M NaOH and kept at -4°C until processed.

After the supernatant was removed, 400 µl 0.1 M NaOH was added to the well. The cells separated from the collagen over ten minutes. This was then aspirated from the well, and stored in an eppendorf at -4°C until processed.

Protein assays were normally performed *en masse* to reduce cost and waste. No samples were kept at -4°C for longer than one month.

BCA solution: reagent A contains BCA, sodium carbonate, sodium tartrate and sodium bicarbonate in 0.1 M NaOH. Reagent B is copper (II) Sulfate Pentahydrate 4% solution.

50 parts reagent A was mixed with 1 part reagent B.

The sample to be assayed was applied to an agitator and 20 µl samples put into adjacent wells of a 96 well plate (duplicate testing).

Stock BSA was used at 1 mg/ml to make up the stock solutions for the standard curve: 1 mg/ml, 0.5 mg/ml, 0.1 mg/ml and 0 mg/ml. Dilution was achieved using 0.1 M NaOH.

20 µl samples of each standard were put into adjacent wells of each 96 well plate.

Subsequently, 200 µl of dye reagent (50:1 reagents A:B) was added to each well. The 96-well plates were cultured at 37°C for 30 minutes and the plates were read on a spectrophotometer at 540 nm and 630 nm.

3.03.04 3-(4,5-dimethylthiazide-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) test

Aim:

To recognise cells with a level of mitochondrial activity.

Principles:

After 3-4 hours of cell incubation with MTT, formazan crystals develop in living and early apoptotic cells. Dead cells do not produce crystals. Crystals absorb light at 550-570 nm but not at 620-650 nm. Absorbency here indicates cell debris. Final optical density (OD) from the formation of formazan crystals can therefore be calculated OD=L1-L2 where L1 is a reading at 550-570 nm and L2 is a reading at 620-650 nm.

Method:

Sixty thousand hepatocytes were seeded per well, using 100 µl hepatocyte suspension in a 96-well plate. After 24 hours incubation 10 µl of MTT solution/well was added, with a final MTT concentration of 0.5 mg/ml.

Hepatocytes were incubated for 3-4 hours, the media removed and formazan crystals solubilised with 200 µl organic solvent. A plate shaker was used for 10-20 minutes and the

plates read on a plate reader at 550-570 nm and again at 620-650 nm.

3.03.05 Annexin-V-Fluos

Aim:

To determine proportion of apoptotic, necrotic, dead and viable hepatocytes.

Principles:

One of the plasma membrane changes seen during apoptosis is translocation of phosphatidylserine (PS) from inner to outer surface. Annexin-V is a Ca2+-dependent phospholipid-binding agent with a high affinity for PS (Vermes *et al*. 1995) and can be used as a probe to identify apoptotic cells.

Membrane integrity is also lost with necrosis. As a consequence, propidium iodide (PI) is also applied as this stains the DNA of necrotic cells only.

Method:

20 µl Annexin-V-Fluos labelling reagent was diluted with 960 µl incubation buffer adding 20 µl PI solution (stock solution 50 µg/ml).

Cryopreserved hepatocytes are thawed and re-suspended at 1 M viable hepatocytes/ 50ml HMM with 20% FCS, in three centrifuge tubes. Assays are performed at zero minutes, 60 minutes and 120 minutes. The latter two tubes were put in an agitating water bath at 37°C.

At the appropriate time hepatocytes were centrifuged at 100 G for 5 mins and the pellet re-suspended in 100 µl labelling solution for 10 mins at 25°C.

During this time the FACScan was prepared as per local protocol and the hepatocytes subsequently analysed.

3.04 Specific experiments

3.04.01 ECVAM pre-validation study

Aim:

To analyse human hepatocyte isolations and cultures with their response to a 72 hour repeated-dose treatment period with various concentrations of either ß-naphthoflavone (BNF), rifampicin (RIF) or phenobarbital (PB) in terms of cellular microsomal CYP-dependent enzyme activities and protein content.

Design :

Laboratoire de Biologie Cellulaire, Faculte de Medecine et de Pharmacie, Besancon, France, UKHTB, Leicester and Laboratoire de Chirurgie Experimentale, Fondation Transplantation, Strasbourg, France performed hepatocyte isolation and culture according to a harmonised protocol. Transferability of the induction test and the between-laboratory variability was assessed. Chemicals were obtained from Sigma-Aldrich (St. Quentin-Fallavier, France), reagents from InVitrogen (Cergy Pontoise, France) and culturing equipment from Becton Dickinson.

I performed all of the Leicester isolations and treatments. After completion of the isolations, the cells were sent to Besancon to be processed *en masse*. I then travelled to France where the described assays, auditing and reporting were performed equally by the research team.

Method:

Human hepatocytes were isolated using ECVAM hepatocyte perfusion buffer and digestion media or InVitrogen Digest Medium. When viability was less than 85% a Percoll® gradient was used. A 3-4 day drug exposure protocol was retained (figure 22).

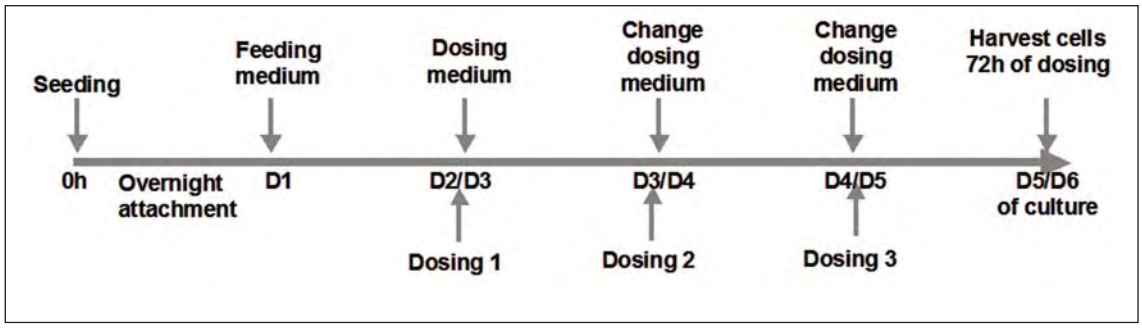


Figure 22 ECVAM timeline.

Hepatocytes with viability higher than 85% were seeded in 60mm Biocoat® dishes overnight at a density of 4 M cells/dish in ECVAM attachment medium. Subsequently ECVAM feeding medium was used for 24-48 hours. Treatment with inducers was started 48-72 hours after plating.

Inducers dissolved in DMSO (0.1%) were added to the feeding media: BNF 2, 10 or 50 µM, RIF 0.1, 1 or 10 µM, PB 50, 250 or 1000 µM. Medium containing DMSO (0.1%) served as control.

All media was changed every day for three days.

Culture dishes were scraped, pooled and frozen at -80°C.

After thawing of hepatocyte homogenates, microsomes were prepared by differential centrifugation as described previously (Richert *et al*. 2002). Microsomes were snap frozen and stored at –80°C.

Total protein concentration was determined 5-6 days following seeding and after 72 hours to test plating efficacy and viability.

Enzyme assays

Microsomes were analyzed to determine the rates of 7-ethoxyresorufin *O*-dealkylation (CYP1A2), testosterone 6 -hydroxylation (CYP3A4/5), and bupropion hydroxylation (CYP2B6) according to previously described methods (Richert *et al*. 2002).

Further details see appendix 16.

3.04.02 Fibrosis and steatosis

Aim:

To determine whether the histological condition of tissue had any impact on isolation outcome.

Principles:

Fibrosis and steatosis were measured using pre-determined scoring systems and analysed against viability and hepatocyte yield.

Method:

Isolation was performed. Where adequate tissue was available, this was formalin fixed and wax blocked within the histology laboratories of Leicester General Hospital. Analysis was performed on haematoxylin and eosin stained slides by two independent histopathologists.

Tissue was graded for steatosis and fibrosis using the liver injury score (Desmet *et al*. 1994), NASH (or Brunt) score (Brunt *et al*. 1999), fibrosis (Rubio *et al*. 1988) and Ishak score (Ishak *et al*. 1995).

3.04.03 Cytotoxicity studies

Aim:

To determine maximal dose of curcumin, ursodeoxycholic acid and ethanol that could be used for apoptosis assays without compromising hepatocyte attachment.

Method:

Hepatocytes were isolated, resuspended in minimal media and plated to a concentration of 60000 cells/well of a 96-well, collagen coated plate. Solution was added as per the cytotoxicity assay tables 14-16, each well containing 250 µl of the appropriate solution. The plates were incubated for 4-24 hours and the BCA and MTT tests performed.

Curcumin cytotoxicity

Molecular weight curcumin 368.39

5 µM stock solution in ethanol 9.2 mg/5 ml ethanol

Column	Concentration curumin (µM)	Volume media (ml)	Volume curcumin stock solution (µl)	Volume ethanol (µl)
1	200	4.8	200	0
2	100	4.9	100	0
3	75	4.9	75	25
4	50	4.9	50	50
5	40	4.9	40	60
6	30	4.9	30	70
7	20	4.9	20	80
8	15	4.9	15	85
9	10	4.9	10	90
10	5	4.9	5	95
11	0	4.9	0	100

Table 12 Curcumin cytotoxicity assay.

Ursodeoxycholic acid (UDCA) cytotoxicity

Molecular weight UDCA 392.56

100 mM stock solution in ethanol 39.25 mg/ml ethanol

Column	Concentration UDCA (µM)	Volume media (ml)	Volume UDCA stock solution (µl)	Volume ethanol (µl)
11	600	4.9	30	70
12	400	4.9	20	80
13	200	4.9	10	90
14	100	4.9	5	95
15	50	4.9	2.5	97.5

Table 13 UDCA cytotoxicity assay.

Ethanol cytotoxicity

Column	Volume media (µl)	Concentration (%)	Volume ethanol (µl)
16	4500	10	500
17	4750	5	250
18	4800	4	200
19	4850	3	150
20	4900	2	100
21	4950	1	50
22	4975	0.5	25
23	4995	0.1	5
24	5000	0	0

Table 14 Ethanol cytotoxicity assay.

3.04.04 Curcumin 'dual isolation'

Aim:

To assess the affect of curcumin on attachment and function of primary and cryopreserved hepatocytes.

Design:

Control isolations were conducted simultaneously without curcumin with ethanol in all solutions. All solutions from the moment the tissue was split contained either curcumin/ethanol or ethanol alone.

Method:

Curcumin stock solution was made and added to all solutions. An equal concentration of ethanol was added to all control solutions (ethanol concentration 0.5%).

The liver was cut in half, ensuring both retained adequate vasculature for perfusion and were 50-100 g weight. Each piece was morphologically similar to minimise perfusion differences. Each piece was randomly allocated to a limb of the study and hepatocyte

isolation was performed.

After a single centrifugation wash step, hepatocytes either underwent x3 centrifugation purification or a Percoll® gradient and pre-incubation. Hepatocytes were cryopreserved based on the post-Percoll® cell count.

Protein and visual attachment were determined on day one, three and five.

3.04.05 Apoptosis studies

Aim:

To cryopreserve hepatocytes in compound enhanced cryopreservation media to improve post-thaw outcomes.

Principles:

Using protein assays, attachment, phase I and II metabolic studies and Annexin-V-Fluos, curcumin, UDCA, ZVAD-fmk and Apoptame Q™ were compared against a control to assess response.

Method:

Hepatocyte isolation was performed with cells undergoing three wash cycles without Percoll® purification or pre-incubation. Hepatocytes were cryopreserved in media B with the adjuncts of either 20 µM curcumin in 2% ethanol, 400 µM UDCA in 2% ethanol (determined by the cytotoxicity studies), ZVAD-fmk or Apoptame Q™ (which were used at the manufacturers recommended dosing of 1 µl/ml cryoprotectant in 20 mM stock diluted in DMSO). Plain media B was used as control.

Hepatocytes were cryopreserved for less than one month.

Following thaw, hepatocytes were plated (attachment, protein, CYP450 function assessed) and Annexin-V-Fluos stained.

3.04.06 ECPLP and hepatocyte isolation

Aim:

To assess whether normothermic perfusion could improve outcome of hepatocyte isolation.

Method:

Porcine livers were obtained as per the tissue used for hepatocyte isolation. Evisceration was performed after death with identification and cannulation of the common bile duct, hepatic artery, portal vein and supra- and infra-hepatic inferior vena cava. Soltran was infused via the portal vein and the liver transported to the laboratory on ice.

A piece of tissue less than 50 g was taken from the most distal aspect of one lobe and pre-perfusion hepatocyte isolation was performed. The cut surface of the liver was sutured to prevent excess 'bleeding' during perfusion. After standard isolation and plating, visual attachment, protein and CYP450 assays were performed at 6 hours.

The liver circuit was a hybrid of those described previously (Grosse-Siestrup *et al*. 2002, Butler *et al*. 2002) and consisted of a centrifugal pump, speed controller, motor head, external drive unit, oxygenator, soft shell reservoir, paediatric flow transducer, water bath, custom built glass reservoir and pressure transducer. A second circuit, labelled comprised of a reservoir of porcine blood circulated using a roller pump. The two circuits were linked such that the circulating blood on either side could exchange substances by diffusion across a hollow fibre filter (Bikhchandani *et al*. 2011) (figure 23). (Appendix 5 solutions were added during perfusion.)

Perfusion was maintained for 6 hours prior to hepatocyte isolation. To ensure fair comparison, a similar sized piece of tissue was used from a second lobe to allow for comparative microvasculature.

Hepatocytes were kept in identical conditions and visual attachment, protein and P450

assays performed at 6 hours post-plating.

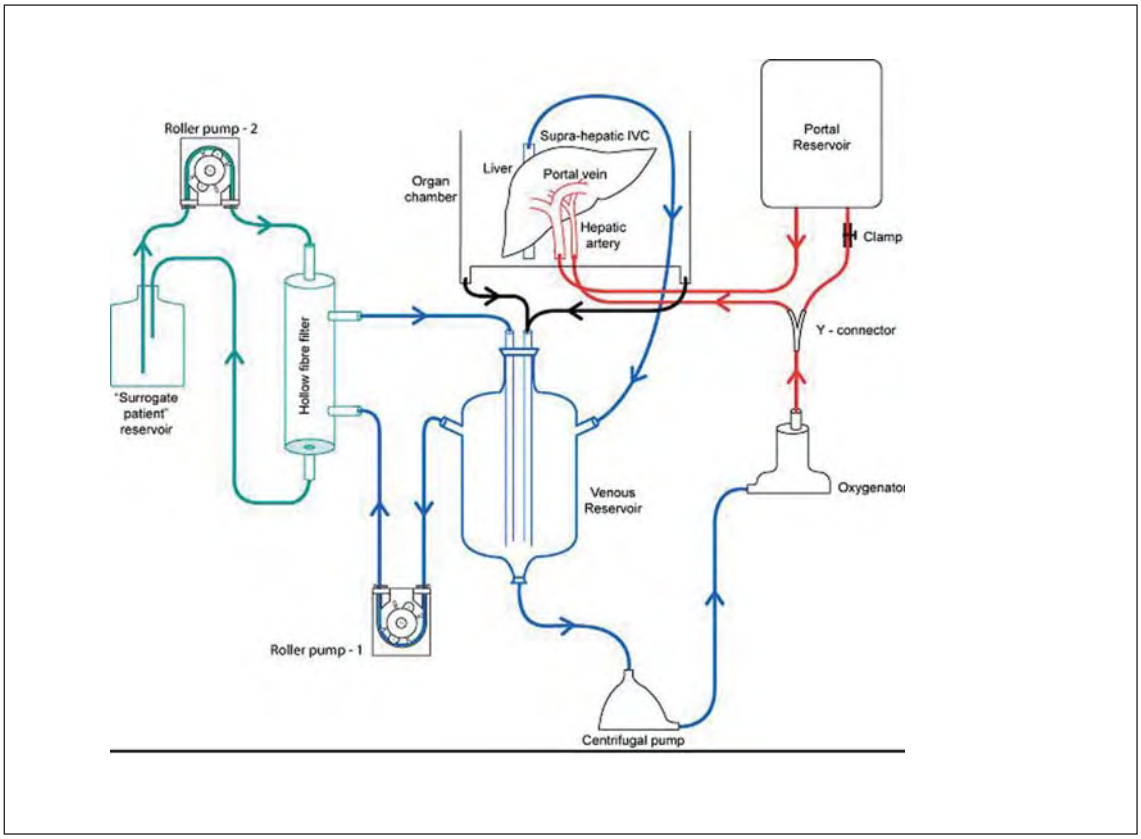


Figure 23 ECPLP circuit.

3.05 Statistical Analysis

All variables were assessed for normal distribution. If so, parametric tests were performed, whereas non-parametric tests were performed in the absence of normal distribution. Statistical analysis was conducted using GraphPad Prism 4.02 and SPSS. Statistical significance was p=0.01 due to the multiple variables analysed.

There is further detail in the results chapters.

Results

4.01 Five years of human donations to UKHTB

In 1999 UKHTB started processing hepatic tissue for hepatocyte distribution to industry. In the five years between October 2000-2005, 433 individuals donated tissue of whom 86 were multi-organ donors. There were 253 male donors and 180 females, with an average age of 58 (range 16-90) most of whom were Caucasian (94%, n=407). Three surgical centres donated tissue, two local and one distant, therefore there was disparity in the amount and quality of demographic information available, as well as variation in CIT. For live tissue the average was 113 minutes (range 15-720).

Of the multi-organ donors, the average weight of donated tissue was 1638 g (41-3260 g), with living donors providing less (average 154 g, range 5.9-1190 g). For those with demographics, surgical procedures were variable (table 15) as was their underlying pathology (table 16).

	n
Wedge excision	1
Segmental resection	6
Right hemihepatectomy	46
Redo right hemihepatectomy	1
Extended right hemihepatectomy	14
Left hemihepatectomy	14
Unknown	307

Table 15 Surgical procedure.

The Pringle manoeuvre was only performed in two of the three centres with times rarely available, but averaging 23 minutes (4-60 minutes in total). Pringle protocol was five minutes clamp on, ten minute recovery time.

		n
Benign	Tumour	11
	Infectious	2
Premalignant		1
Primary cancer	Gallbladder	5
	Cholangiocarcinoma	14
	HCC	9
Metastatic disease	CRC	356
	Carcinoid	5
	Renal cell carcinoma	2
	Breast	2
	Other	12
Unknown		16

Table 16 Donor pathology.

In view of the interest in cholestasis documented elsewhere, patients with an elevated bilirubin were isolated. Thirty patients had documented levels, only ten of these were clinically jaundiced.

Live donors typically produced more hepatocytes per gram of tissue, with better viabilities (table 17).

		Live donor	MOD
Viability (%)	Average	75.25	64
	Range	0-94.7	0-86
Total viable cells (M)	Average	438	199
	Range	0-6800	0-1802
Viable cells/g tissue (M)	Average	4.23	
	Range	0-20.9	

Table 17 Isolation outcomes.

In summary, over this time, using average values where data is incomplete, 67.78 kg of liver was donated, producing 169335 million viable hepatocytes.

4.02 Human isolation variables and their effect on fresh culture and cryopreservation

Hepatocyte isolation is a stepwise procedure with gradual purification via washing or Percoll®. Fresh culture and cryopreservation is affected by both steps, with pre-incubation another potential influence on cell quality. These factors were reviewed both for fresh and thawed hepatocytes (table 18).

	n		Viability (%)	Total number of viable hepatocytes (M)	Viable hepatocytes per gram of liver (M)	Viable hepatocytes per gram of liver digested (M)	Attachment (%)	Monolayer protein (mg/M)	Hepatocytes lost (%)
x1 wash, fresh culture	46	Mean	60.29	580.03	6.27	30.42	63.87	0.31	
		Range	0-90.2	0-2437.5	0-16.35	0-114.64	0-100	0-0.58	
x1 wash, then Percoll® purification, fresh culture	26	Mean	75.03	66.50	2.57	16.79	77.50	0.37	60.27
		Range	59.1-86.7	3.25-171.25	0.22-4.97	0.5-55.64	10-100	0.08-0.59	17.78-82.44
x3 washes, fresh culture	229	Mean	73.79	397.11	3.51	21.99	77.86	0.42	42.05
		Range	0-94.7	0-6800	0-20.93	0-736	0-100	0-0.95	0-86.91
Pre-incubated, fresh culture	9	Mean	66.45	40.34	3.17	13.18	76.36	0.46	20.71
		Range	47.3-81.2	17-70	0.53-8.78	2.2-30.14	50-100	0.3-0.68	0-63.7
x3 washes, then Percoll® purification, then fresh culture	7	Mean	53.56	141.44	4.06	27.72	47.35		71.43
		Range	0-85	0-482.5	0-22.35	0-344.26	0-90		1.79-100
Percentage cells lost, pre-incubating, when the wash steps were included increased to 91.9% mean, ranging from 75-98.55%.									

Table 18 Isolation summary data.

Group codes are assigned to each condition (table 19).

Group code	Condition
1	x1 wash, fresh culture
2	x1 wash, then Percoll® purification, fresh culture
3	x3 washes, fresh culture
4	Pre-incubated, fresh culture
5	x1 wash, then cryopreserved
6	x1 wash, Percoll® purification, then cryopreservation
7	x3 washes, then cryopreserved
8	Pre-incubated, then cryopreserved
9	Historical media
10	x3 washes, cryopreserved then Percoll® gradient

Table 19 Key for human isolations.

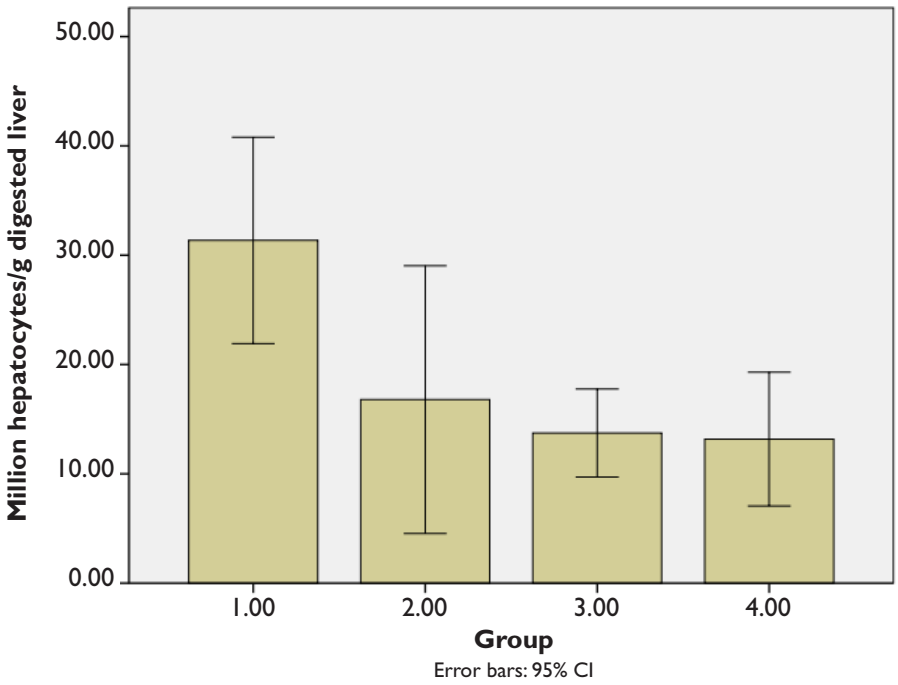


Figure 24 Viable hepatocytes/g liver in fresh culture.

As purification progresses, hepatocytes are sequentially lost (figure 24) with statistical significance seen when the x1 wash is compared with x3 washes ($p<0.0001$) and pre-incubated hepatocytes ($p<0.005$).

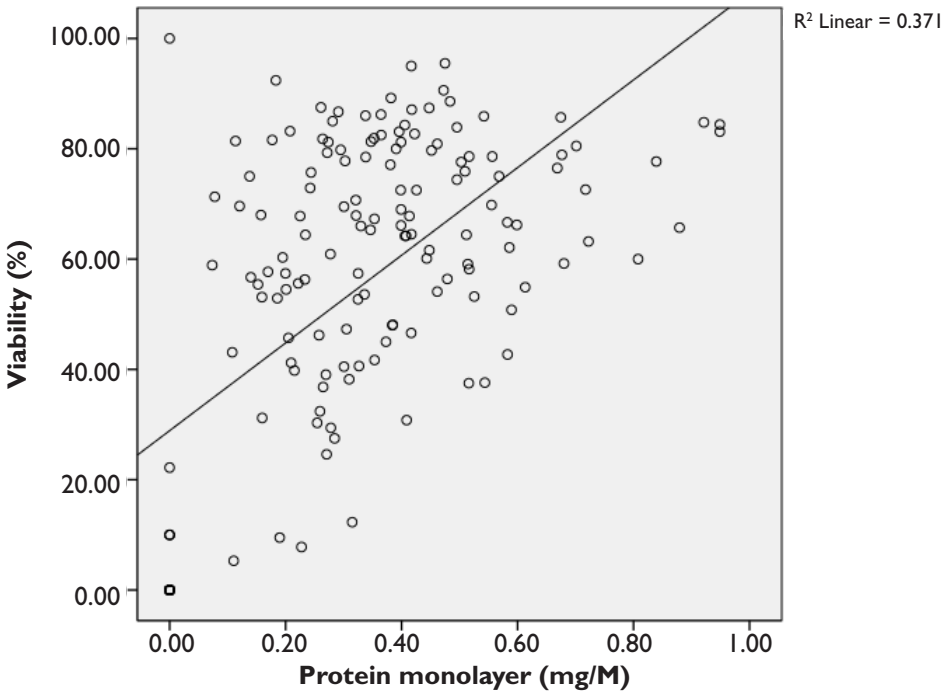


Figure 25 Viability as compared with protein monolayer.

As viability increases there is a significant improvement in monolayer protein ($p<0.001$) (table 25) which equates to attachment ($p<0.0001$).

Group code	Condition
1	x1 wash, fresh culture
2	x1 wash, then Percoll® purification, fresh culture
3	x3 washes, fresh culture
4	Pre-incubated, fresh culture
5	x1 wash, then cryopreserved
6	x1 wash, Percoll® purification, then cryopreservation
7	x3 washes, then cryopreserved
8	Pre-incubated, then cryopreserved
9	Historical media
10	x3 washes, cryopreserved then Percoll® gradient

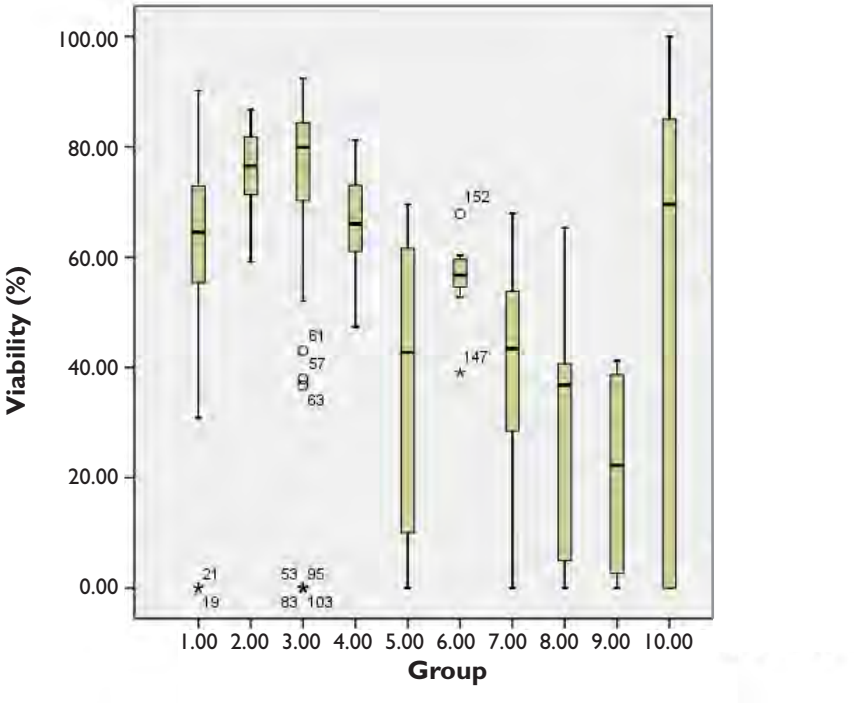


Figure 26 Viability across all groups.

There is variance in terms of viability across the groups (figure 26, table 20). The x1 wash with Percoll® purification demonstrates significantly improved viability ($p<0.01$), with the fresh preparations having insignificant differences. After cryopreservation the only conditions maintaining viability involve a pre- or post-isolation Percoll® gradient at the expense of hepatocyte yield (figure 27). Viability increasingly deteriorates as more steps are involved in the cryopreservation process: x1 wash ($p<0.01$), x3 washes ($p<0.0005$), x3 washes and pre-incubation ($p<0.0001$).

	n		Viability (%)	Attach-ment (%)	Monolayer protein (mg/M)	Hepatocytes lost (%)
x1 wash, then cryopreserved	9	Mean	36.42	28.89	0.29	70.81
		Range	0-69.6	0-90	0-0.88	38.89-100
x1 wash, Percoll® purification, then cryopreservation	8	Mean	47.17	42.22	0.24	61.51
		Range	0-67.8	0-100	0-0.48	26-100
x3 washes, then cryopreserved	34	Mean	38.22	49.87	0.31	44.24
		Range	0-67.9	0-100	0-0.67	0-100
Pre-incubated, then cryopreserved	10	Mean	26.52	33.18	0.21	71.43
		Range	0-65.3	0-100	0-0.59	31.25-100
Historical media	14	Mean	25.93	34.33	0.21	63.22
		Range	0-61	0-100	0-0.54	0-100
x3 washes, cryopreserved then Percoll® gradient	18	Mean	51.26	40.28	0.21	74.41
		Range	0-100	0-90	0-0.64	23.24-100
x3 washes, cryopreserved in historical media, then Percoll® gradient	5	Mean	46.84	37.00	0.24	68.40
		Range	0-72	0-75	0-0.62	52.5-100
x3 washes, pre-incubated, then cryopreserved, then Percoll® gradient	5	Mean	65.10	37.00	0.35	68.22
		Range	1-90.6	43.14-75	0-0.56	0.1-90
x1 wash, then cryopreserved, then Percoll® gradient	5	Mean	63.00	51.00	0.30	70.02
		Range	7.4-95.5	46.64-100	0-0.47	0-100
x1 wash, then Percoll® purification, then cryopreserved then Percoll® gradient	5	Mean	52.04	39.00	0.23	51.56
		Range	7.7-81.6	0-100	0-0.40	0-100

Table 20 Thaw outcomes.

Group code	Condition
1	x1 wash, fresh culture
2	x1 wash, then Percoll® purification, fresh culture
3	x3 washes, fresh culture
4	Pre-incubated, fresh culture
5	x1 wash, then cryopreserved
6	x1 wash, Percoll® purification, then cryopreservation
7	x3 washes, then cryopreserved
8	Pre-incubated, then cryopreserved
9	Historical media
10	x3 washes, cryopreserved then Percoll® gradient

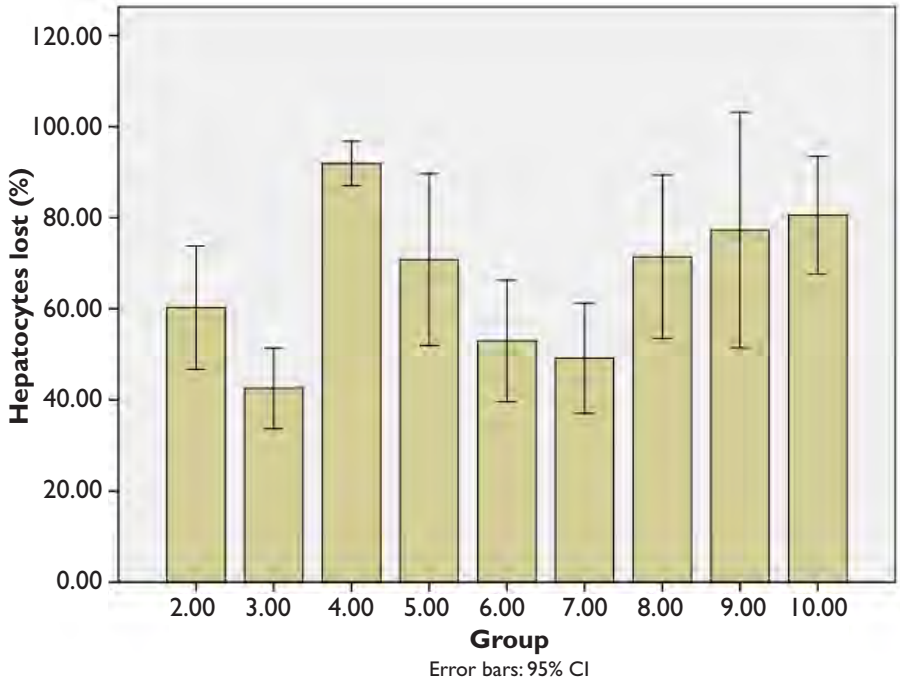


Figure 27 Percentage of hepatocytes lost at each stage of the purification process.

4.02.01 Cryopreservation solution validation

Initally hepatocytes were cryopreserved in 20% FBS, 10% DMSO based on historical laboratory data (Lloyd *et al*. 2003). Other groups use more complex media, with PVP a globally acknowledged adjunct to DMSO (de Souza *et al*. 2001). To ensure maximal return was achieved from cryopreservation, hepatocytes were cultured in this historical media (TC) and 'media B' (Alexandre *et al*. 2002).

Table 21 Cryopreservation solution comparison

	AGE	GENDER	PATHOLOGY	Media B post-cryopreservation				Media TC post-cryopreservation			
				VIABILITY (%)	HEPATOCYTES LOST (%)	ATTACH-MENT (%)	MONOLAYER PROTEIN (mg/M)	VIABILITY (%)	HEPATOCYTES LOST (%)	ATTACH-MENT (%)	MONOLAYER PROTEIN (mg/M)
4847	60	FEMALE	CRC	47.4	-0.35	75	0.29	33.8	46.53	50	0.26
4672	55	FEMALE	INFECTIOUS	35.6	19.38	75	0.67	26.3	48.19	50	0.42
4722	45	MALE	SAH	44	19.44	75	0.29	26	53.70	25	0.25
4541	57	FEMALE	CRC	36.2	46.76	25	0.30	23.8	70.83	50	0.22
4428	60	MALE	AML	46.67	61.11	50	0.23	33.7	88.19	25	
4228	60	FEMALE	SAH	40.74	63.33	75	0.49	15.6	84.72	50	
4619	68	FEMALE	CRC	44.7	54.95	50	0.22	22.7	75.35	25	0.15
4371	74	FEMALE	HCC	63	3.70	75	0.42	61	-60.49	75	0.30
4120	55	MALE	SAH	55.6	-12.13	100	0.22	37.6	28.15	100	0.54
5862	52	FEMALE	CRC	43.4	43.21	50		5.3	95.37	5	0.11
5296	72	MALE	CRC	9.5	96.30	10	0.19	39.8	59.17	10	0.22
5301	73	FEMALE	CRC	67.9	49.07	90	0.32	41.2	62.22	50	0.21
5444	75	FEMALE	CRC	29.4	55.56	50	0.28	0	100	0	0
5996	48	MALE	CRC					0	100	0	0
5681	66	FEMALE	CRC	30.3	75.30	25	0.25	22.2	96.30	0	0

It is clear that media B is a superior cryopreservative (table 21). The historical media has more significant viability drop ($p<0.0001$ vs $p<0.0005$) and inferior attachment ($p<0.005$ vs $p<0.05$). As a result, the historical media was discarded and all experiments thereafter used media B.

4.02.02 Hepatocyte loss

With fresh hepatocytes, fewest are lost with x3 washes in contrast to pre-incubation ($p<0.005$). Post-thaw, there is large volume hepatocyte loss with least seen in the pre-cryopreservation Percoll® purification and x3 washes groups.

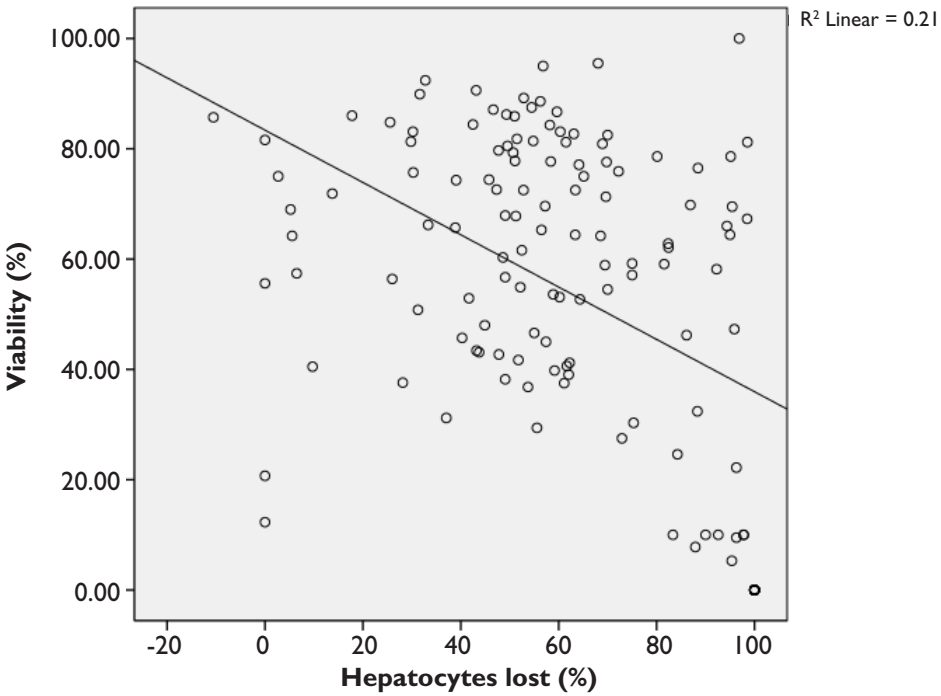


Figure 28 Viability and hepatocyte loss.

There is an inverse relationship between viability and hepatocyte loss ($p<0.0001$) (figure 28).

Group code	Condition
1	x1 wash, fresh culture
2	x1 wash, then Percoll® purification, fresh culture
3	x3 washes, fresh culture
4	Pre-incubated, fresh culture
5	x1 wash, then cryopreserved
6	x1 wash, Percoll® purification, then cryopreservation
7	x3 washes, then cryopreserved
8	Pre-incubated, then cryopreserved
9	Historical media
10	x3 washes, cryopreserved then Percoll® gradient

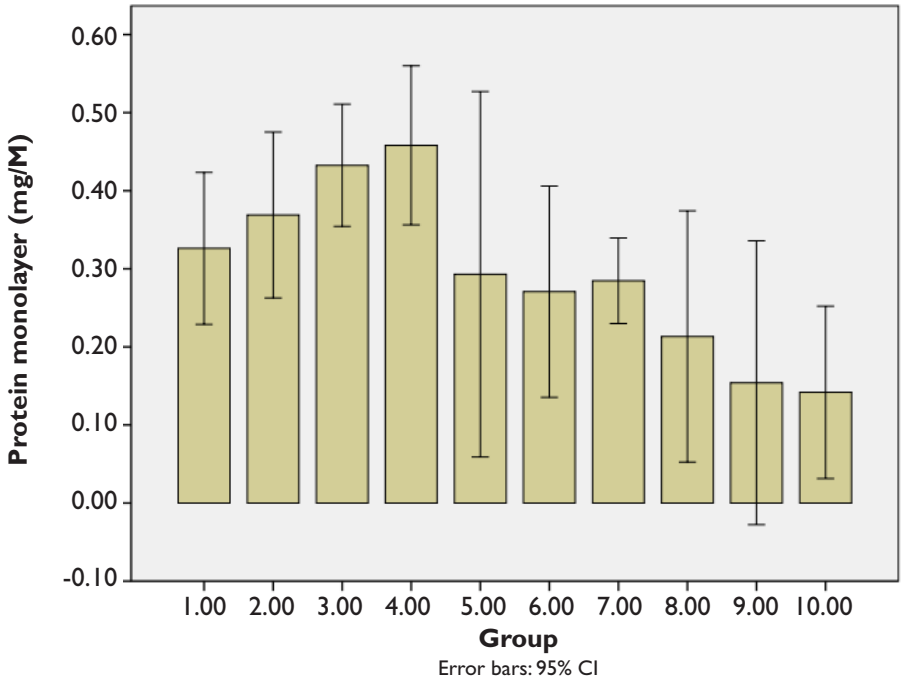


Figure 29 Protein monolayer across all groups.

Monolayer protein is reduced post-thaw when compared with fresh hepatocytes (figure 29), only approaching significance in the pre-incubation and post-cryopreservation Percoll® gradient groups.

4.02.03 Metabolic studies

Group code	Condition
1	x1 wash, fresh culture
2	x1 wash, then Percoll® purification, fresh culture
3	x3 washes, fresh culture
4	Pre-incubated, fresh culture
5	x1 wash, then cryopreserved
6	x1 wash, Percoll® purification, then cryopreservation
7	x3 washes, then cryopreserved
8	Pre-incubated, then cryopreserved
9	Historical media
10	x3 washes, cryopreserved then Percoll® gradient

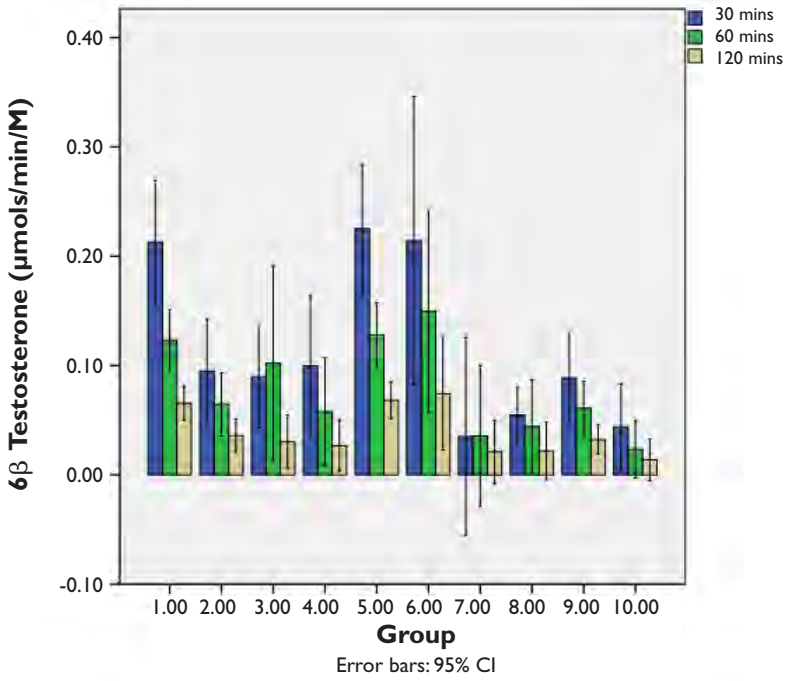


Figure 30 Testosterone activity.

CYP450 activity was measured in µmols/min/M cells throughout.

Assays were performed to assess CYP3A4, CYP2C9, CYP1A2, CYP2D6 and phase II sulfation and glucuronidation. CYP3A4 hydroxylates testosterone and demonstrated significant differences across all groups and time lines (figure 30). Most groups followed a similar pattern of activity, with maximal hydroxylation in the first 30 minutes. The x1 wash and x1 wash with pre-cryopreservation Percoll® purification groups performed well post-cryopreservation.

Group code	Condition
1	x1 wash, fresh culture
2	x1 wash, then Percoll® purification, fresh culture
3	x3 washes, fresh culture
4	Pre-incubated, fresh culture
5	x1 wash, then cryopreserved
6	x1 wash, Percoll® purification, then cryopreservation
7	x3 washes, then cryopreserved
8	Pre-incubated, then cryopreserved
9	Historical media
10	x3 washes, cryopreserved then Percoll® gradient

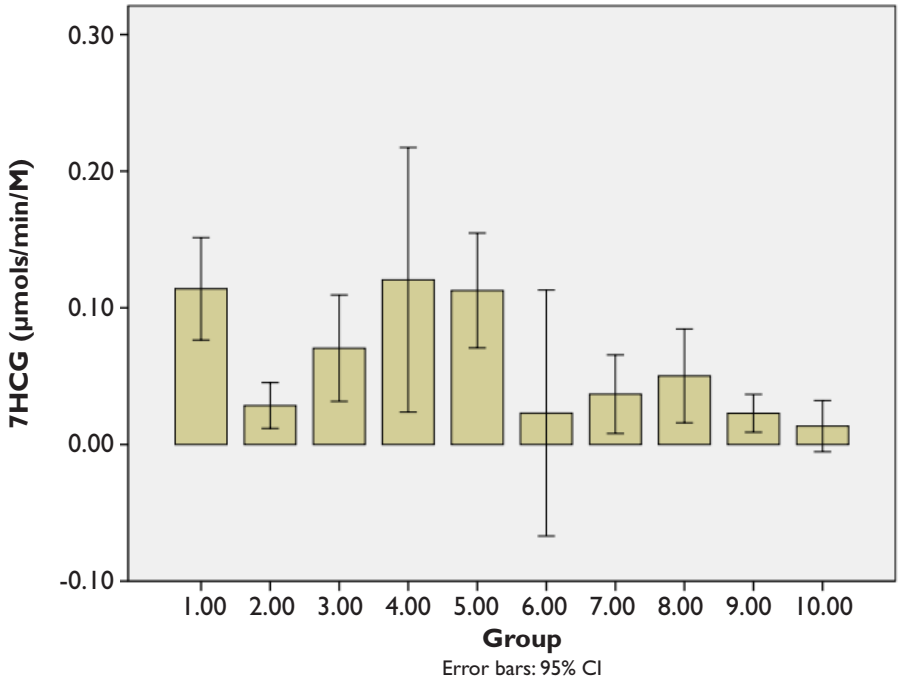


Figure 31 7HCG at T120.

The only other significant finding was the 120 minute 7HCG results (figure 31). Percoll® performed uniformly badly with comparable results between the x1 wash pre- and post-cryopreservation groups.

4.02.04 Statistics

Analysis was performed using a 2-way ANOVA. Where significant differences were identified, student's t-test was used to compare individual groups and identify significance.

4.03 Fibrosis and steatosis

The liver injury, NASH and fibrosis score systems are all measures of chronic injury, with the Ishak score a measure of acute hepatocyte injury (Goodman 2007) (table 22).

Liver injury score	0=Absent 1=Mild 2=Moderate 3=Severe	Cellular Ballooning
		Necrosis
		Regeneration
		Microvesicular Steatosis
		Macrovesicular Steatosis
		Portal Inflammation
		Lobular Inflammation
		Cholestasis
NASH (Brunt)		Steatosis
		Ballooning
		Lobular Inflammation
		Portal Inflammation
Fibrosis		NASH (Brunt) Fibrosis
		Metavir Fibrosis
		Ishak Fibrosis
		9 Point LIS Fibrosis
Misc.		Sinusoidal Dilatation
		Peliosis
Ishak Score		Interface hepatitis
		Confluent Necrosis
		Focal Necrosis
		Portal Inflammation
		Architecture

Table 22 Histological scoring systems.

The most significant endpoint was determined as number of viable hepatocytes produced per gram of tissue used for isolation. Although viability and purity may be important, live hepatocytes will usually attach to culture media, with a viable hepatocyte equating to a functional hepatocyte. As such, number isolated is more important in terms of success.

Sixty isolations from 54 donors were analysed (figures 32-35).

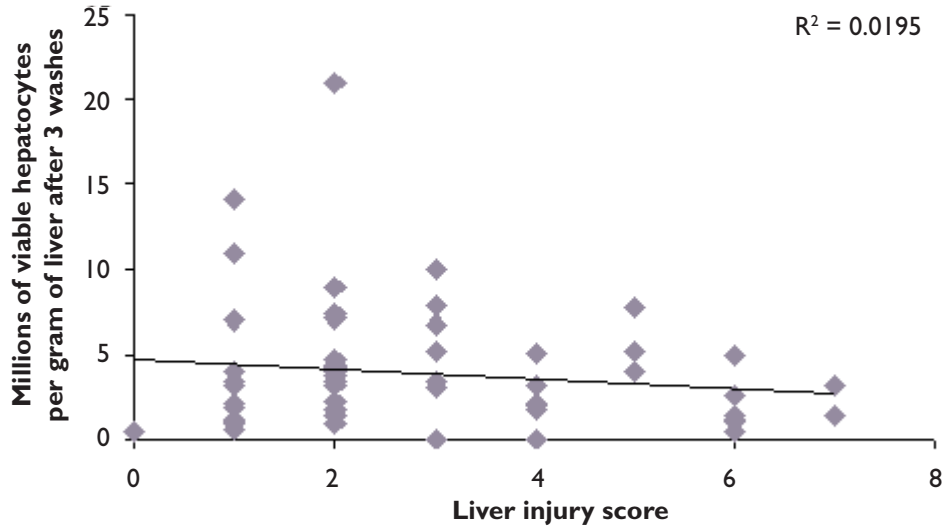


Figure 32 Liver injury score and number of millions of viable hepatocytes per gram isolated (x3 wash).

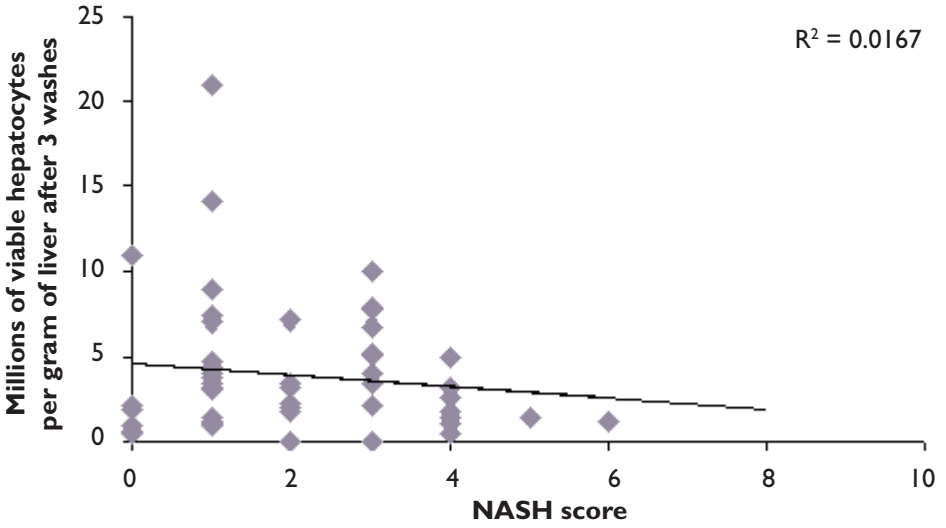


Figure 33 NASH score and number of millions of viable hepatocytes per gram isolated (x3 wash).

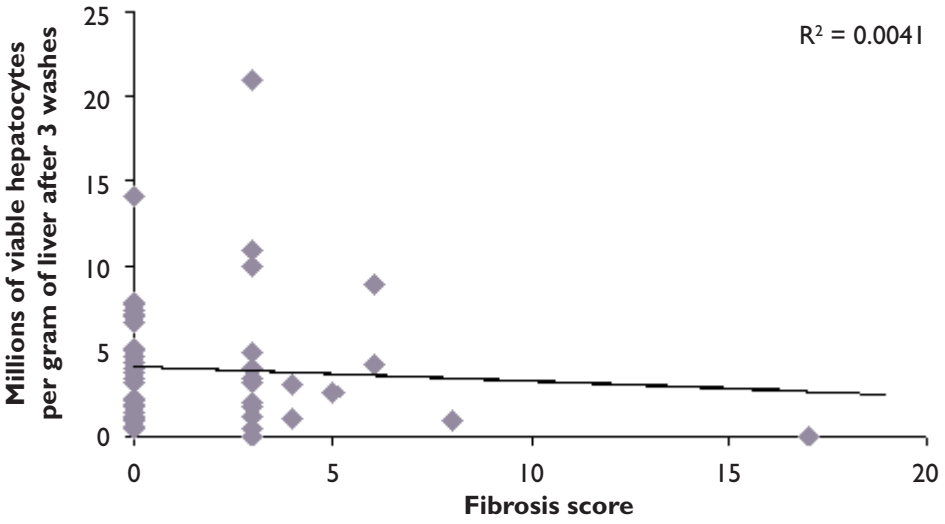


Figure 34 Fibrosis score and number of millions of viable hepatocytes per gram isolated (x3 wash).

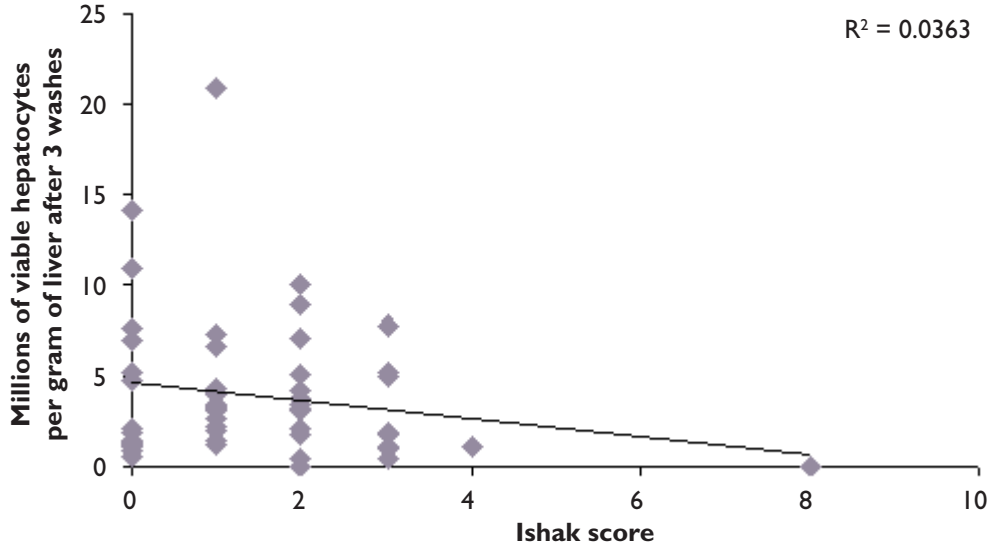


Figure 35 Ishak score and number of millions of viable hepatocytes per gram isolated (x3 wash).

There was a weak negative correlation with all scores measuring acute and chronic injury when compared with isolation outcome. With the solitary wash (x1) isolation process numbers were too small to analyse.

4.03.01 Validation of histology scores

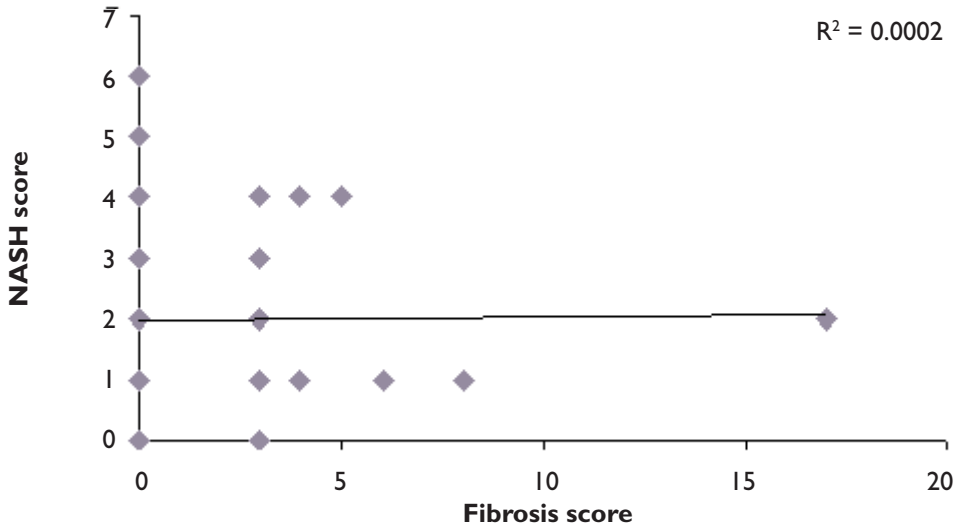


Figure 36 Fibrosis and NASH scores.

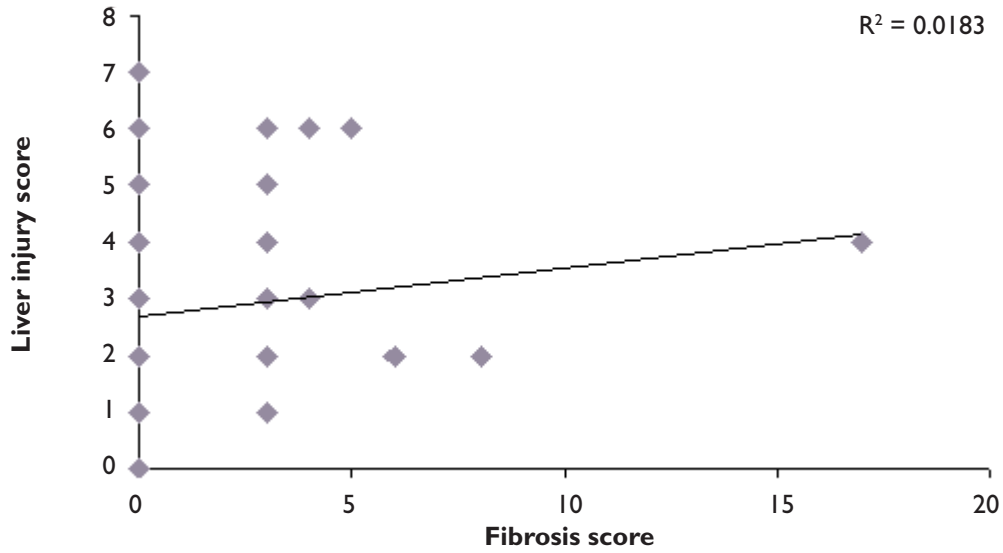


Figure 37 Fibrosis and liver injury scores.

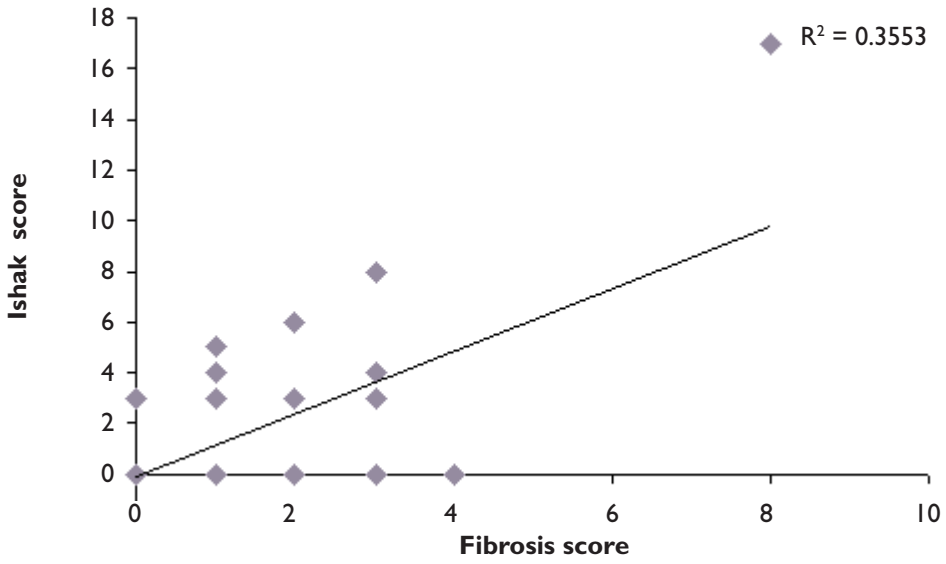


Figure 38 Fibrosis and Ishak scores.

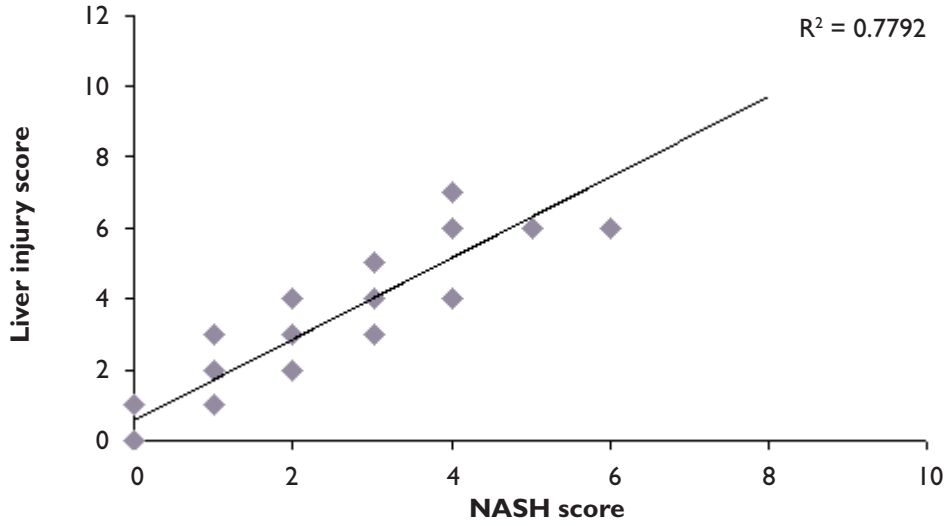


Figure 39 NASH and liver injury scores.

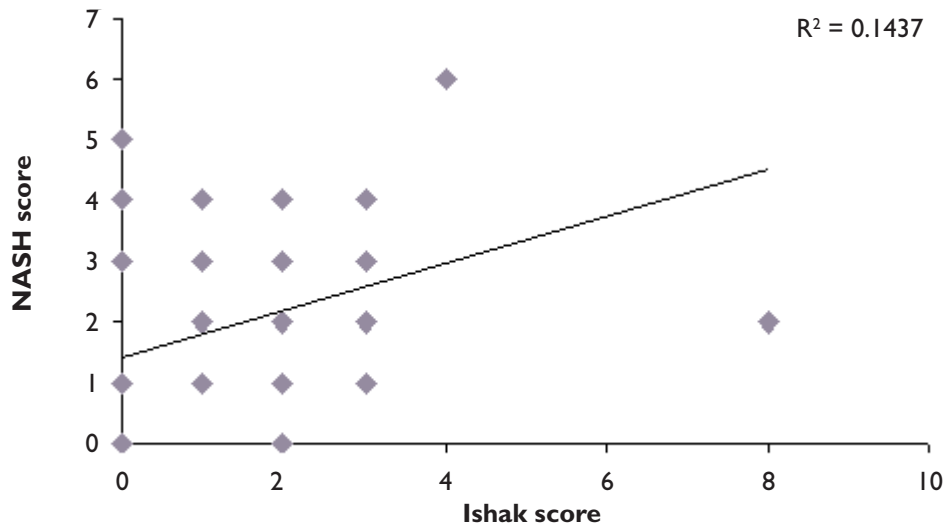


Figure 40 NASH and Ishak scores.

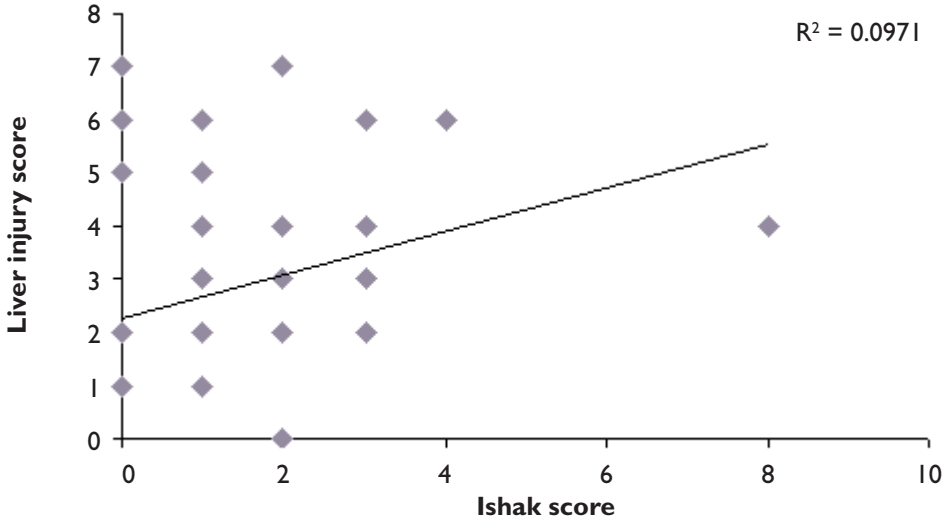


Figure 41 Liver injury and Ishak scores.

To support validity of these histological scoring systems they were cross-referenced for score outcome (figures 36-41). The liver injury and NASH scores, and ISHAK and fibrosis scores correlate well.

4.03.02 Statistics

Pearson correlation assumed the normal distribution of the sample and two-tailed test demonstrated significance.

4.04 ECVAM

Initially the yield of viable hepatocytes obtained with two different Sigma powdered collagenase batches (400-600 U/mg protein) and perfusion buffers consisting either of HBSS, 0.5% BSA and 0.5 mM EGTA or PBS and 0.5 mM EGTA were compared. In all conditions, an average yield of 7.21±1.21M viable hepatocytes/g liver with viability between 70-80% was obtained after Percoll® purification, with no significant difference either between donors or laboratories.

In the Leicester arm, the InVitrogen Hepatocyte Digest Medium was compared with the 0.05% collagenase preparation (n=18). The yield of viable hepatocytes/g liver was significantly lower (3.87±1.07M), but the high viability of the preparations (89.46±2%) required no Percoll® purification before plating. With these results, it was accepted that InVitrogen Hepatocyte Digest Medium could continue to be used in Leicester and collagenase digest media used in France. The criteria for the harmonized protocol of human hepatocyte isolation used in the present study are summarized in table 23.

	Criteria
Source of human livers	Surgical liver biopsies from patients with benign liver tumours, primary liver cancer or metastatic cancer involving liver
Flushing in theatre	1. Not necessary if transport time is less than 1 hour 2. Yes with Soltran if transport time is longer than 1 hour
Transport time	0 - 5 hours
Hepatocyte isolation - Glue - Number of cannulae - Flow rate/cannula - Collagenase - Digestion time	 + 2-4 cannulae 20-40 ml/min/cannula Supplied as powder or as a ready-to-use digestion medium Not longer than 20 minutes

Table 23 Criteria for tissue origin, collection, transport and hepatocyte isolation.

Isolation outcome is summarized in table 24.

Microsomal protein content of attached cultured hepatocytes harvested after 5-6 days

Table 24 EVCAM data

Year	Date	Code	>1 Isolation	Age	Cause donation	Chemo	Pringle	WIT	CIT	Tissue mass (g)	Digested tissue mass (g)	Un-digested tissue mass (g)	Cannulae	Volume wash buffer (ml)	Time perfusion (mins)	Speed	Collagenase	Volume digestion (ml)	Digestion time (mins)	Speed	Digestion
2	15.06.04	001/A/04629	NO	65	CRC	1	16	3	45	163	90	73	3	1000	20	30	GIBCO	1000	20	30	Good
2	22.06.04	001/A/04938	NO	57	CRC	0	10	10	50	181	21	160	3	1000	20	30	GIBCO	1000	25	30	Good
2	26.06.04	001/A/04506	NO	64	CRC	1	23	10	60	174	35	139	3	1000	20	30	GIBCO	1000	20	30	Moderate
2	26.06.04	001/A/04391	NO	54	CRC	1	28	5	80	114	37	77	3	1000	20	30	GIBCO	1000	25	30	Moderate
2	20.07.04	001/A/04454	NO	73	CRC	1	18	5	60	66	49	17	3	500	15	25	0.05%	500	20	25	Good

Pre-Percoll					Post Percoll					Conclusion			
Yield viable hepatocytes (M)	Viable hepatocytes/g liver	Viable hepatocytes/g digested liver	Viability (%)	Percoll	Viable hepatocytes yield (M)	Viable hepatocyte yield/g liver (M)	Viable hepatocyte yield/g digested liver (M)	Final viability (%)	Hepatocytes lost at Percoll (%)	Viable hepatocyte yield/g liver (M)	Viable hepatocyte yield/g digested liver (M)	Final viability (%)	Attachment (%)
1147	7	12.7	78.6	0						7	12.7	78.6	85
1343	7.4	63.9	71.5	1	305	6.1	52.7	81	25	6.1	52.7	81	90
580	3.3	16.6	87.2	0						3.3	16.6	87.2	90
257	2.25	6.9	86	0						2.25	6.9	86	80
930	14.1	19	60	1	482.5	7.3	9.8	85	48	7.3	9.8	85	85

of culture was used as a test of plating efficacy and viability. First, the effect of the treatments on the protein level was analysed for each donor in each laboratory (figure 42).

Controls were hepatocytes in plain culture

BNF 2	**β -naphthoflavone 2 µM**	**PB 50**	**Phenobarbitol 50 µM**	**RIF 0.1**	**Rifampicin 0.1 µM**
BNF 10	**β -naphthoflavone 10 µM**	**PB 250**	**Phenobarbitol 250 µM**	**RIF 1**	**Rifampicin 1 µM**
BNF 50	**β -naphthoflavone 50 µM**	**PB 1000**	**Phenobarbitol 1000 µM**	**RIF 10**	**Rifampicin 10 µM**

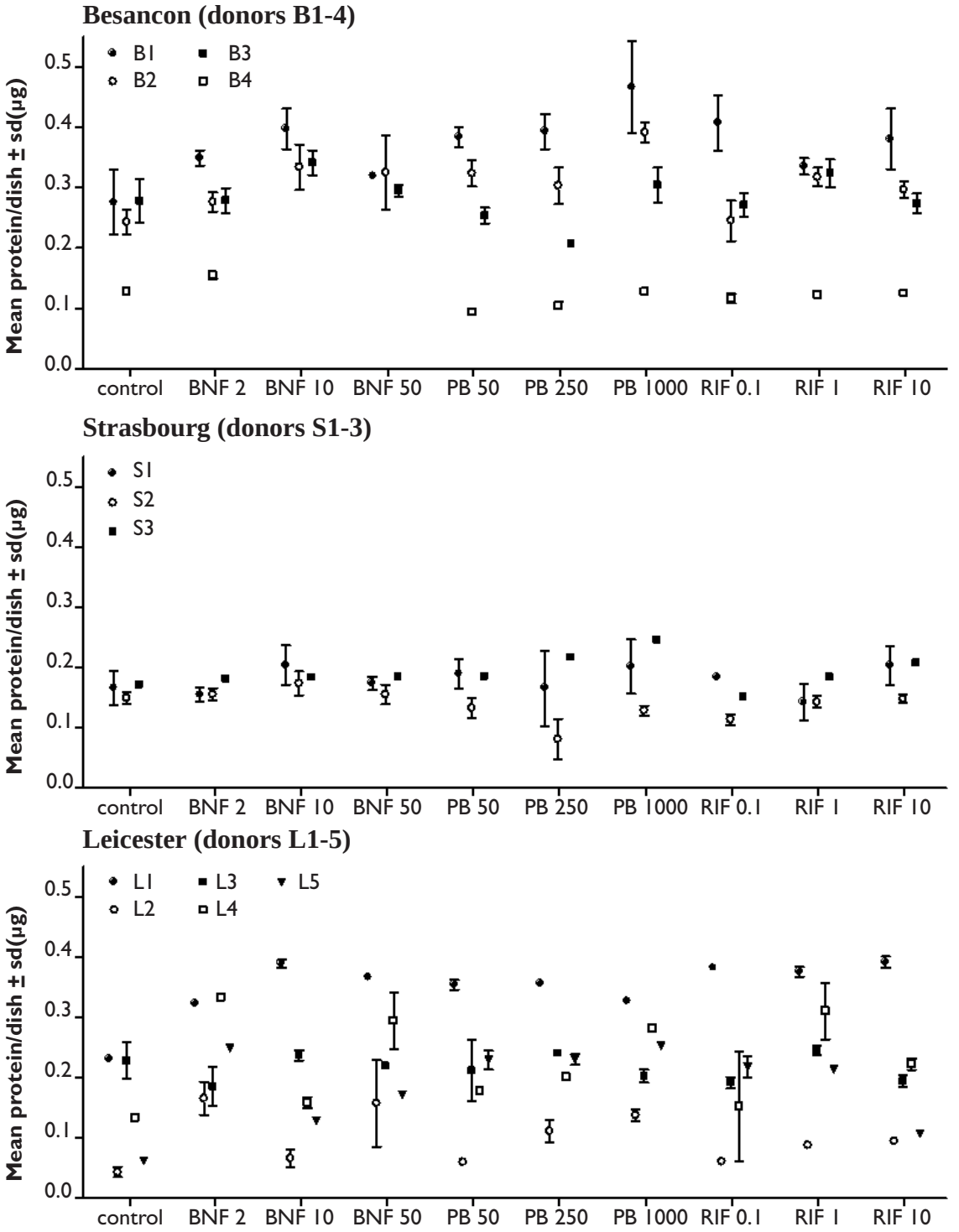


Figure 42 Protein data for each donor, treatment and laboratory.

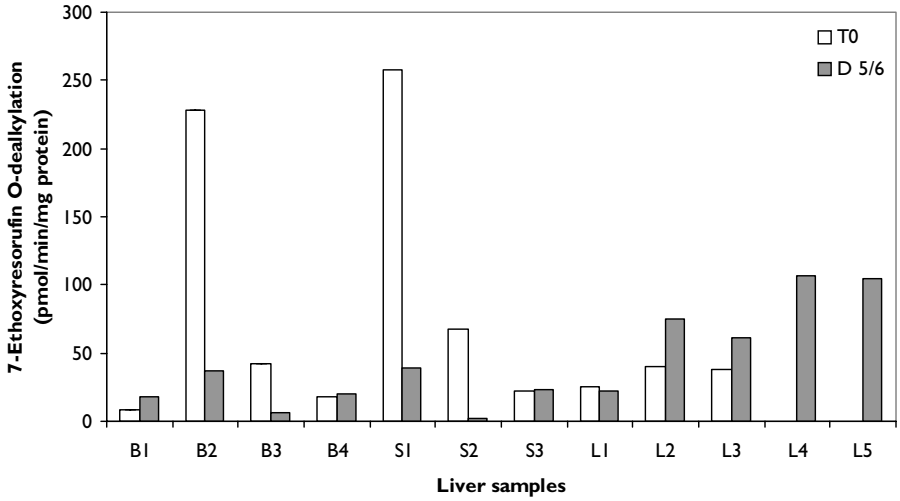
At Besancon testing for treatment effects by a 1-way ANOVA with a post-hoc Dunnett test versus the control ($p=0.01$) revealed that CYP450 induction had a significant effect on protein concentration across all donors ($p<0.01$). For Donor B1, seven of the nine treatments resulted in significantly increased protein concentration, with a response range of 0.2 µg. Less significant treatment effects were seen in the other donors. At the other two laboratories the results were more variable. For donors S1 and L3, all treatments were comparable to the control. Overall, response levels ranged between 43-467 µg microsomal protein. No patterns in compounds or concentration were obvious in terms of induction agent.

The donors per laboratory were compared to assess between-donor variability and within- and between-laboratory reproducibility. Reviewing the demonstrated protein data for each donor and each laboratory makes it obvious that donors responded on an individual level. At Besancon, donor B4 gave consistently the lowest response for all treatments, while in Strasbourg the three donors showed very similar response levels. The protein levels of the Leicester donors varied between 40-400 µg protein/dish. To account for donor specific response levels, the differences between the three laboratories was analysed by a 2-way ANOVA for repeated measurements. Excluding donor B4 because of missing data, no significant laboratory effect ($p=0.07$) and no significant interaction between treatments and laboratories ($p=0.22$) were found. The results of the analysis of each individual donor were confirmed by a significant treatment effect ($p<0.01$).

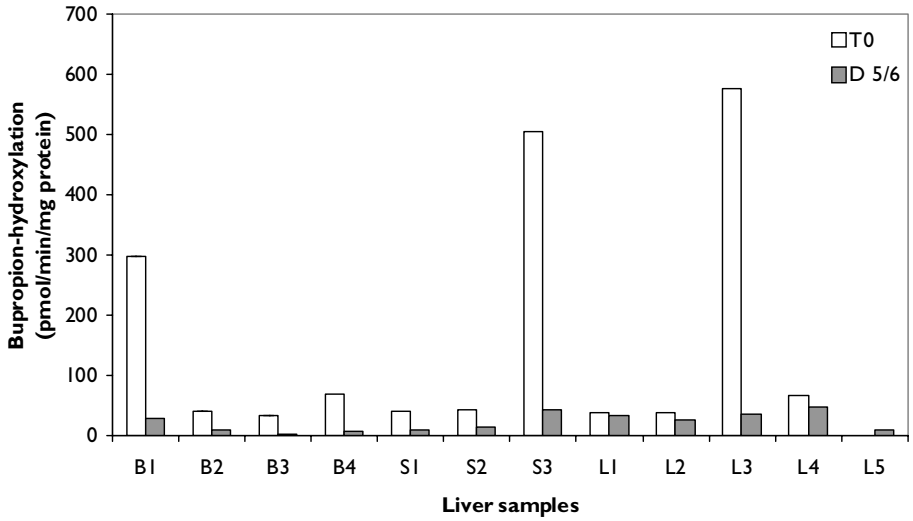
Regarding induction therapy, there was no clear pattern regarding compound or concentration on protein content. Non-significant laboratory effects were present, indicating acceptable between-laboratory reproducibility, from which satisfactory within-laboratory reproducibility can be inferred (figure 43). (Values are means from duplicated experiments.)

B1-4	**Besancon donors 1-4**
S1-3	**Strasbourg donors 1-3**
L1-5	**Leicester donors 1-5**

A



B



C

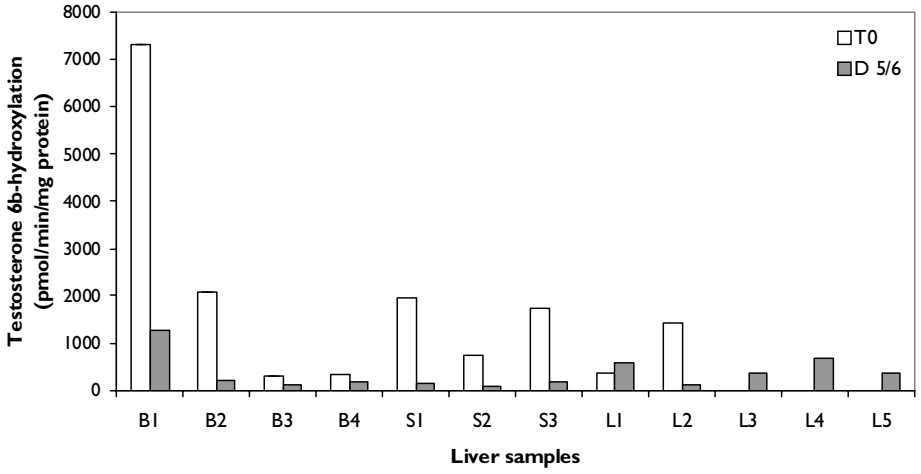


Figure 43 Microsomal CYP-dependent monooxygenase activities after 5-6 days of culture.

There is high variability in microsomal CYP1A1 activity in the ten freshly isolated hepatocyte samples ranging between 8.66-258.25 pmol/min/mg protein. After the culture period, the activities were either equivalent, higher or lower in equal proportion compared to freshly isolated cells. At 5-6 days of culture (n=12) the range was between 1.84-106.56 pmol/min/mg protein.

There is high variability in microsomal CYP2B6 activity in freshly isolated hepatocytes (n=11), ranging between 33.43-576.80 pmol/min/mg protein. After culture, activities dropped in all but one with a range of 2.50-47.70 pmol/min/mg protein.

Microsomal CYP3A4/5 activity in freshly isolated hepatocytes (n=9) ranged between 293.18-7307.15 pmol/min/mg protein. After culture, the activities dropped in all but one with a range of 90.71-1261.25 pmol/min/mg protein.

High between-donor variability was observed in freshly isolated hepatocytes for all CYP450 activities tested, which was sustained over the 5-6 day culture period. For each CYP450 measured, the relative induction compared to their negative controls is displayed for each donor in each laboratory (figure 44).

Statistical analysis, was performed with the corresponding logarithmically transformed raw data, including the negative controls. Accounting for the data dependence for a given donor, a 2-way ANOVA was carried out for each drug-CYP combination, treatment and laboratory (table 25). As this technique cannot cope with missing data, some donors had to be excluded.

There was insignificant laboratory effect. Furthermore, only the treatment effect of PB on CYP2B6 resulted in a significant interaction indicating different concentration-dependent responses in the three laboratories. Regarding induction, no significant effects of BNF on CYP3A4/4 and of PB and RIF on CYP1A2 were found. While BNF significantly induced CYP1A1 and PB significantly induced CYP3A4/5, CYP2B6 was increased by all three treatments.

BNF 2	**β-naphthoflavone 2 µM**	**PB 50**	**Phenobarbitol 50 µM**	**RIF 0.1**	**Rifampicin 0.1 µM**
BNF 10	**β-naphthoflavone 10 µM**	**PB 250**	**Phenobarbitol 250 µM**	**RIF 1**	**Rifampicin 1 µM**
BNF 50	**β -naphthoflavone 50 µM**	**PB 1000**	**Phenobarbitol 1000 µM**	**RIF 10**	**Rifampicin 10 µM**

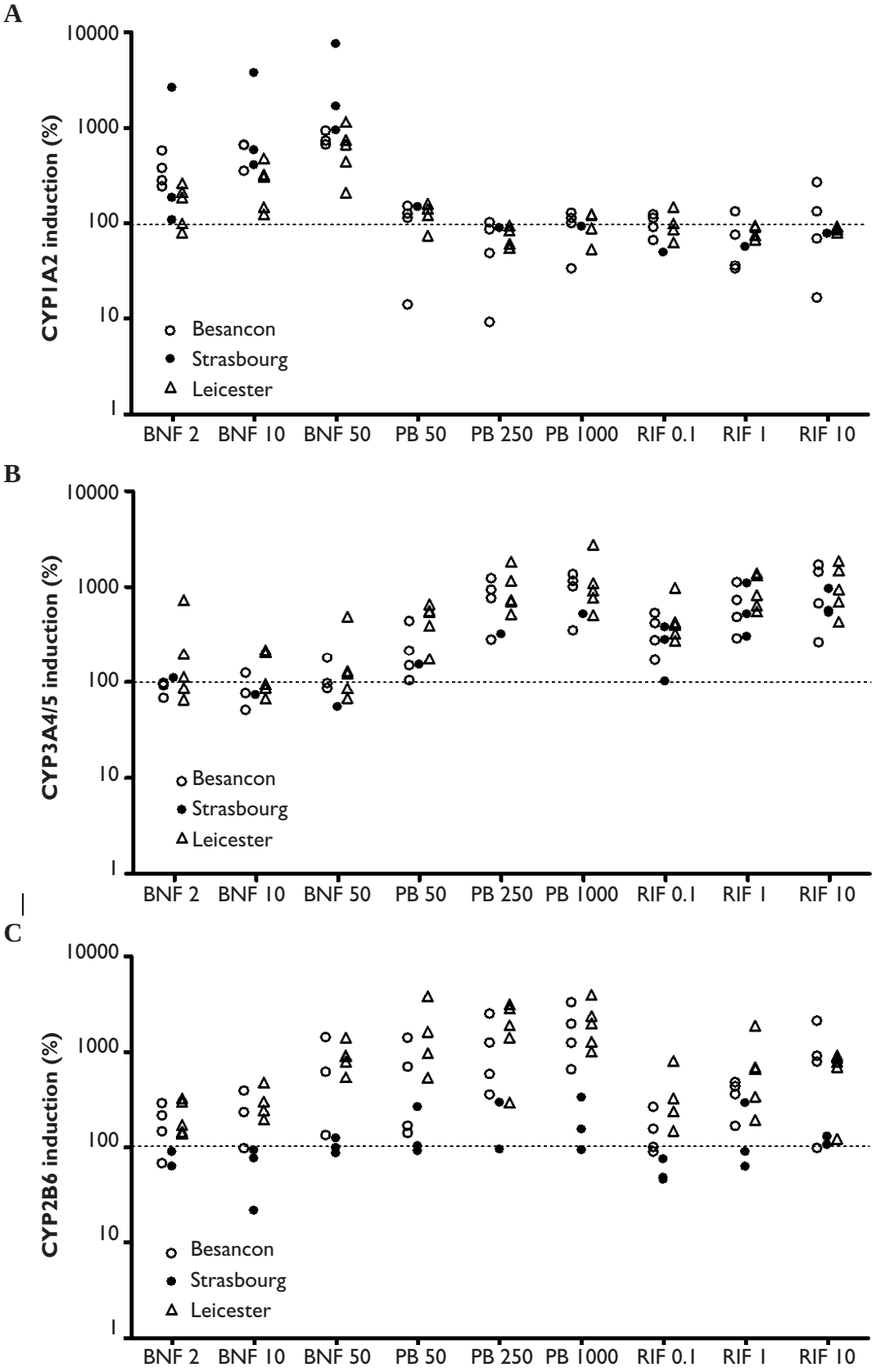


Figure 44 CYP450 induction.

CYP	Drug	Number of donors included			ANOVA p-values		
		Besancon	Strasbourg	Leicester	laboratory	treatment	interaction
1A2	BNF	3	3	5	0.1334	**0.0001**	0.0683
	PB	4	1	4	0.101	0.2436	0.7342
	RIF	4	1	4	0.0736	0.4357	0.9207
3A4/5	BNF	3	1	5	0.3914	0.7415	0.5325
	PB	4	1	5	0.1382	**0.0001**	0.314
	RIF	4	3	5	0.0537	**0.0001**	0.6847
2B6	BNF	3	2	4	0.1216	**0.0001**	0.0113
	PB	4	2	4	0.022	**0.0001**	**0.0011**
	RIF	4	3	4	0.0163	**0.0001**	0.013

Table 25 4 p-values.

The inductions with significant effect were further analysed with a Bonferroni post-test to compare concentration-dependent responses between the laboratories (table 26).

CYP1A2 was significantly induced in all laboratories by BNF, RIF significantly increased CYP3A4/5. PB only induced CYP3A4/5 in Besancon and Leicester in high concentration. For CYP2B6, clear differences between the laboratories became evident. In contrast to the absence of any treatment effect at Strasbourg, all treatments induced CYP2B6 in a similar concentration-dependent response in the other two laboratories. In Leicester significant effects usually were seen at lower concentrations.

4.04.01 Statistics

Analysis was performed as described above (both 1- and 2-way ANOVA with a post-hoc Dunnett test and Bonferroni post-test).

CYP	Drug	Concentration	Laboratory		
			Besancon	Strasbourg	Leicester
1A2	BNF	2	ns	**	ns
		10	***	***	ns
		50	***	***	***
	PB	50	na	na	na
		250	na	na	na
		1000	na	na	na
	RIF	0.1	na	na	na
		1	na	na	na
		10	na	na	na
3A4/5	BNF	2	na	na	na
		10	na	na	na
		50	na	na	na
	PB	50	ns	ns	***
		250	***	ns	***
		1000	***	ns	***
	RIF	0.1	***	ns	***
		1	***	***	***
		10	***	***	***
2B6	BNF	2	ns	ns	ns
		10	ns	ns	**
		50	***	ns	***
	PB	50	***	ns	***
		250	***	ns	***
		1000	***	ns	***
	RIF	0.1	NS	ns	**
		1	**	ns	***
		10	***	ns	***

ns: not significant, p-value >0.01

na: not analysed as overall no significant treatment effect was seen

**: significant to the level of 0.01

***: significant to the level of 0.001

Table 26 Results of Bonferroni post-test.

4.05 Cytotoxicity evaluations

Using the BCA protein assay (figures 45-47) and MTT looking for injured or early apoptotic cells (figures 48-50), appropriate concentrations for the later assays were chosen. Results for each donor are represented (n=6).

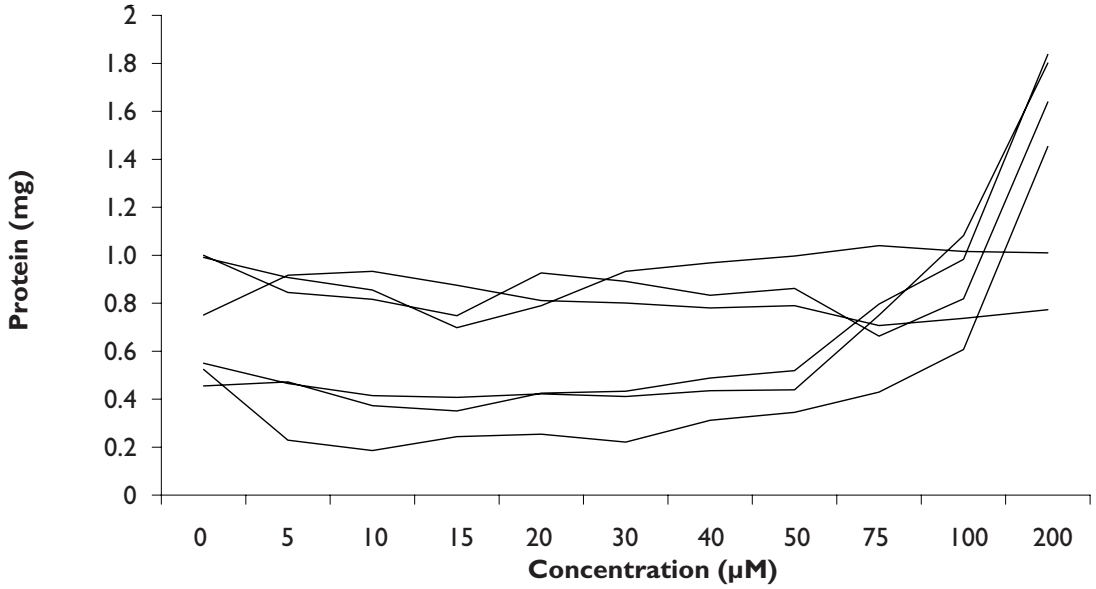


Figure 45 Protein cytotoxicity assays: curcumin.

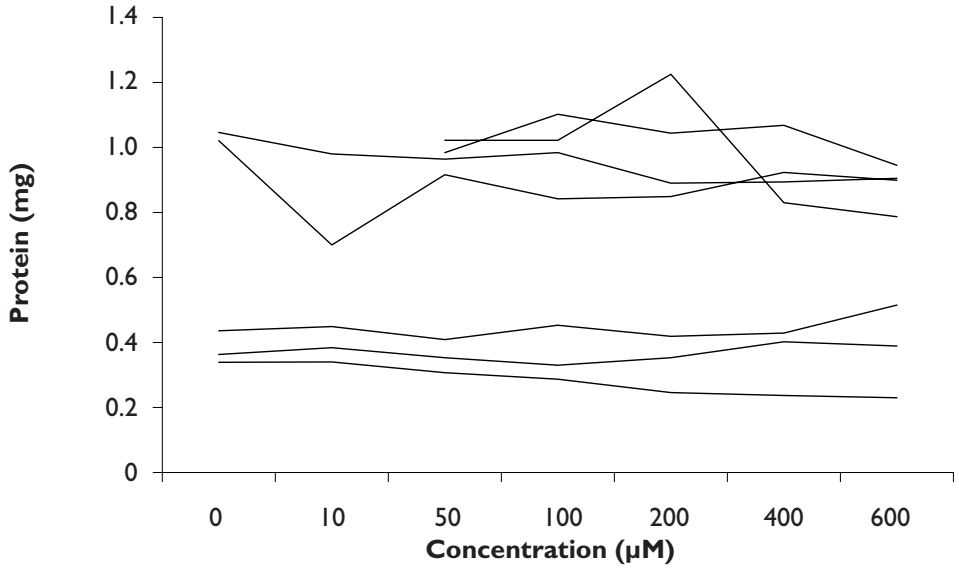


Figure 46 Protein cytotoxicity assays: UDCA.

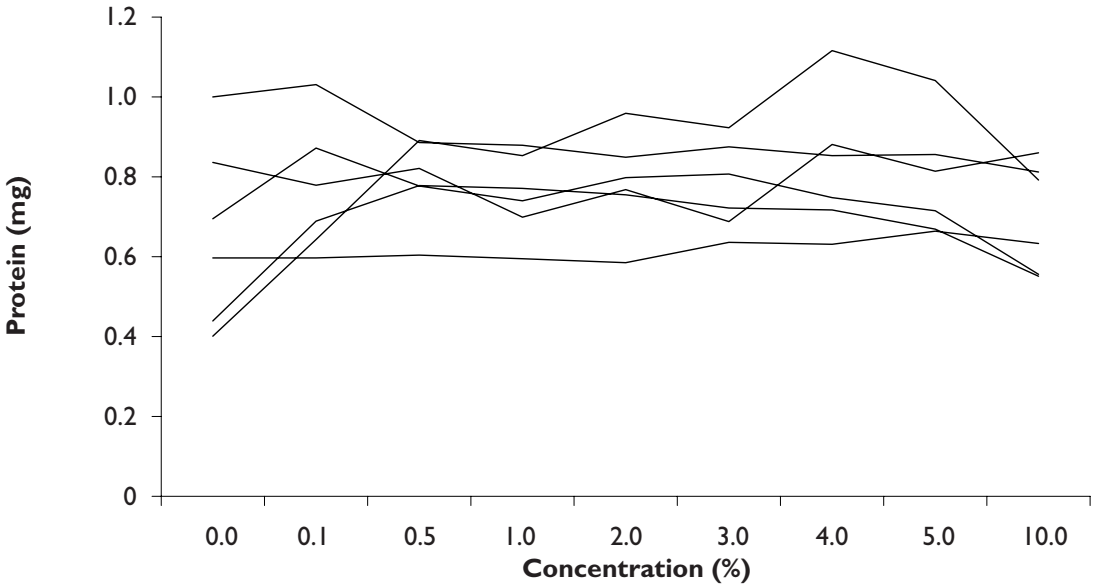


Figure 47 Protein cytotoxicity assays: ethanol.

Reviewing the protein data, curcumin demonstrates dose response until the concentration is greater than 100 μM, secondary to the colour of tumeric interfering with ELISA specificity. Regarding UDCA, again results are unremarkable, with a slight peak in protein attachment at approximately 200 μM. Ethanol gives best results at low concentration (0.5-3%).

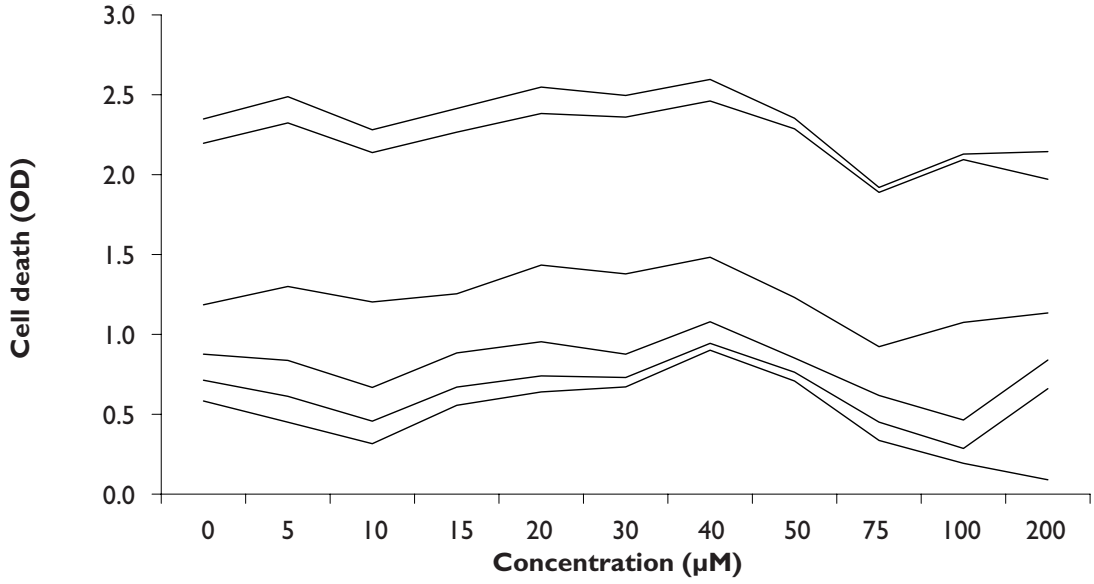


Figure 48 MTT cytotoxicity assays: curcumin.

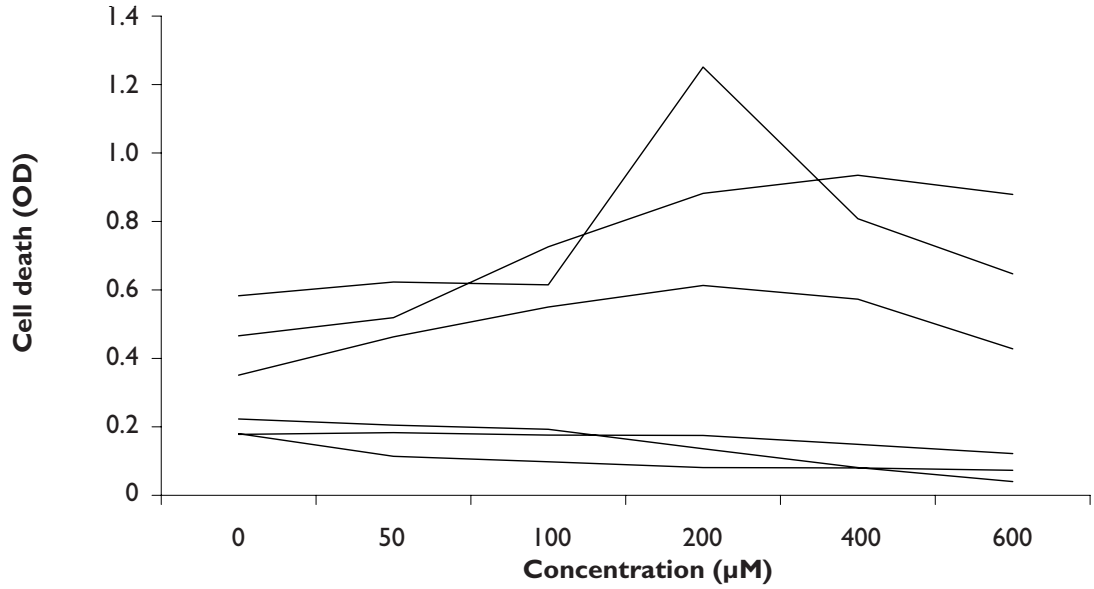


Figure 49 MTT cytotoxicity assays: UDCA.

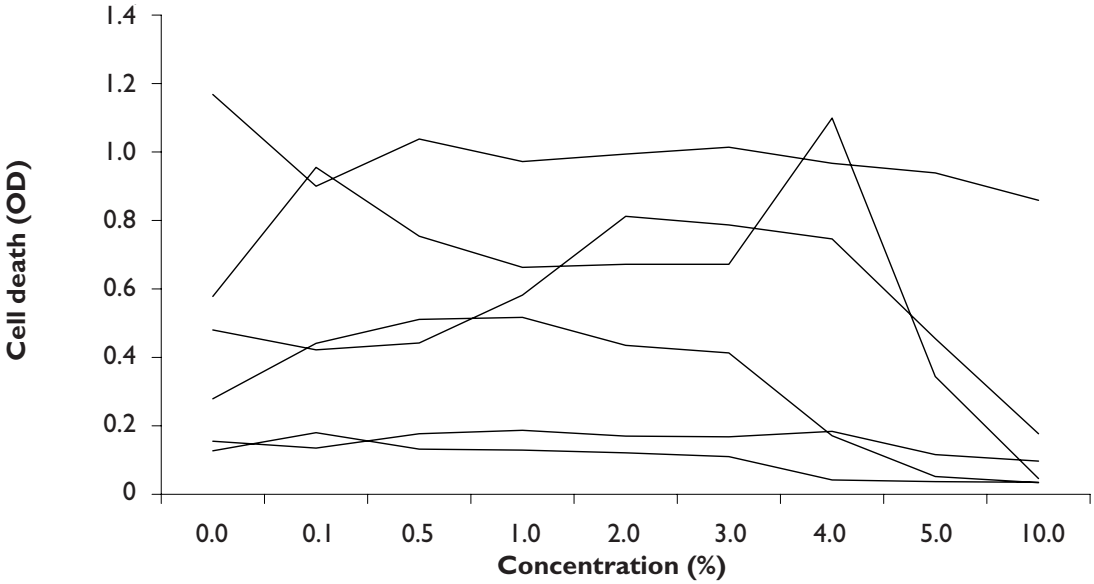


Figure 50 MTT cytotoxicity assays: ethanol.

Curcumin has the fewest damaged hepatocytes at lower concentrations (20 μM) and most at higher concentrations probably exhibiting similar artifact as explained above. UDCA demonstrates a biphasic pattern (favourable results with 50 μM and 400 μM, maximal injury with 200 μM). Finally, with ethanol it is suggested the best results are at high dose, but at these concentrations hepatocytes die. There is another apparent biphasic pattern with poor results with concentrations of 0.1% and 4%.

Taking these together, a concentration of 20 µM curcumin and 400 µM UCDA, in 2% ethanol was used for the comparative apoptosis studies. After discussion with the French team for the dual isolation project, a slightly higher curcumin concentration of 25 µM in 0.5% ethanol was used.

4.06 The affect of curcumin on human hepatocyte isolation and culture

Continuing from earlier work, hepatocyte loss secondary to necrosis and apoptosis throughout the isolation process leads to dramatically reduced output from any one isolation. Using curcumin dissolved in low concentration ethanol throughout the experiments in dual isolation, fresh hepatocytes were isolated during each stage of this process (table 27). It is clear that viability influences outcome and that attached hepatocytes are functional hepatocytes, but the effect of curcumin is questionable. If antioxidants can improve outcome in terms of viability, overall yield or reduced hepatocyte loss, then further investigation into its use in this context might be worthwhile.

		Pre-Percoll®		
		Viable hepatocytes/g liver (M)	Viability (%)	
Curcumin 0.025 mM	Average	10.33	56.08	
	Range	1.9-32	21.4-79	
Plain + 0.5% alcohol	Average	12.80	61.43	
	Range	3.8-28	43.9-80	
		Post-Percoll®		
		Viable hepatocytes/g liver (M)	Viability (%)	Hepatocytes lost at Percoll® (%)
Curcumin 0.025 mM	Average	4.10	71.58	65.46
	Range	0.6-15.7	46-83.9	53-78.74
Plain + 0.5% alcohol	Average	5.28	75.72	59.77
	Range	1.16-13.9	62.6-82	46.8-70
		Post-Preincubation		
		Viable hepatocytes/g liver (M)	Final viability (%)	Hepatocytes lost at pre-incubation (%)
Curcumin 0.025 mM	Average	2.84	65.03	15.80
	Range	0.8-11.2	50.4-76.8	Gain of 24.9-55.42
Plain + 0.5% alcohol	Average	3.46	59.97	39.63
	Range	0.36-10.4	32-84.3	9.32-65.9

Table 27 Viability at each stage of the dual isolation process.

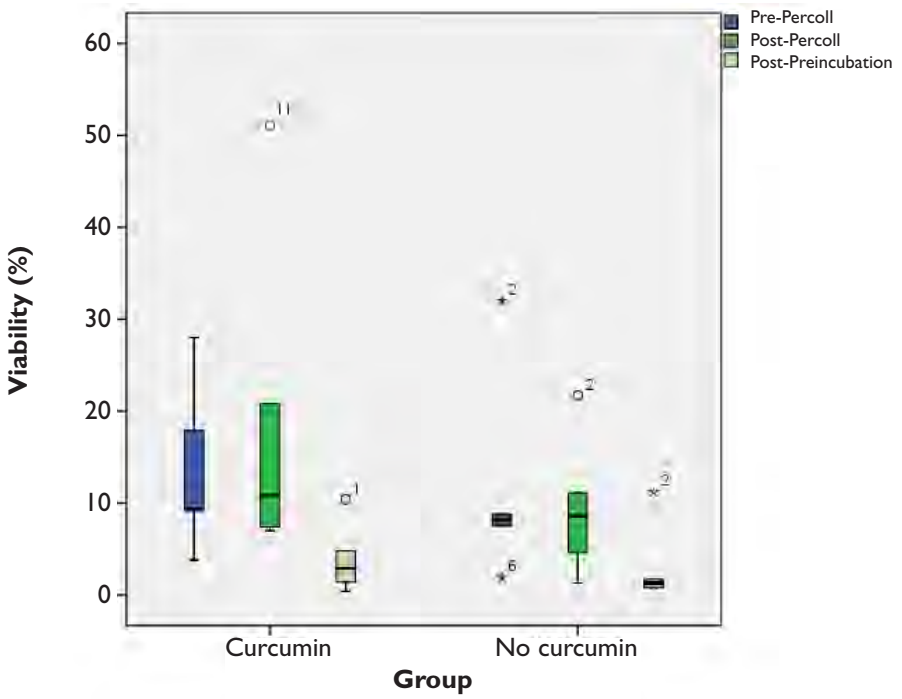


Figure 51 Viability at each stage of the isolation process.

It is evident that percentage viability drops steeply between the Percoll® purification step and the pre-incubation resuscitation step (p=0.05). This is more obviously seen in the curcumin group (figure 51). There is no statistical difference in outcome when the two groups are directly compared with each other despite initial appearances. Hepatocyte loss at this step is also statistically significant (p=0.02) (figure 52). This is aimed at improving hepatocytes prior to cryopreservation, but it seems to simply damage them further. The pre-incubation stage is hepatocyte culture in suspension rather than monolayer culture and such a deterioration would be unexpected.

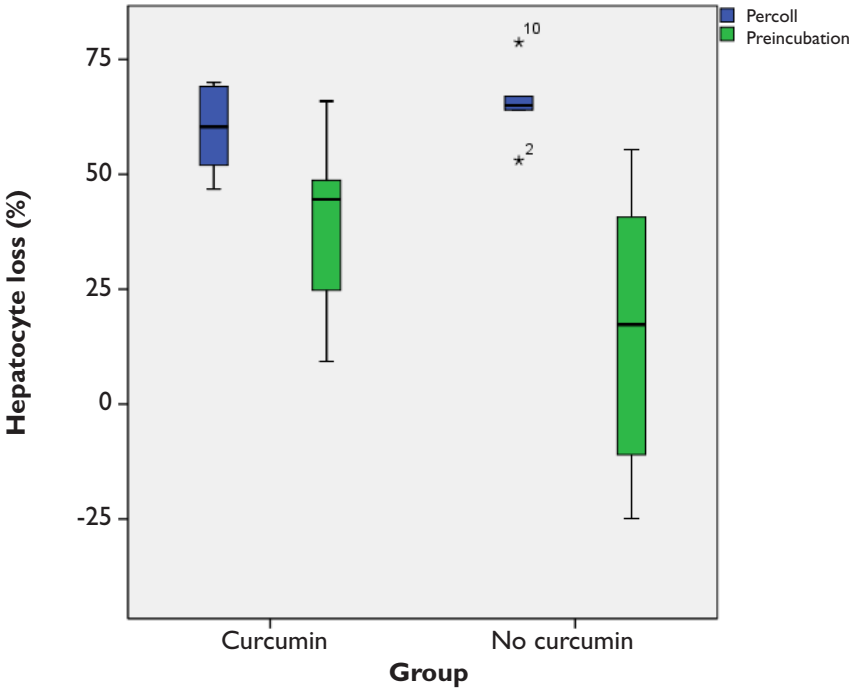


Figure 52 Percentage hepatocyte loss with Percoll® purification and pre-incubation.

Reviewing the fresh culture monolayer protein and attachment data, at different time points over three days, there were no statistically significant differences between the three groups.

4.06.01 Statistics

The Mann Whitney U test ensured the samples were independent of one another and two-tailed test was used to assess significance.

4.07 Apoptosis studies

Comparing two caspase inhibitors (ZVAD-fmk and Apoptame), curcumin and UDCA against a plain control (table 28), no significant differences were seen whether viability, attachment, monolayer protein or any of the phase I or II assays were compared.

	MEDIA B			
CODE	VIABILITY (%)	HEPATOCYTES LOST AT THAW (%)	ATTACHMENT (%)	MONOLAYER PROTEIN (mg/M)
5296	16.9	92.2	100	0.190
5301	44.9	56.9	75	0.321
5371	40.5	9.7	10	0.301
6302	40.7	45.1	50	0.315
6244	27.5	72.9	75	0.284
6993	66.2	33.3	100	0.599
	CURCUMIN			
CODE	VIABILITY (%)	HEPATOCYTES LOST AT THAW (%)	ATTACHMENT (%)	MONOLAYER PROTEIN (mg/M)
5301	42.4	30.6	25	0.369
5371	31.1	8.3	25	0.318
6302	20.3	96.1	25	0.343
6244	51.5	38.9	50	0.402
6993	52.5	56.3	75	0.357
	ZVAD-fmk			
CODE	VIABILITY (%)	HEPATOCYTES LOST AT THAW (%)	ATTACHMENT (%)	MONOLAYER PROTEIN (mg/M)
6302	19.2	98.6	25	0.320
6244	35.8	55.6	50	0.254
6993	57.5	58.3	50	0.263
	APOPTAME			
CODE	VIABILITY (%)	HEPATOCYTES LOST AT THAW (%)	ATTACHMENT (%)	MONOLAYER PROTEIN (mg/M)
6302	36.7	56.9	50	0.311
6244	48.8	41.7	75	0.465
6993	72.1	35.4	75	0.315
	UDCA			
CODE	VIABILITY (%)	HEPATOCYTES LOST AT THAW (%)	ATTACHMENT (%)	MONOLAYER PROTEIN (mg/M)
6302	39.3	60.4	50	0.355
6244	55.7	31.9	50	0.340
6993	59.6	53.3	50	0.265

Table 28 Thaw data.

Using the Annexin-V-Fluos assay (table 29), the majority of hepatocytes were necrotic or dead, with fewest living hepatocytes. There was no clear pattern demonstrated over time for any of the four groups studied (living, dead, necrotic or apoptotic). Living hepatocyte count did not appear to correlate with measured viability.

			Living hepatocytes	Apoptotic hepatocytes	Necrotic hepatocytes	Dead hepatocytes
Donor	Agent	Time	% total			
5301	CURC	0	1.82	1.48	60.18	36.56
5301	CURC	60	9.92	23.42	55.18	9.88
5301	CURC	180	14.28	7.68	66.08	9.84
5301		0	12.5	4.2	48.06	30.16
5301		60	15.44	14.18	47.02	23.1
5301		180	18.94	21	47.38	8.9
5296		0	15.52	7.12	56.6	17.86
5296		60	8.36	4.68	58.94	31.22
5296		180	20.66	11.2	22.76	41.18
5371	CURC	0	21.94	10.28	38.48	28.58
5371	CURC	60	13.3	13.54	60.4	7.04
5371	CURC	180	7.22	7.1	70.38	11.78
5371		0	13.08	16.78	62.26	8.78
5371		60	11.08	12.56	62.92	6.92
5371		180	10.34	3.24	69.76	14.04
6244	APOP	0	49.94	3.62	32.22	10.08
6244	APOP	60	21.66	22.74	51.12	7.96
6244	APOP	180	12.3	22.84	49.38	6.78
6244	CURC	0	69.81	13.45	6.63	2.18
6244	CURC	180	47.46	27.4	3.74	3.6
6244	redo CURC	0	12.94	9.42	57.48	22.46
6244	redo CURC	60	11.18	23.02	65.56	7.72
6244	redo CURC	180	17.4	10	22.96	16.18
6244	UDCA	0	20.4	22.7	22.24	25.1
6244	UDCA	60	21.64	11.24	39.52	16.28
6244	UDCA	180	12.82	10.82	61.92	11.48
6244	ZVAD	0	42.66	10.02	33.34	6.64
6244	ZVAD	0	16.8	15.3	39.36	12.78
6244	ZVAD	60	15.26	17.52	58.34	11.04
6244	ZVAD	180	19.82	7.02	20.44	41.26
6244		0	18.36	17.02	23.52	41.48
6244		60	24.62	11.66	39.8	20.52
6244		180	9.9	13.04	63.72	5.66
6302	APOP	0	29.5	5.56	25.7	30.54
6302	APOP	60	22.42	5.02	39.06	15.48
6302	APOP	180	7.78	4.5	52.76	30.2
6302	CURC	0	29.56	4.44	23.42	34.86
6302	CURC	60	19.94	7.96	47.48	14.16
6302	CURC	180	12.96	4.84	41.94	29.52
6302	UDCA	0	28.94	5.16	47.48	16.22
6302	UDCA	60	14.62	19.3	24.72	30.32
6302	UDCA	180	8.76	6.64	56.86	22.86
6302	ZVAD	0	17.8	3.54	46.76	30.54
6302	ZVAD	60	16.06	5.48	56.16	13.5
6302	ZVAD	180	16.62	7.94	53.98	10.92
6302		0	15.98	2.18	45.6	32.56
6302		60	17.06	4.4	51.6	16.6
6302		180	12.6	7.6	57.76	12.68

Table 29 Annexin-V-Fluos assay data.

4.07.01 Statistics

The Kruskal-Wallis test was used to ensure equity of the hepatocyte populations and Chi-squared test to assess significance.

4.08 Porcine isolation variables and their effect on fresh culture and cryopreservation

Porcine hepatocytes cells did not cryopreserve well although fresh hepatocytes were comparable.

Group code	Condition	n
1	x1 wash, fresh culture	11
2	x1 wash, then Percoll® purification, fresh culture	11
3	x3 washes, fresh culture	16
4	Pre-incubated, fresh culture	10
5	x1 wash, then cryopreserved	11
6	x1 wash, Percoll® purification, then cryopreservation	11
7	x3 washes, then cryopreserved	16
8	Pre-incubated, then cryopreserved	9

Table 30 Key for porcine isolations.

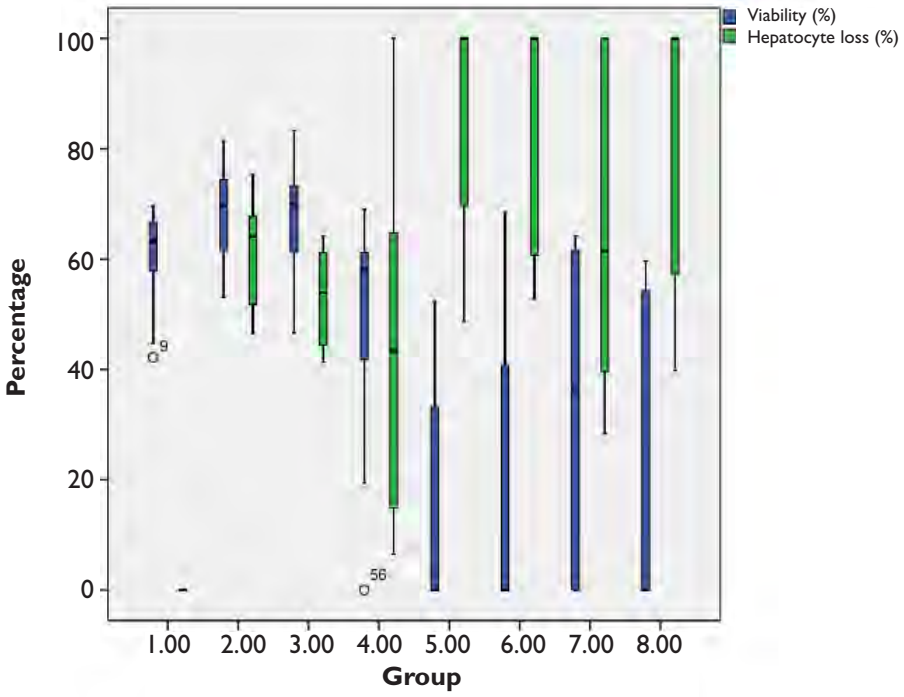


Figure 53 Viability and percentage lost.

There was significant deterioration in viability in all groups when cryopreserved and compared with their fresh equivalent in the x1 wash and Percoll® purification, x3 wash

(p<0.001) and even the fresh pre-incubation cells (p<0.01) (figure 53). Hepatocyte loss on thawing was significant in the x1 wash and x1 wash with Percoll® purification groups (p<0.001).

4.08.01 Metabolic studies

Group code	Condition
1	x1 wash, fresh culture
2	x1 wash, then Percoll® purification, fresh culture
3	x3 washes, fresh culture
4	Pre-incubated, fresh culture
5	x1 wash, then cryopreserved
6	x1 wash, Percoll® purification, then cryopreservation
7	x3 washes, then cryopreserved
8	Pre-incubated, then cryopreserved

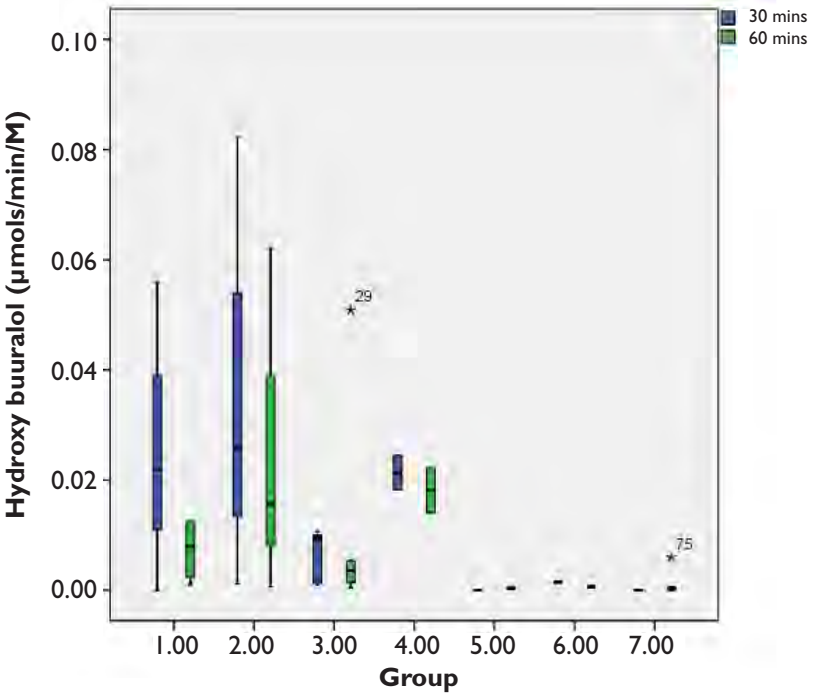


Figure 54 Bufuralol hydroxylation at 30 and 60 minutes.

There are statistically significant differences between the groups at 30 and 60 minutes (figures 54-55) with minimal activity in the cryopreserved hepatocytes (p<0.01).

Group code	Condition
1	x1 wash, fresh culture
2	x1 wash, then Percoll® purification, fresh culture
3	x3 washes, fresh culture
4	Pre-incubated, fresh culture
5	x1 wash, then cryopreserved
6	x1 wash, Percoll® purification, then cryopreservation
7	x3 washes, then cryopreserved
8	Pre-incubated, then cryopreserved

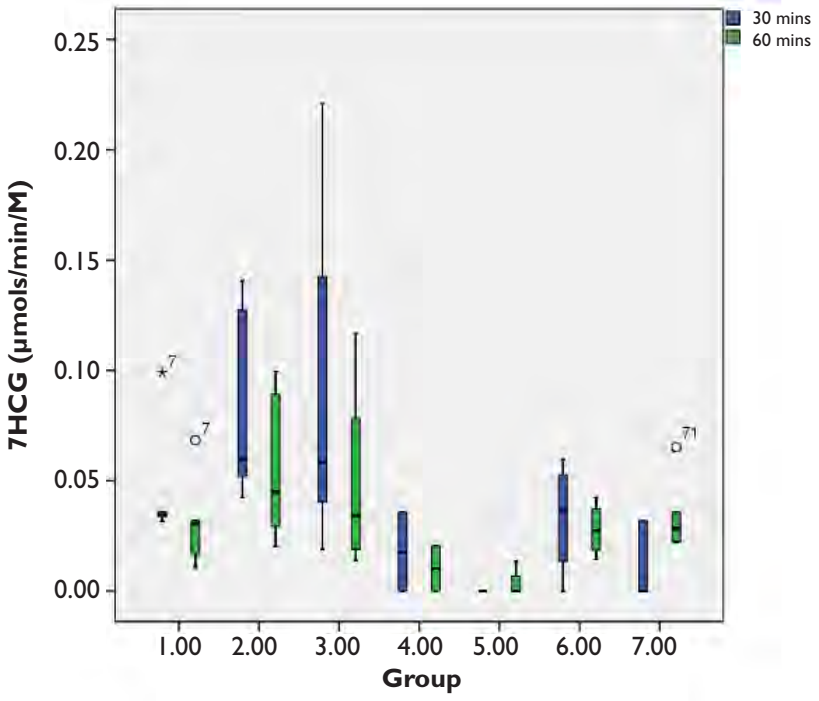


Figure 55 7HCG at 30 and 60 minutes.

There are subtle differences between all groups at 30 minutes ($p<0.005$) with no differences seen after this point. Cryopreserved hepatocytes have reduced activity.

Essentially there was no or minimal difference in CYP450 activity across the groups, at all time points. Importantly no preincubated cryopreserved cells survived the thaw process.

4.08.02 Statistics

The Chi-squared test was used to identify significance.

4.09 The affect of whole organ porcine liver perfusion on isolation outcome

Organ retrieval involved WIT of approximately five minutes (mean 5.4+1, 4-7.8). Extracorporeal perfusion of liver required transport of organ back to the perfusion laboratory, bench preparation of liver and priming of the circuit giving a CIT of about three hours (Bikhchandani *et al*. 2011).

The post-perfusion isolation was significantly better across all measurable endpoints other than total monolayer protein (table 31).

Table 31 Whole organ perfusion and hepatocyte isolation data.

Perfusion	Time started	Hours pump perfusion	Start weight (g)	Number cannulae	Perfusant	Volume perfusant (ml)	Perfusion time (mins)	Speed pump	Digestion time (mins)	Quality digest	Digest media
PRE	16:49	0	19	3	GIBCO	500	15	40	15	Excellent	GIBCO
POST	0:40	6	20	2	GIBCO	500	15	40	15	Excellent	GIBCO

Perfusion	Volume Digest (ml)	Weight digested (g)	Weight undigested (g)	% digested	Wash buffer	Spins (100G for 3 mins)	% viability
PRE	500	9	10	47.4	UKHTB	3	37.7
POST	500	14	6	70	UKHTB	3	76.6

Perfusion	Yield total hepatocytes	Yield viable hepatocytes	Yield total hepatocytes/g digested	Yield total hepatocytes/g	Yield viable hepatocytes/g digested	Yield viable hepatocytes/g	Day 1 monolayer total protein	Day 1 supernatant total protein	Day 1 visual monolayer %
PRE	1392.573	525	154.73	73.29	58.333	27.632	0.2	0.38	50
POST	1314.458	1006.875	93.89	65.723	71.92	50.343	0.172	0.036	90

	Testosterone				Diclofenac				Phenacetin			
	T0	T30	T60	T120	T0	T30	T60	T120	T0	T30	T60	T120
PRE	0	0	0	0	0	0.028		0	0	0	0.002	0.002
POST	0	0	0	0	0	0.174		0.018	0	0.029	0.016	0.004

	Bufuralol				7HCG				7HCS			
	T0	T30	T60	T120	T0	T30	T60	T120	T0	T30	T60	T120
PRE	0	0	0	0	0	0.22	0.11	0.023	0	0	0	0
POST	0	0	0	0	0	0.067	0.033	0.012	0	0.007	0.003	0.001

4.10 Summary

5 years of human donations to UKHTB

14 kg of liver is donated per year.

More than 33 billion viable hepatocytes are produced per year.

Human isolation variables and their effect on fresh culture and cryopreservation

PVP as an adjunct to DMSO gave superior results post-thaw.

All purification methods cause unacceptable hepatocyte loss that is compounded by cryopreservation.

Percoll® universally improves viability.

Pre-incubation confers no benefit and may cause harm.

All cells had CYP450 activity.

A viable hepatocyte will attach to collagen and have metabolic activity.

Fibrosis and Steatosis

There was a weak negative correlation between histological injury and isolation outcome.

Most scoring systems demonstrated a degree of cross correlation.

ECVAM prevalidation study

There was high between-donor variability in CYP450 activity.

Protocols were reproducible between laboratories.

There was no inter-laboratory variability.

Curcumin 'dual isolation'

Pre-incubation significantly reduced viability.

Curcumin had no effect on isolation outcome.

There was no difference in monolayer protein across the three groups.

Apoptosis studies

There was no difference in outcome irrespective of anti-apoptotic compound.

Apoptotic hepatocytes were seen less often than necrotic or dead cells.

Porcine isolation variables and their effect on fresh culture and cryopreservation

There was no reproducible difference across all groups.

Porcine hepatocytes were especially sensitive to pre-incubation with marked hepatocyte loss.

Porcine hepatocytes failed to cryopreserve well.

Microbiological contamination is a concern with slaughterhouse organs.

ECPLP and hepatocyte isolation

Normothermic resuscitation resulted in observable improvement across all criteria for a successful isolation.

4.11 Discussion

4.11.01 Human isolation variables

Fresh cell viability is higher than that of cryopreserved cells (Donini *et al*. 2001, Baccarani *et al*. 2005). Unlike other studies (Terry *et al*. 2005), pre-incubation failed to confer benefit to hepatocytes post-thaw, rather reducing viability and increasing hepatocyte loss. Experience with spinner flasks was observational, with this procedure leading to hepatocyte clumping and spontaneous spheroid formation, with subsequent measurement of hepatocyte viability and number impossible (Darr & Hubel 2001). Thus groups could not be fairly compared and this method of pre-incubation was not used.

Percoll® purification caused cell loss with significant viability gain on many occasions (6.27M to 2.57M viable hepatocytes/g, 60% cell loss) compatible with other groups (Vondran *et al*. 2007). There was insignificant difference across most groups with regard to CYP450 activity and attachment, confirming attachment as an accurate marker of function (Richert *et al*. 2004, LeCluyse *et al*. 2005).

Human tissue has inter-donor variability impossible to control for. Underlying pathologies vary, as do surgical procedures and peri-operative tissue insult such as duration of Pringle manoeuvre. Regarding the latter point, data was not even available for every specimen used. Each piece of liver had different macroscopic and therefore microscopic anatomy, pertaining especially to the vasculature. Cannulation and thus the extent of perfusion varied between isolations, both in terms of number and size of cannulae and subsequent adequacy of perfusion. Patchy or incomplete perfusion depended on vasculature and could not be controlled for.

Soltran (hyperosmolar citrate) was used with all living donor tissue for reasons of cost

and historical methodology. This is not used in the field of transplantation and could have conferred additional injury to the ischaemic tissue, especially when stored for prolonged time periods prior to isolation.

Collagenase and collagenase preparations are well known to vary between batches with degradation seen over time, potentially resulting in between-isolation variability. Using a pre-prepared digest media also removes any in-house quality control. Contamination may also be seen, although primarily efficacy is affected.

Where possible dual samples were taken and a mean obtained; this was not always possible either due to constraints of finance or practicality.

4.11.02 Fibrosis and steatosis

No two donors produce identical hepatocytes, with no useful evidence suggesting any donor should be excluded from donation to research (Alexandre *et al*. 2002, Vondran *et al*. 2007). One large pool of potentially useable organs is non-transplantable livers and lobes (those not used following downsizing of an organ for paediatric transplantation). One concern about these organs is the reason for exclusion from the transplant program and the assumption that these are all steatotic, fibrotic and cirrhotic, thus worthless for isolation (Gerlach *et al*. 2003, Barbich *et al*. 2004).

One observation seen during hepatocyte isolation is that tissues with the best perfusion yield the most hepatocytes, with optimal digestion. This is an expected finding as maximal collagenase activity can occur only when it is maximally perfused throughout the tissue. Based on observation, it appeared that tissue that felt fibrotic produced fewer hepatocytes at worse viabilities. This would affect the use of multi-organ donor tissue as it would be of lower quality.

There is however minimal actual evidence correlating histological findings analysed

by a pathologist and isolation outcome. Histological grading is challenging, both maintaining individual accuracy and minimising between-observer error. In this study, data was often assessed by a solitary histopathologist only, potentially resulting in inaccuracy. Secondly, samples for analysis were often taken from liver proximal to diseased tissue. This could result in histological abnormalities being detected that are not representative of the whole organ. Despite this, the histology would be fairly characteristic of the tissue used for hepatocyte isolation as the tissue used was always proximal to the diseased tissue.

In this study, across all groups it was expected that fibrotic tissue, with an element of pre-existing chronic disease would yield fewer hepatocytes. There was a negative correlation between all scores for liver injury and isolation outcome although this was so weak one could not justify using these scores to determine the value of undertaking hepatocyte isolation.

It is interesting to note that the liver injury score and fibrosis scores do not correlate well, unlike liver injury score and NASH and Ishak and fibrosis scores (moderate correlation in the latter). NASH and fibrosis score bear no relationship, justified as they measure disparate pathological processes. The association between Ishak and NASH and fibrosis scores is weak if indeed truly present and this could be explained by the specimens being taken in proximity to the diseased tissue in most cases.

4.11.03 ECVAM prevalidation study

High between-donor variability in the attachment rate of viable seeded hepatocytes was observed, measured by mean microsomal protein/dish for control cultures. CYP-dependent activities after culture are in the range of those previously described for CYP1A2-dependent 7-ethoxyresorufin O-deethylation, CYP3A4/5-dependent testosterone

6 -hydroxylation (Madan *et al*. 2003, Parkinson *et al*. 2004) and CYP2B6-dependent bupropion hydroxylation (Wang *et al*. 2003).

The effect of BNF on CYP1A2-dependent activity was highly significant, with good between-laboratory reproducibility of the concentration-dependent response. This corresponds with previous reports (LeCluyse *et al*. 2000, Madan *et al*. 2003). In accordance with our results, RIF has reproducibly induced CYP3A4/5 in primary human hepatocyte cultures (Madan *et al*. 2003).

The effects of PB, RIF and BNF on CYP2B6 dependent activity were all significant. This improved activity was associated with increased protein content, confirming the induction of this specific CYP450 as observed elsewhere (Madan *et al*. 2003). No inducing effects by RIF or BNF were observed in Strasbourg. A laboratory effect cannot be excluded but this is probably a donor-dependent response to inducers.

The maximum response of a CYP450 to a specific inducer was always observed with the highest concentration tested in accordance with other work (Faucette *et al*. 2004).

4.11.04 Curcumin 'dual isolation'

The benefit of 'dual isolations' is that tissue from a single donor, of similar size and macroscopic anatomy can be used for hepatocyte isolation either concurrently or in sequence to assess a question whilst removing the inter-donor variability that we have seen (Richert *et al*. 2010). It must be accepted that there will always be differences between the tissues but this experimental method can give a more reliable control and thus reduce the number of isolations required for a meaningful result.

All hepatocytes attached as efficiently as each other and therefore it can be safely assumed they would function in a roughly similar fashion (Richert *et al*. 2004, LeCluyse

et al. 2005). This is relevant as the important issues become hepatocyte loss with prolonged purification and resuscitation procedures. If hepatocytes are lost without gain, as seen in these experiments then the argument for undergoing these steps is weakened.

Equally, the failure for curcumin to demonstrate any effect in this study may confirm that this dose is in the mid-point of its biphasic behaviour, neither demonstrating benefit nor harm.

4.11.05 Apoptosis studies

For any potentially pro- or anti-apoptotic agent there is a group that have established or contradicted its effects. All four agents used potentially affected the mitochondrial pathway, either by direct caspase inhibition or inhibition of ROS. It is accepted that compensatory mechanisms may circumvent the beneficial effect of caspase inhibition (Zheng *et al*. 2000). Equally, inhibiting caspases may induce oxidative stress, thus rendering any positive effects neutralised (Cauwels *et al*. 2003).

These unremarkable results are probably a result of small study number, although this is not uncommon with human cellular research. Equally, the Annexin-V-Fluos data is suboptimal, with cell populations overlapping. This data suggests most post-thaw hepatocytes are injured, yet CYP450 data suggests there is moderate activity with monolayer protein comparable across all groups.

One significant criticism of these results is the absence of a control fresh hepatocyte population sample looking at cytotoxic injury immediately post-isolation. Many of the isolations took place out of office hours and as such the necessary equipment was not available. With these values it would be possible to differentiate between cryopreservation related injury and any abnormality with the initial hepatocyte population.

As the total number of cryopreserved hepatocytes was low with each condition, solitary

rather than multiple readings were obtained, also potentially compromising their value.

4.11.06 Porcine isolation variables

The initial five porcine isolations were all grossly infected on day one of culture thus discarded and none of these cryopreserved hepatocytes survived thaw. Laboratory contamination was excluded and to minimise contamination, the sequential porcine perfusion buffers were exchanged for Invitrogen hepatocyte perfusion and digestion media. This also brought the human and porcine protocols into line. Simultaneously it was decided that instead of procuring whole liver, a lobe would be taken to minimise abattoir contamination. This latter step was seen to improve infection rates.

Cryopreserved porcine hepatocytes have altered enzyme function (de Souza *et al*. 1991) and in contrast to many reports they appear more sensitive to cryopreservation than human hepatocytes (Lawrence & Benford 1991, Adams *et al*. 1995). Certainly porcine hepatocytes were easily obtained from abattoir organs (Donini *et al*. 1997) although at lower viability and yield than in some reports (Sielaff *et al*. 1995).

4.11.07 ECPLP and hepatocyte isolation

The only negative result post-normothermic perfusion was a lower monolayer protein value at 6 hours following fresh cell culture. It is difficult to explain this in view of the other observed differences as this would imply reduced cell attachment. However severe the IR injury to this tissue has been, even six hours of resuscitation results in improved cellular outcome and clearly ECPLP requires further assessment. No other groups have reported pre- and post- perfusion hepatocyte isolations although one has compared cold perfusion to ECMO, with subsequent hepatocyte isolation. Here ECMO also appeared to confer viability and attachment benefit, without mention of yield (Noormohamed *et al*. 2010).

Having demonstrated inter-donor variability elsewhere, using the donor as its own control is an attractive prospect.

As with whole organs for transplantation purposes, resuscitation may allow a greater pool of organs to be used for all purposes, reducing waste and improving marginal tissue to transplant, whole organ *ex vivo* perfusion or producing hepatocytes for research and BAL development (St Peter *et al*. 2002, Reddy *et al*. 2009).

4.12 Implications

Hepatocyte isolation is time efficient but economically draining. Large scale laboratories are in a better position to maximise output from tissue without waste. Centralisation of services makes live donor tissue more readily available and easier to co-ordinate, whilst improved awareness by transplant co-ordinators may increase MOD tissue availability. Organs declined for transplantation should be used for cell isolation with vastly prolonged ischaemia times only affecting that decision. Human hepatocytes are a readily available resource and should be used for all primary hepatocyte culture whether for drug or BAL development.

Superior viable hepatocyte yield is achieved with minimal purification (x1 wash only) and as such extraction of debris and red blood cells is better achieved by avoiding recirculation of perfusion media. Although viabilities are lower than following further purification, the viable hepatocytes attach and function, therefore outcome is maximised. In the context of a BAL, the cells will be a co-culture of parenchymal cells and may actually be advantageous in appropriate culture conditions. Across all groups, a viable hepatocyte is an attached and functional cell.

The digestion phase of the isolation process requires Glisson's capsule to be recreated such that there is no escape of collagenase. Glue is an easy way of achieving this.

Pre-incubation is a reasonable premise, potentially allowing resuscitation prior to cryopreservation, but in reality appears to reduce viability and hepatocyte yield and therefore cannot be encouraged.

Anti-apoptotic agents in larger studies may confer benefit, but do not appear to have great significance. Successful storage technology remains elusive and tissue banks must advance this science to permit reproducible good recovery and viability. Using DMSO

alone as a single cryoprotectant is outmoded and compounds such as PVP clearly give improved outcomes. Larger scale laboratories with greater tissue turnover are the only way of developing this service.

4.13 Future work

Performing hepatocyte isolations within the setting of a tissue bank meant the focus was high viability hepatocytes potentially at the expense of hepatocyte number per isolation. The benefit of hindsight demonstrates this attitude slows advancement of the process of hepatocyte isolation and is potentially deleterious in the longer term. Future work in this field should occur within large tissue banks with research interests and technical experience that minimises the number of hepatocytes required for experimental work.

Ideally all work should be duplicated to allow validation of results, limitations on equipment availability resolved and fresh cultured hepatocytes should always be available for each cryopreserved condition to act as a control. Experimental number is always limited with human tissue work, but where possible this should be increased, to allow for statistically significant outcomes.

ECPLP is a relatively new area with tremendous scope for development, whether the end purpose is whole organ, lobar or cell transplantation or BAL development. Certainly the effect of perfusion on hepatocyte biology is a fascinating field worthy of further study. With establishment of porcine models, the next step in the development of this model would be to use human livers turned down for transplantation.

4.14 Completing the cycle

It is interesting that although in excess of $50 million has been spent, a BAL has yet to confer clinical benefit (Selden & Hodgson 2004) and the notion that one day a financially viable and functional bioartificial liver will be available to the general population within any health care unit is hopeful but entirely unrealistic. In terms of availability to tertiary referral centres again this is an optimistic notion, based largely on desire and wishful thinking rather than reality.

BAL development over the last ten years has in reality advanced very little and transplantation remains the gold standard treatment of ALF. The initial studies have only led to a small number of randomised, controlled trials generating level 1 evidence. None have been published since 2007 (Hassanein *et al*. 2007) and this a MARS study. ELAD and HepatAssist remain the only BAL to have undergone randomised, controlled studies (Ellis et al. 1996, Demetriou *et al*. 2004). Reviews and meta-analyses predominate (Stutchfield *et al*. 2011), with artificial livers (MARS) the only mainstream liver support still discussed (Banares *et al*. 2010). Tissue engineering and 3D scaffolds remain of interest although the technology to transform this into realistic liver replacement therapy is no closer.

Stem cell research is the future if a BAL is ever to be established (Muraca *et al*. 2010) although cell or gene therapies directly delivered to the patient may be a more cost effective alternative.

Bioreactor design is another debateable issue. On the most basic level, whether it should be a bioreactor at all. Thereafter, there are issues of dialysis perfusate, filtration and hepatocyte configuration/type/co-culture, whether the reactor or its component parts should be reusable and the list goes on.

Trials have been performed historically with bioartificial systems so a ‘successful’

hepatic replacement therapy randomised trial would not be a true precedent but as previous therapies have failed to prove themselves this is a long way off.

Cost clearly is extortionate, both in terms of the research prior to trial work, trials themselves and subsequent acceptance of the model as a therapeutic agent. In the UK, NICE guidelines determine resource allocation for some of the more expensive therapies available within the NHS. Acceptance of such a therapy on anything other than an experimental basis seems unlikely in the current political climate, without very significant demonstration of positive and economically beneficial outcomes. Unfortunately for the proponents of these systems, there is currently little financial backing for the multi-million pound development of such a bioreactor.

Hepatocyte transplantation is promising as it carries relatively few postoperative complications when compared with whole organ transplant (Funaki *et al*. 2002). This is a developing field and this may be a more sensible route of investigation, accepting immunosuppression to allow function of the transplant. The burden of medical therapy may be less significant than with whole organ transplant with the hepatocytes acting as an *in situ* bridge to recovery in the group of patients with potential for regeneration and recovery.

The two dimensional and static nature of monolayer culture does not reflect the structure of an intact liver. The major obstacles to overcome with hepatocyte culture models is loss of differentiated phenotype which requires manipulation of co-culture, media and extracellular matrix to produce the best cells. Bio-reactors strive to achieve the sinusoidal architecture of hepatic tissue. This being the case, a whole organ may provide better liver support.

Ex vivo perfusion of a functioning organ is more attractive and certainly less expensive, but the caveats of the use of transplantable organs exist, with cross-contamination concerns

(if porcine organs were used or an organ used for more than one recipient). Additionally here, issues arise regarding the requirement for immunosuppression whilst the treatment is in use although this may be no higher risk than using a BAL. Porcine models are being developed to maintain organs for transplantation, with non-transplanted organs a potential future source of tissue.

Successful hepatocyte preparation is technically quite difficult and still remains largely an art

Seglen 1976

Appendices

Study Number: 5960

PATIENT INFORMATION SHEET

The development of a bioartifical liver.

Principle investigator: Mr Ashley Dennison, FRCS, MD
You may contact: Mr Ashley Dennison, Department of Surgery, Leicester General Hospital, Leicester, LE5 4PW

Investigators: Mr David Berry, FRCS, MD
Dr Clare Pattenden, MB ChB
Dr Severine Illouz, PhD

We would like to invite you to participate in the above named study that is being carried out at Leicester General Hospital.

Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

Thank you for reading this.

What is the purpose of the study?

We would like to ask you to consider allowing some of the liver tissue removed at your operation to be used for medical research.

Researchers in universities and hospitals use human liver tissue to try to find better ways of treating liver disease that affect many people. Liver tissue may be used to discover better ways of treating cancer patients or patients with other serious liver disease.

Our project is looking at ways of keeping liver cells alive and how these cells can be used to develop a bioartificial liver/liver dialysis machine.

The type of operation you are having means that tissue donation may be possible.

Do I have to take part?

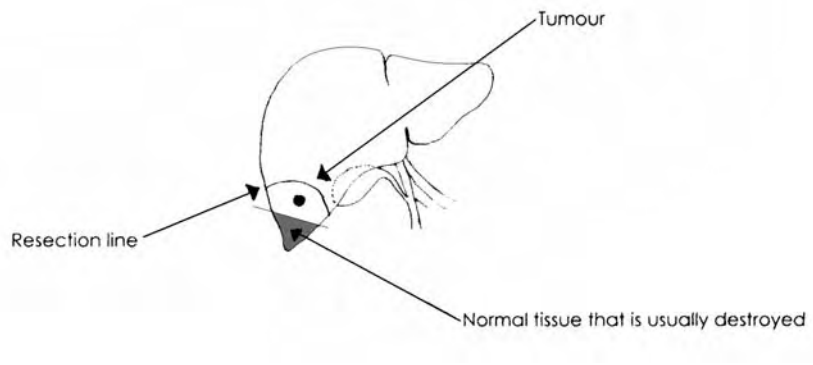
It is up to you to decide whether or not to take part. If you do decide to take part you will be asked to sign a consent form. You will be given a copy of this information sheet, a signed consent form and a copy of the information collected about you to keep.

If you decide to take part you are still free to withdraw at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive.

Version two, 04/04/04

What will be involved if I take part in the study?

Based on the results of a number of medical tests you have undergone, it has been determined that you have a tumour(s) in your liver and that the best clinical



treatment for you is to have an operation to remove the cancer from your liver. Your test results have shown that the cancer is in a part of the liver where it can be removed (see diagram).

Once the tissue has been removed, a small piece from the edge of the tumour will be taken for tests to ensure that all the diseased tissue has been removed, while the rest of the tissue would normally be destroyed.

This tissue that is normally destroyed, could instead be used for medical research purposes. Only the tissue that would otherwise be destroyed will be used.

If you agree to allow the use of your liver tissue in this project the surgeon will perform the operation in exactly the same way as if you had not consented for this to happen. No extra tissue will be taken from your liver. You will not be requested to undergo any other additional tests as part of the project.

What are the possible disadvantages and risks of taking part?

None. Your treatment, diagnosis and care will be the same.

What are the possible benefits of taking part?

The information we get from this study may help us to treat future patients needing liver surgery or with liver disease in the future. You will receive no direct benefit.

What if something goes wrong?

If you are harmed by taking part in this research project, there are no special compensation arrangements. If you are harmed due to someone's negligence, then you may have grounds for a legal action but you may have to pay for it. Regardless of this, if you wish to complain, or have any concerns about any aspect of the way you have been approached or treated during the course of this study, the normal National Health Service complaints mechanisms are available to you.

Version two, 04/04/04

Will my taking part in this study be kept confidential?

Inclusion into the study requires consent for the use of tissue which would otherwise be destroyed. All information which is collected about you during the course of the research will be kept strictly confidential. No record of patient name is kept. We do ask for some information which is important for the functional studies of the liver cells. You cannot be recognised from any information we obtain and you will be given a copy of this data.

In accordance with all medical consultations, any discussion with you relating to this study will be kept confidential.

What will happen to the results of the research study?

We intend on publishing the results of our research. As information obtained from you is anonymous, you will not be identified in any report or publication.

Who is organising and funding the research?

The Hepatobiliary and Pancreatic Surgery Department is sponsoring this research.

Your doctor is not being paid for including you in this study.

Version two, 04/04/04

Version two, 04/04/04

Study Number: 5960
Patient Identification Number for project:

PATIENT CONSENT FORM

The development of a bioartifical liver.

Principle investigator: Mr Ashley Dennison, FRCS, MD
You may contact: Mr Ashley Dennison, Department of Surgery, Leicester General Hospital, Leicester, LE5 4PW

Investigators: Mr David Berry, FRCS, MD
Dr Clare Pattenden, MB ChB
Dr Severine Illouz, PhD

Please tick box

1. I confirm that I have read and understand the information sheet dated 4th April 2004 (version 2) for the above study and have had the opportunity to ask questions. O

2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. I understand how the donation will be collected and that giving a donation for this research is voluntary. O

3. I understand that sections of any of my medical notes may be looked at by medically qualified researchers involved in this project or from regulatory authorities where it is relevant to my taking part in research. I give permission for these individuals to have access to my records. O

4. I agree to take part in the above study. O

_____	_____	_____
Name of Patient	Date	Signature

I confirm I have explained the nature of this project, as described in the Patient Information Sheet, in terms which in my judgement are suited to the understanding of the patient.

_____	_____	_____
Researcher	Date	Signature

1 for patient; 1 for researcher; 1 for medical notes

Version two, 04/04/04

Reagents and equipment

Reagent and equipment	Supplier
0.3 ml crimp top fixed insert vials	Chromacol
11 mm aluminium crimp cap type 7 rubber/PTFE	Chromacol
14-18 G cannulae	NHS logistics
2 ml crimp top vial/ 11 mm crimp cap type 7 rubber/PTFE liner combination	Chromacol
6 -hydroxytestosterone	Sigma
7-hydroxycoumarin	Sigma
7-hydroxycoumarin glucuronide	Sigma
7-hydroxycoumarin sulphate	Sigma
Acetonitrile 190 FAR UV	Rathburn Chemicals
Annexin-V-Fluos	Roche
Apoptag® Peroxidase In Situ apoptosis detection kit	Chemicon International
Apoptame	MP Biomedicals
Arthroscopy set	Medi Plus
Ascorbic acid	Sigma
BCA assay	Sigma
BD FACSCalibur	Becton Dickinson
BD Falcon 12 well plates	BD Bioscience
BD Falcon 96 well plates	BD Bioscience
BCA assay	Sigma
Bilirubin	Sigma
Bio-console 560 speed controller	Medtronic
BioMedicus BP560 centrifugal pump	Medtronic
BSA	Sigma
Bufuralol	Sigma
Calcium chloride	Sigma
Caspase-3 activity assay	Roche
Cefuroxime	Sigma
Cell death detection ELISA plus	Roche
Clinimix	Baxters
Collagenase	Sigma
Cryogenic vials 2.0 ml	Corning
Curcumin	Sigma
Dexamethasone	Sigma
D-Fructose	Sigma
Diclofenac	Sigma
Diclofenac	Sigma
D-MEM high glucose	Gibco
DMEM powder	Sigma
DMSO	Sigma
Dulbecco's PBS 10x	Gibco
Dulbecco's PBS 1x	Gibco
Dulbecco's PBS 1x	Gibco
EDTA	Sigma
EGTA	Sigma
Enzcheck caspase-3 assay kit #1	In Vitrogen
Ethanol	Sigma
Evacure EC4A	Kuraray Inc.
FBS	In Vitrogen
FCS	Sigma
Flolan	GlaxoSmithKlein
Fungizone	In Vitrogen
GD120 water bath	Grant instruments
Gentamicin	In Vitrogen

Reagents and equipment cont.

Reagent and equipment	Supplier
Haematocytometer	Sigma
HBSS x10	In Vitrogen
Heparin	Sigma
Hepatozyme-SFM	Gibco
HEPES sodium salt	Sigma
HMM	Cambrex
Human albumin fraction V	Sigma
Hydroxybufuralol	Sigma
Hydroxydiclofenac	Sigma
Instant adhesive	Loctite
Insulin	Sigma
Isopropanol	Sigma
Liver Digest Media	In Vitrogen
Liver Perfusion Media	In Vitrogen
Medtronic Bioprobe TX50P paediatric flow transducer	Medtronic
Medtronic external drive unit 540-T motor head	Medtronic
Medtronic MinimaxTM 138 oxygenator	Medtronic
Medtronic MVR800 soft shell reservoir	Medtronic
Methanol	Sigma
Methanol 99.8% for HPLC	Fisher Scientific
Mr Frosty	Sigma
MTT	Sigma
Multi-Skam Ex spectrophotometer	Labsystems
Paracetamol	Sigma
Penicillin	Sigma
Perchloric acid 60%	Fisher Scientific
Percoll®	Sigma
Phenacetin	Sigma
Phosphoric acid solution	Sigma
PI	Roche
Polyvinylpyrrolidone K25	Fluka
Potassium chloride	Sigma
Ringer's lactate	Baxters
Roller Pump	Watson Marlow
Sieve: 1000 µm 200 mm diameter	Christianson Scientific
Sieve: 100 µm 200 mm diameter	Christianson Scientific
Sieve: 500 µm 200 mm diameter	Christianson Scientific
Sodium bicarbonate 7.5%	In Vitrogen
Sodium chloride	Sigma
Sodium hydroxide	Sigma
Sodium perchlorate	Sigma
Sodium taurocholate	Sigma
Soltran kidney perfusion perfusion solution	Baxters
Streptomycin	Sigma
Testosterone	Sigma
Tetrabutyl ammonium phosphate	Sigma
Triethylamine	Sigma
Trigene	Medichem
Trypan blue	Sigma
Ursodeoxycholic acid	Sigma
Viaspan	Bristol-Myers Squibb
Vybrant apoptosis assay kit #1	In Vitrogen
William's E	Gibco
Z-VAD-fmk	MP Biomedicals

Solutions

Solution	Contents
AB-AM	Penicillin 100 U/ml Streptomycin 100 µg/ml Fungizone 0.25 µg/ml
Culture media	500 ml HMM 5 ml AB-AM (100X)
Curcumin stock solution 5 mM	36.8 mg Curcumin (MW 368.39) 20 ml pure filtered (0.2 micro-filtered) ethanol Dual isolation protocol: 2.5 ml of this stock solution should be added to each 500 ml solution giving a 2525 µM final curcumin concentration
Curcumin working solution 20 µM	20 µl stock solution in 5ml pure filtered ethanol
Dual isolation attachment/suspension medium	(4°C) 500 ml DMEM 25 ml FBS 16 µl insulin 100 IU/ml (equivalent to 4 mg/ml) 50 µl of 10 mM stock dexamethasone 5 ml AB-AM (100X) DMEM, 5% FBS, 4 µg/ml insulin, lµM dexamethasone, AB-AM (penicillin 100 U/ml, streptomycin 100 µg/ml, Fungizone 0.25 µg/ml)
Dual isolation HBSS	(4°C) 200 ml HBSS l0x 1800 ml sterile purified water 10 ml sodium bicarbonate 7.5% solution
ECVAM digestion media	DMEM 0.5% w/v BSA 50 µg/ml Ascorbic acid 0.05% w/v collagenase type IV (500 mg/l) Filtered through polysulphone 0.2 µm filter
ECVAM perfusion media	HBSS 0.5 mM EDTA (208.1 mg/l) 0.5% w/v BSA Filtered through polysulphone 0.2
FACS scan dye	20 µl Annexin V solution 20 µl PI stain 960 µl buffer
Freezing Medium A	(-20°C) 100 ml DMEM 6.25 g BSA 5 g PVP (POLYVINYLPYRROLIDONE K25) 5 ml AB-AM (100X) (DMEM, 6.25%w/v BSA, 5%w/v PVP, l% AB-AM) Spin until PVP dissolved then filter. Store at -20°C

Solutions cont.

Solution	Contents
Freezing Medium B	(4°C) 10 ml Medium A 10 ml UW 10 ml FBS 5 ml filtered DMSO (DMEM, 2.5% BSA, 2% PVP, 28.5% UW, 28.5% FBS, 14.3% DMSO, 0.3% AB-AM)
Hepatocyte suspension media	4.4 g $NaHCO_3$ 4.68 g Hepes 4 g HAS fraction V 0.8 g D-Fructose DMEM powder equivalent x2 Make up to 2l with Mili-Q water Adjust pH to 7.4 with 1M HCl
Hepatocyte wash buffer	NaCl 120 mM KCl 6.2 mM $CaCl_2$ 0.9 mM Hepes 10 mM Albumin 0.2% Distilled water 5 l Adjust to pH 7.4 Filter (0.22 µl) under vacuum suction in a Biosafety Class II cabinet
Krebs	5.3 l water Sodium Chloride 79.72 g Potassium Chloride 2.05 g Sodium Phosphate 1.90 g Sodium Bicarbonate 24.03 g Calcium Chloride 14.7 g in 100 ml water Magnesium sulphate 24.65 g in 100 ml water
MTT	Dissolve 125 mg MTT in 5 ml HBSS to obtain a final concentration of 5 mg/ml. Sonicate solution and filter. Store at -20°C for 1 month
Organic solvent for MTT test	Mix 15 ml DMSO with 15 ml Ethanol to give a 1:1 solution. Store at room temperature
Percoll® (90% isotonic solution)	(4°C) 180 ml Percoll® 20 ml PBS 1x Ratio Percoll®:hepatocyte solution 3:1 for centrifugation

Solutions cont.

Solution	Contents
Porcine digestion buffer	Porcine stock buffer 100 ml $CaCl_2$ 5 mM (1.08 g) Collagenase 500 mg Add this solution to a further 700 ml porcine stock buffer. Filter (0.22 µl) under vacuum suction in a Biosafety Class II cabinet.
Porcine perfusion buffer I	Porcine stock buffer 500 ml EGTA 1 mM Dexamethasone 20 mg (Dexamethasone dissolved first in a small volume of methanol) Filter (0.22 µl) under vacuum suction in a Biosafety Class II cabinet.
Porcine perfusion buffer II	Porcine stock buffer 500 ml EGTA 1 mM Filter (0.22 µl) under vacuum suction in a Biosafety Class II cabinet.
Porcine perfusion buffer III	Porcine stock buffer 100 ml Filter (0.22 µl) under vacuum suction in a Biosafety Class II cabinet.
Porcine stock buffer	Ionised water 5 l Sodium Chloride (NaCl) 154 mM Potassium Chloride (KCl) 5.6 mM Glucose 5 mM Sodium Bicarbonate ($NaHCO_3$) 25 mM Hepes 20 mM Adjust to pH 7.4 Filter (0.22 µl) under vacuum suction in a Biosafety Class II cabinet.
UDCA stock solution 100mM	39.25 mg UDCA 1ml pure filtered (0.2 micro-filtered) ethanol Dual isolation protocol: 2.5 ml of this stock solution should be added to each 500 ml solution giving a 25 µM final curcumin concentration
UDCA working solution 400µM	20 µl stock solution in 5 ml pure filtered ethanol
ZVAD stock solution	Dissolve 1 mg of ZVAD-FMK in 214 µl DMSO (10 mM solution). 2 µl/1 ml media used to give a final 20 µM concentration. Store at -20°C

HPLC conditions for analysis of metabolite production

TESTOSTERONE 6 -HYDROXYLASE

Column:	Hypersil 5 C18 15x4.6 mm
Temperature:	45°C
Mobile phase:	55% Methanol: 45% water
	0.05% phosphoric acid (500 µl/l mobile phase)

Isocratic

Detection:	UV 254 nm
Flow:	1.5 ml/min

(Note: warm column at 45°C at flow of 1 ml/min to avoid high pressure)

Testosterone RT approx. 8 mins
6B hydroxyl-testosterone RT approx. 3 mins

DICLOFENAC 4'-HYDROXYLASE METHOD

Column:	Hypersil 5 C18 250x4.6 mm
Temperature:	45°C
Mobile phase A:	50 Mm Sodium perchlorate
	pH 2.5 with perchloric acid (60%)
	40% acetonitrile
Mobile phase B:	Methanol
Gradient	0 70% A
	20 100% B
Flow rate:	1 ml/min
Detection:	280 nm

PHENACETIN O-DEETHYLASE METHOD

Column:	Agilen Zorbax xdb C18 250x4.6 mm
Temperature:	45°C
Mobile phase A:	0.1 N phosphoric acid
	(6.4 ml conc. phosphoric acid in 1 l water)
Mobile phase B:	Acetonitrile
Gradient	0 90% A
	2 90% A
	4 75% A
	6 75% A
	7 90% A
	10 90% A
Flow rate:	1.5 ml/min
Detection:	244 nm

Phenacetin RT approx. 5 mins
Paracetamol RT approx. 2 mins

HPLC conditions for analysis of metabolite production cont.

BUFURALOL 1'-HYDROXYLASE ASSAY

Column:	Agilent zorbax XDB C8 column
Temperature:	45°C
Mobile phase:	60% 50 mM Sodium Perchlorate
	(7 g sodium perchlorate in 1 l water)
	pH 2.5 with perchloric acid (60%)
	40% acetonitrile
Flow rate:	1.5 ml/min
Detection:	Fluorescence excitation 252 nm
	emission 302 nm

7-HYDROXYCOUMARIN GLUCONURIDATION AND SULPHATION

Column:	Supelco LC8 3.3 cm	
Temperature:	45°C	
Mobile phase A:	900 ml 0.25% acetic acid	
	1.7 g/l tetrabutyl ammonium phosphate	
	100 ml acetonitrile	
	pH 4.7 with sodium hydroxide	
Mobile phase B:	500 ml 0.25% acetic acid	
	1.7 g/l tetrabutyl ammonium phosphate	
	500 ml acetonitrile	
	pH 4.7 with sodium hydroxide	
Flow rate:	1.5 ml/min	
Gradient	0	15% B
	2	15% B
	7	73% B
	7.1	15% B
	10	15% B

7HC glucuronide RT approx. 1.5 mins
7HC RT approx. 2 mins
7HC sulphate RT approx. 6 mins
Standards will only last for one week (deterioration is significant as 7HCG has the same retention as 7HC).

Substances added to the perfusate during ECPLP

One time boluses	
Flolan	20 ml (500 µg in 50 ml of diluent, diluted with 200 ml 0.9% saline)
Sodium Bicarbonate (8.4%)	40 ml
Cefuroxime	750 mg in 5 ml of water
Calcium Chloride	10 ml
Scheduled boluses	
Heparin	1000 units/hr
Clinimix (aminoacids only)	15 ml/hr
Infusions	
Sodium Bicarbonate (8.4%)	40 ml/hr
Flolan	20 ml/hr (500 µg in 50 ml of diluent, diluted with 200 ml of N/S)
Sodium taurocholate	10 ml/hr (2% w/v, 2 g in 100 ml 0.9% saline): 200 mg/hr
Insulin	50 U/hr

Audit and Governance

Logistics of using human tissue for research

Materials and Methods:

All patients potentially undergoing liver resection between October 2003-2004 were identified pre-operatively and consent for tissue donation requested from all conforming to LREC guidelines (n=121). Data was collected prospectively and included demographics, outcome from surgery and outcome of each donation.

Results:

During the first twelve months of this thesis (October 2003-4) 121 patients were identified as potentially undergoing hepatic resection, of whom 105 could be approached according to LREC guidelines. Of these, 104 (99%) patients agreed to donate surplus surgically resected tissue. Following surgery, 44 patients (42%) yielded specimens with enough normal tissue to perform hepatocyte isolation.

Tissue mass ranged between 32-515 g (mean 159), the number of viable cells produced between 28-5491M (mean 614) and viabilities between 53-95% (mean 77).

A total of 6316 g of tissue in total was donated, with isolations yielding an average of 4M viable cells per gram of tissue

Summary:

The vast majority of surgical patients consent for tissue donation for research. Greater than 500 billion viable hepatocytes could be produced if all units donated surplus tissue.

Discussion:

Reorganization of hepato-pancreato-biliary services would currently require 12 centres nationwide serving populations of approximately 5M (BSG 2001). If all units used their

surplus surgical tissue this would potentially yield in excess of 150 kg of tissue per year, with greater than 500 billion viable hepatocytes.

UK transplant co-ordinators experience of tissue donation for research

Materials and Methods:

157 transplant and tissue co-ordinators in the United Kingdom were identified from UK Transplant. Each co-ordinator regardless of donor/recipient status received identical questionnaires requesting information regarding their personal experiences of tissue and organ donation for research.

Results:

Organ and tissue donation whether for research or transplantation relies principally on transplant co-ordinators. There had been no prior evaluation of the views and knowledge of co-ordinators who are often the first to approach families about donation of organs.

There was a 65% (n=102) response to the questionnaire with one percent of donor co-ordinators having never consented donors for research. Recipient co-ordinators rarely request research consent as Doctors perform this role (explanted lungs, hearts and livers).

27% (28) of co-ordinators have been approached by families asking about donation for research and 88% of co-ordinators consenting donors for research use tissue banks.

27% (28) did not feel they know enough about research to obtain informed consent and the consensus in 50% (51) was that donors' families want to know what research will be undertaken on the donated tissues.

Using the comments offered on the questionnaire there is 100% consent for explants to be used for research, with research consent always sought and rarely declined in the context of corneal donation.

There was clear confusion as to what can be donated for research and what work is

then performed with this tissue. 61% (62) of co-ordinators want to know more about the use of tissues and organs in research.

Summary:

Most transplant co-ordinators are willing to request consent for research.

The majority do not know enough about research to obtain informed consent.

Discussion:

Some co-ordinators expressly confirmed their belief in the benefits of research and others felt families are fairly unanimous in their support for unused transplanted tissue being directed towards research. It is routine practice to approach all families regarding tissue donation and they are given the option of donation for research as well as transplantation. One co-ordinator was able to comment that last year (2003) 95% gave consent for research. Importantly, co-ordinators sometimes have to use intuition (for some families) as donation for research could be a step too far and when this happens consent for transplantation has to be protected. Some routinely request consent but feel few people agree to research with the majority of families declining if organ donation is not going to take place. Equally, where no organs are suitable for transplantation, families are not asked as there is no-one to retrieve organs.

Some do not know which organs can be donated for research and whom to donate this tissue to. Others have simply never thought about explanted organs being used for research. A number of co-ordinators described when asking for permission for research the request starts to sound like a shopping list. One found consent only for research despicable.

Co-ordinators expressed concern when they were unable to tell the family specifically what the tissue will be used for, explaining since Alder Hey more families are concerned about organ retention. Tissue banks may benefit by producing information for transplant co-ordinators to cover this much needed information.

There is no literature to compare these results with as most discussion regarding cadaveric donation of tissue for research concerns tissue taken at post-mortem (Savulescu 2002, van Diest 2002).

Patient views on tissue donation for research

Materials and Methods:

All patients (n=87) in a twelve month period (February 2004-5), with LREC criteria for consent, undergoing potential liver resection were asked if they were happy for tissue surplus to diagnostic requirement obtained during their surgery to be anonymously donated for biomedical research. Potential donors approached by a single doctor were included (n=83). They were then asked if they would be happy to complete a postal questionnaire after discharge from hospital. Anonymous questionnaires were sent by post followingdischarge home to all patients with operable disease (n=58).

Results:

There is debate about the use of retained tissue both post-operatively and post-mortem and with the Human Tissue Act 2004 the law has been rewritten to account for this. All patients (n=83) approached agreed to donate the surplus tissue obtained during their surgery and all agreed to have a postal questionnaire sent to them on discharge: 58 questionnaires were sent.

Eighty-one percent (47) of patients returned the questionnaire. Although only 47% (22) could identify the researcher requesting consent by name, 85% (40) could by appearance. All described their surgery appropriately and all but 2% (1) could recall agreeing to donate tissue to research. Ninety-eight percent (46) of patients were happy about their decision and these would donate tissue again if requested, curiously the others did not comment that they were dissatisfied.

Eighty-seven percent (41) of patients read and kept the associated information booklet and only 4% (2) wanted more information. 55% (26) correctly identified research projects their donation would be used for; describing drug development studies and the study of normal tissue appropriately.

Sixty-four percent (30) of patients were interested in receiving more information about biomedical research and 60% (28) wanted to know results of research involving their own tissue specifically. 85% (40) of patients were happy for their tissue to be stored for long periods before use with only 4% (2) unhappy with a ten year storage time.

Motives behind tissue donation were predominantly to help others (95%), with a smaller number seeing the surplus tissue as waste (43%).

Summary:

Patients felt adequately informed to consent for tissues to be donated to research.

They felt very strongly about having contributed to biomedical research.

They did not regret their decision.

Discussion:

Patients in our centre felt adequately informed regarding consent for their tissues to be donated to biomedical research, feeling very strongly about having positively contributed to biomedical sciences. Surgical patients elsewhere are not as enthusiastic (Jack & Womack 2003), but again little work has been done to evaluate this further.

Researcher satisfaction with banked and processed tissues from UKHTB

Materials and Methods:

A review of tissue dispatches in 2003 was undertaken. Dispatched specimens were accompanied by self-explanatory feedback forms. Hepatocyte viabilities were compared pre- and post- dispatch. Satisfaction as assessed by the information given was then

reviewed.

Results:

In 2003 processed and unprocessed organs, tissues, cells and cellular fractions were sent to 42 different research centres: 368 different dispatches from more than 149 donors. Although the majority were liver, hepatocytes or microsomes, 120 other organs and tissues were dispatched over this time period. There was a 22.6% (n=83) response rate. 3.6% (3) reported hepatocyte viability below requirements for their planned study and 4.8% (4) reported less than the required cell number. However, the average loss of hepatocyte viability was only 3.27%. 44.6% (37) requested further equivalent tissue specimens and researchers were satisfied with donor information received. No research units used cryopreserved cells.

Summary:

Hepatocytes travel well in solution or monolayer culture.

Researchers are not proactive supplying meaningful feedback.

No researcher used cryopreserved cells.

Discussion:

Trying to obtain information to allow continued development of any tissue bank is challenging, despite the fact that consumers appear largely satisfied with the specimens they receive. Development of tissue banking requires improved communication between researcher and tissue bank and to ensure quality tissue and responsible auditing, novel methods of information acquisition including electronic forms need implementing.

Peer reviewed work

Published papers

Richert L, Viollon-Abadie C, Bonet A, Heyd B, Mantion G, Alexandre E, Bachellier P, Kingston S, **Pattenden C**, Illouz S, Dennison A, Hoffmann S, Coecke S. *Inter-laboratory Evaluation of the response of primary human hepatocytes cultures to model CYP inducers – a European Center for Validation of Alternative Methods-funded pre-validation study*. Toxicology in Vitro. 2010;24(1):335-45

Illouz S, Alexandre E, **Pattenden C**, Mark L, Bachellier P, Webb M, Berry D, Dennison A, Richert L. *Differential effects of curcumin on cryopreserved versus fresh primary human hepatocytes*. Phytother Research. 2008; 22(12): 1688-91

Keynote presentation

SLTB Scientific Meeting
London, September 2004
Invited keynote speaker

Towards banking of human hepatocytes.

CJ Pattenden1, TDR Lloyd2, J Trafford3, S Orr3, DP Berry1 and AR Dennison1
1Department of Hepatobiliary Surgery, Leicester General Hospital, Leicester LE5 4PW
2Department of Surgery, Pilgrim Hospital, Sibsey Road, Boston, Lincs
3UK Human Tissue Bank, De Montfort University, Leicester LE1 5XY

Human hepatocytes are used for pharmacotoxicological research, transplantation and bioartificial liver development. They may be isolated from multi-organ donor tissue or surgically resected specimens, the latter being obtained most commonly as a consequence of major liver surgery for malignant disease.
Hepatocyte isolation and subsequent research is often conducted during antisocial hours due to the timing of donation and evidence suggesting reduced ischaemic time positively affects yield and viability. Additionally, supply of liver is often erratic and geographically challenging with demand presenting similar difficulties.
To alleviate the nocturnal aspect of hepatocyte biomedical research, to ensure maximal use of each donation and to build a bank of hepatocytes, these cells could be cryopreserved. Tissue banking is the recovery, processing, sterilisation (where appropriate), storage, labelling and distribution of tissues for research and therapeutic use. It is the duty of the tissue bank to ensure informed consent for use of the donation is obtained and that the research performed has ethical approval. It requires a co-ordinated approach, liaising between transplant and tissue co-ordinators and surgical teams for acquisition of tissue and researchers, pharmaceutical companies, drivers and local and regional ethics committees for distribution.
The challenges affecting continued development of a bank of cryopreserved hepatocytes include: ethics, informed consent, logistics, legislation, practicalities, consumer demand. This presentation reviews these difficulties and recent changes within this field.

Published abstracts

Mann CD, Neal CP, Metcalfe MS, **Pattenden CJ**, Johnson NA, Dennison AR, Berry DP. *Major resection of hepatic colorectal metastases in elderly patients – an aggressive approach is justified.* British Journal of Surgery 2007;94(S5):18

Mann CD, Neal CP, **Pattenden CJ**, Thomasset SC, Johnson NJ, Dennison AR, Berry DP. *The results of hepatectomy performed for all indications in a UK tertiary Hepatobiliary referral centre*. European Journal of Surgical Oncology 2006; 32(9): 1060

Mann CD, Neal CP, **Pattenden CJ**, Metcalfe MS, Jeyapalan I, Musto P, Dennison AR, Berry DP. *Hepatic resection in elderly patients – ageism is unjustified*. European Journal of Surgical Oncology 2006; 32(9): 1060

Neal CP, **Pattenden CJ**, Mann CD, Metcalfe MS, Garcea G, Dennison AR, Berry DP. *Perioperative factors predict morbidity but not mortality following extended hepatic resection*. HPB 2006; 8(S2): 166

Richert L, Abadie C, Bonet A, Heyd B, Mantion G, Halkic N, Alexandre E, Bachellier P, Jaeck D, Orr S, Patel R, Kingston S, **Pattenden C**, Dennison A, Hoffmann S, Coecke S. *Response of primary human hepatocyte cultures to model CYP inducers- an European Center for Validation of Alternative Methods (ECVAM) funded pre-validation study.* Drug Metabolism Reviews 2006; 38(S1): 151-2

Riyad K, **Pattenden CJ**, Subar D, Trafford J, Brailsford C, Kingston S, Crees G, Patel R, Peak S, Vadeyar H, Sherlock D. *Neoadjuvant therapy with oxaliplatin based regimens for downstaging of colorectal liver metastasis does not reduce viability of hepatocytes*. Gut 2006; 55(S1): 184

Garcea G, **Pattenden CJ**, Berry DP. *Tissue banking of pancreatic samples; are they benign or malignant?* Pancreatology 2006; 6: 198

Pattenden CJ, Pollard CA, Metcalfe MS, Lloyd TDR, Berry DP, Dennison AR. *Patient satisfaction with access to information pre-hepatic resection.* European Journal of Surgical Oncology 2005; 31(9): 1081-2

Neal CP, **Pattenden C**, Mann C, Stephenson J, Garcea G, Barlow A, Dennison A, Berry D. *Extended hepatic resection: results of a single UK hepatobiliary centre.* European Journal of Surgical Oncology 2005; 31(9): 1078

Neal CP, **Pattenden C**, Mann C, Stephenson J, Garcea G, Dennison A, Berry D. *Extended hepatectomy: results of a single UK hepatobiliary centre*. HPB 2005; 7(S1): 23

Pattenden CJ, Lloyd TDR, Trafford J, Orr S, Berry DP, Dennison AR. *UK transplant co-ordinators experience of tissue donation for research.* Cell and Tissue Banking 2005; 6(2): 139

Pattenden CJ, Granger CA, Lloyd TDR, Orr S, Berry DP, Dennison AR. *Researcher satisfaction with banked and processed tissues: UK Human Tissue Bank 2003*. Cell and Tissue Banking 2005; 6(2): 140-141

Pattenden CJ, Lloyd TDR, Trafford J, Orr S, Berry DP, Dennison AR. *Religious teachings and donation of surplus surgical tissue*. Cell and Tissue Banking 2005; 6(2): 140

Presented abstracts

Pattenden CJ, Neal CP, Porter E, Kingston S, Berry DP, MacGregor A, Dennison AR. *Patient and histological factors influencing primary hepatocyte isolation from surgically resected tissue*. Presented at the 9th Congress of the European Hepato-Pancreato-Biliary Association, Athens June 2009 as a poster

Illouz S, Alexandre E, **Pattenden C**, Berry D, Dennison AR, Richert L. *Does curcumin prevent oxidative damage during human hepatocyte isolation, culture or cryopreservation?* Presented at the Hepatocyte User's Group biannual meeting, Stansted November 2006 as a poster

Mann CD, Neal CP, **Pattenden CJ**, Thomasset SC, Johnson NJ, Dennison AR, Berry DP. *Hepatectomy for all indications – the results of a UK tertiary Hepatobiliary referral centre*. Presented at the 7th World Congress of the International Hepato-Pancreatico-Biliary Association, Edinburgh September 2006 as a poster

Pattenden CJ, Trafford J, Vadeyar H, Berry DP, Sherlock D, Kingston S, Dennison AR. *5 years of hepatocyte banking: the UK Human Tissue bank experience*. Presented at the 9th Annual Congress of the British Transplantation Society, Edinburgh March 2006 as a poster

Rees A, **Pattenden C**, Trafford J. *The legal aspects of surgically resected human tissue donation in the United Kingdom*. Presented at the 14th Annual Meeting of the European Association of Tissue Banking, Florence December 2005 as a poster

Pattenden CJ, Neal CP, Garcea G, Trafford J, Berry DP, Kingston S, Dennison AR. *One year's hepatocyte supply using surplus surgically resected tissue from a single hepatobiliary unit*. Presented at the 14th Annual Meeting of the European Association of Tissue Banking, Florence December 2005 as an oral presentation

Pattenden CJ, Neal CP, Garcea G, Lloyd TDR, Berry DP, Dennison AR. *Patient views on tissue donation for research*. Presented at the 14th Annual Meeting of the European Association of Tissue Banking, Florence December 2005 as an oral presentation

Richert L, Viollon-Abadie C, Bonet A, Heyd B, Mantion G, Alexandre E, Jaeck D, Orr S, Patel R, Kingston S, **Pattenden C**, Dennison A, LeCluyse E. *The European Center for Validation of Alternative Methods (ECVAM) -funded pre-validation study on the response of primary human hepatocyte cultures to model CYP inducers.* Presented at the Hepatocyte User's Group Scientific meeting, Valencia, Spain November 2004 as a poster

Pattenden CJ, Trafford J, Orr S, Berry DP, Dennison AR. *Researcher satisfaction with banked and processed tissues, UK Human Tissue Bank 2004.* Presented at the 13th Annual Meeting of the European Association of Tissue Banking, Prague October 2004 as a poster

Pattenden CJ, Trafford J, Orr S, Berry DP, Dennison AR. *Quality control and local audit: UK Human Tissue Bank 2004*. Presented at the 13th Annual Meeting of the European Association of Tissue Banking, Prague October 2004 as a poster

Rees A, **Pattenden C**, Trafford J. *Is payment for human tissue from surgical resections for non-therapeutic research an ethical dilemma?* Presented at the 14th Annual Meeting of the European Association of Tissue Banking, Florence December 2005 as an oral presentation

Pattenden CJ, Lloyd TDR, Trafford J, Berry DP, Dennison AR. *Patient views on research participation: preliminary results.* Presented at the 13th Annual Meeting of the European Association of Tissue Banking, Prague October 2004 as a poster

Rees AN, **Pattenden CJ**, Trafford J, Dennison AR. *Is it right that surgically resected tissue be used for research without the specific consent of the patient? An ethical viewpoint.* Presented at the 13th Annual Meeting of the European Association of Tissue Banking,

Prague October 2004 as a poster

Pattenden CJ, Garcea G, Lloyd TDR, Bowers S, Sutton CD, Berry DP, Dennison AR. *UK Colorectal lead clinicians referral practice for patients with metastatic colorectal cancer.* Presented at the 8th Annual Scientific Meeting of the Association of Upper Gastrointestinal Surgeons of Great Britain and Ireland, Cambridge September 2004 as a poster

References

(2003). Human Tissue Bill

(2004). Human Tissue Act 30

Abatangelo G., Brun P., Cortivo R. (1994) Hyaluronic acid. An overview. In: Williams DF, ed. Novel Biomaterials based on Hyaluronic acid and its derivatives. Proceedings of the Annual Meeting of the European Society of Biomaterials, Liverpool 8-18

Abouna GM., HG. Boehmig *et al*. (1970). Long-term hepatic support by intermittent multi-species liver perfusions. Lancet 2(7669):391-6

Abouna GM., P. Ganguly *et al*. (2001). Successful *ex vivo* liver perfusion system for hepatic failure pending liver regeneration or liver transplantation. Transplant Proc 33(1-2):1962-4

About cancer. Segmental anatomy of the liver. http://www.aboutcancer.com/ Accessed 12.02.11

Acker JP., A. Larese *et al*. (1999). Intracellular ice formation is affected by cell interactions. Cryobiology 38(4):363-71

Adams RM., M. Wang *et al*. (1995). Effective cryopreservation and long-term storage of primary human hepatocytes with recovery of viability, differentiation, and replicative potential. Cell Transplant 4(6): 579-86

Advisory Committee on Dangerous Pathogens (ACDP) (1995) Protection against blood borne infections in the workplace: HIV and hepatitis. DoH

Alexandre E., M. Cahn *et al*. (2002) Influence of pre- intra- and post-operative parameters of donor liver on the outcome of isolated human hepatocytes. Cell Tissue Bank 3(4):223-33

Alexandre E., P. David *et al*. (1999) Expression of Cytochromes P450 2E1, 3A4, and 1A1/1A2 in growing and confluent human HepG2 hepatoma cells - effect of ethanol. Tox In Vitro 13:427-435

Alexandre, E., C. Viollon-Abadie, *et al*. (2002) Cryopreservation of adult human hepatocytes obtained from resected liver biopsies. Cryobiology 44(2):103-13

Alison M. (2002) Liver regeneration with reference to stem cells. Cell Dev Biol 13:385-387

Amersham Biosciences (2001) Percoll®: methodology and applications. Uppsala, Sweden, Amersham Biosciences:1-81

Anchordoguy TJ., CA. Cecchini *et al*. (1991) Insights into the cryoprotective mechanism of dimethyl sulfoxide for phospholipid bilayers. Cryobiology 28(5): 467-73

Anonymous (1999) BRI inquiry paper on medical and clinical audit in the NHS. Bristol, Bristol Royal Infirmary Inquiry:530

Anonymous (2001) The Royal Liverpool Children's Inquiry: summary and recommendations. London, The House of Commons:1-32

Anonymous (2011) www.landesbioscience.com (Accessed 21st January 2011)

Ash, SR., CA. Caldwell *et al*. (2002) Treatment of acetaminophen-induced hepatitis and fulminant hepatic failure with extracorporeal sorbent-based devices. Adv Ren Replace Ther 9(1):42-53

Ash SR., J. Steczko *et al*. (2000) Push-pull sorbent-based pheresis and hemodiabsorption in the treatment of hepatic failure: preliminary results of a clinical trial with the BioLogic-DTPF System. Ther Apher 4(3):218-28

Auth MK., D. Woitaschek *et al*. (2005). Preservation of the synthetic and metabolic capacity of isolated human hepatocytes by coculture with human biliary epithelial cells. Liver Transpl 11(4):410-9

Baccarani U., A. Donini *et al*. (2001) Steatotic versus cirrhotic livers as a source for human hepatocyte isolation. Transplant Proc 33(1-2):664-5

Baccarani U., A. Sanna *et al*. (2005) Cryopreserved human hepatocytes from cell bank: *in*

vitro function and clinical application. Transplant Proc 37(1):256-9

Baccarani U., A. Sanna *et al*. (2003) Isolation of human hepatocytes from livers rejected for liver transplantation on a national basis: Results of a 2-year experience. Liver Transpl 9(5):506-12

Bader A., L. De Bartolo *et al*. (2000) High level benzodiazepine and ammonia clearance by flat membrane bioreactors with porcine liver cells. Journal of Biotechnology 81(2-3 SU):95-105

Bakala A., W. Karlik *et al*. (2003) Preparation of equine isolated hepatocytes. Toxicol In Vitro 17(5-6):615-21

Banares R. *et al*. (2010) Extracorporeal Liver Support With the Molecular Adsorbent Recirculating System (MARS) in Patients With Acute-on-Chronic Liver Failure (AOCLF). The RELIEF trial. Presented at the International Liver Congress 2010. Abstract 118

Baquerizo A., A. Mhoyan *et al*. (1997) Xenoantibody response of patients with severe acute liver failure exposed to porcine antigens following treatment with a bioartificial liver. Transplant Proc 29(1-2):964-5

Barbich M., A. Lorenti *et al*. (2004) Culture and characterization of human hepatocytes obtained after graft reduction for liver transplantation: a reliable source of cells for a bioartificial liver. Artif Organs 28(7):676-82

BATB (1999) General standards for tissue banking. www.batb.org.uk/general1.htm. (Accessed 05.02.04)

Baust J., R. Van Buskirk *et al*. (2000) Cell viability improves following inhibition of cryopreservation-induced apoptosis. In Vitro Cell Dev. Biol. 36:262-270

Bell BP., VJ. Navarro *et al*. (2001) The epidemiology of newly diagnosed chronic liver disease in the United States: findings of population-based sentinel surveillance. Hepatology 34(2):468A

Berry M.N. and D.S. Friend (1969) High-yield preparation of isolated rat liver parenchymal cells: a biochemical and fine structural study. J Cell Biol 43(3):506-20

Bhandari RN., LA. Riccalton *et al*. (2001) Liver tissue engineering: a role for co-culture systems in modifying hepatocyte function and viability. Tissue Eng 7(3):345-57

Bikhchandani J., M. Metcalfe *et al*. (2011) Extracorporeal porcine liver perfusion system with a semi-permeable membrane for artificial liver support. Unpublished work

Bischof JC., CM. Ryan *et al*. (1997) Ice formation in isolated human hepatocytes and human liver tissue. Asaio J 43(4):271-8

Bissell DM., Hammaker LE., Meyer UA. (1973) Parenchymal cells from adult rat liver in non-proliferating monolayer culture. Fundamental studies. J Cell Biol 59:722-34

Blaauboer BJ., Boobis AR. *et al*. (1994) The Practical Applicability of Hepatocyte Cultures in Routine Testing. ECVAM Workshop Report 1. ATLA 22:231-241

Block GD., J. Locker *et al*. (1996) Population expansion, clonal growth, and specific differentiation patterns in primary cultures of hepatocytes induced by HGF/SF, EGF and TGF alpha in a chemically defined (HGM) medium. J Cell Biol 132(6):1133-49

Boyle R. (1683) New experiments and observations touching cold, or, an experimental history of cold, begun. London, Richard Davis Bookseller

Bracken MB. (2009) Why animal studies are often poor predictors of human reactions to exposure. J R Soc Med 102(3):120-2

Bramhall SR., U. Gur *et al*. (2003) Liver resection for colorectal metastases. Ann R Coll Surg Engl 85(5):334-339

Bruni S. and TM. Chang (1999) Comparison of bilirubin conjugation in encapsulated hepatocytes, hepatocyte homogenate and intact hepatocytes. Artif Cells Blood Substit Immobil Biotechnol 27(4):357-65

Brunner G., Losgen H. (1987) Benefits and dangers of plasma with fulminant hepatic

failure. In: Oda T., Shiokawa Y., Inoue N. eds. Therapeutic Plasmapheresis VI. Cleveland, OH: ISAO Press 187–191

Brunt EM., CG. Janney *et al*. (1999) Nonalcoholic steatohepatitis: a proposal for grading and staging the histological lesions. Am J Gastroenterol. 94(9):2467-74

BSG (2001) Specialised services for hepatology, hepatobiliary and pancreatic surgery, definition no. 19 (adult). http://www.bsg.org.uk/ pdf_word_docs/natspec.pdf. (Accessed 01.03.11)

Buitrago A., E. Gylfe *et al*. (1977) Rapid isolation of pancreatic islets from collagenase digested pancreas by sedimentation through Percoll® TM at unit gravity. Biochem Biophys Res Comm 79:823-828

Burkitt HG., Young B., Heath JW. (1994) Wheater's Functional Histology, 3rd ed. Churchill Livingston: London

Burnell JM., JK. Dawborn *et al*. (1967) Acute hepatic coma treated by cross-circulation or exchange transfusion. N Engl J Med 276(17):935-43

Butler AJ., MA. Rees *et al*. (2002) Successful extra-corporeal porcine liver perfusion for 72 hr. Transplantation 73:1212-8

Caldwell J., I. Gardner *et al*. (1995) An introduction to drug disposition: the basic principles of absorption, distribution, metabolism, and excretion. Toxicol Pathol 23(2):102-14

Canaple L., N. Nurdin *et al*. (2001) Development of a coculture model of encapsulated cells. Ann N Y Acad Sci 944:350-61

Castaneda F., RK. Kinne (2000) Cytotoxicity of millimolar concentrations of ethanol on HepG2 human tumor cell line compared to normal rat hepatocytes *in vitro*. J Cancer Res Clin Oncol 126(9):503-10

Castilla R., R. Gonzalez *et al*. (2004) Dual effect of ethanol on cell death in primary culture

of human and rat hepatocytes. Alcohol Alcohol 39(4):290-6

Catapano G., L. De Bartolo *et al*. (1996) The effect of oxygen transport resistances on the viability and functions of isolated rat hepatocytes. Int J Artif Organs 19(1):61-71

Catapano G., L. De Bartolo *et al*. (2001) Morphology and metabolism of hepatocytes cultured in Petri dishes on films and in non-woven fabrics of hyaluronic acid esters. Biomaterials 22(7):659-65

Cauwels A., B. Janssen *et al*. (2003) Caspase inhibition causes hyperacute tumor necrosis factor-induced shock via oxidative stress and phospholipase A2. Nat Immunol 4(4):387-93

Chang TM. (1972) Haemoperfusions over microencapsulated adsorbent in a patient with hepatic coma. Lancet 2(7791):1371-2

Chaudry IH., MG. Clemens *et al*. (1981) Alterations in cell function with ischemia and shock and their correction. Arch Surg 116(10):1309-17

Chen Z., Y. Ding *et al*. (2001) Cryopreservation of suckling pig hepatocytes. Ann Clin Lab Sci 31(4):391-8

Chen HL., HL. Wu *et al*. (1998) Long term culture of hepatocytes from human adults. J Biomed Sci 5:435-440.

Cheng PY., M. Wang *et al*. (2003) Rapid transcriptional suppression of rat cytochrome P450 genes by endotoxin treatment and its inhibition by curcumin. J Pharmacol Exp Ther 307(3):1205-12

Chesne C., A. Guillouzo (1988) Cryopreservation of isolated rat hepatocytes: a critical evaluation of freezing and thawing conditions. Cryobiology 25(4):323-30

Chesne C., C. Guyomard *et al*. (1993) Viability and function in primary culture of adult hepatocytes from various animal species and human beings after cryopreservation. Hepatology 18(2):406-14

Cima LG., DE. Ingber *et al*. (1991) Hepatocyte culture on biodegradable polymeric substrates. Biotech Bioeng 38(2):145-58

Clavien PA., CE. Oberkofler *et al*. (2010) What is critical for liver surgery and partial liver transplantation: size or quality? Hepatology 52(2): 715-29

Clemens MG., M. Bauer *et al*. (1997) Remodeling of hepatic microvascular responsiveness after ischemia/reperfusion. Shock 8(2):80-5

Cotterell AH., BH. Collins *et al*. (1995) The humoral immune response in humans following cross-perfusion of porcine organs. Transplantation 60(8):861-8.

Couinaud C. (1957) *Le Foie: Etudes Anatomiques et Chirurgicales*. Paris

Councilman WT. (1890) Report on etiology and prevention of yellow fever. Public Health Bull 2:151–9

Coundouris JA., MH. Grant *et al*. (1993) Cryopreservation of human adult hepatocytes for use in drug metabolism and toxicity studies. Xenobiotica 23(12):1399-409

Court FG., PE. Laws *et al*. (2004) Subtotal hepatectomy: a porcine model for the study of liver regeneration. J Surg Res 116(1):181-6

Court FG., SA. Wemyss-Holden *et al*. (2002) The mystery of liver regeneration. Br J Surg 89(9):1089-95

Court FG., SA. Wemyss-Holden *et al*. (2003) Bioartificial liver support devices: historical perspectives. ANZ J Surg 73(9):739-48.

Cunningham CC., Van Horn CG. (2003) Energy availability and alcohol-related liver pathology. Alcohol Research & Health 27(4):281–299

Darr TB., Hubel A. (1997) Freezing characteristics of isolated pig and human hepatocytes. Cell Transplant 6(2):173-83

David P., C. Viollon *et al*. (1998) Metabolic capacities in cultured human hepatocytes obtained by a new isolating procedure from non-wedge small liver biopsies. Hum Exp

Toxicol 17(10):544-53

De Bartolo L., Bader A. (2001) Review of a flat membrane bioreactor as a bioartificial liver. Ann Transplant 6(3):40-6

De Bartolo L., G. Jarosch-Von Schweder *et al*. (2000) A novel full-scale flat membrane bioreactor utilizing porcine hepatocytes: cell viability and tissue-specific functions. Biotechnol Prog 16(1):102-8

De Bartolo L., S. Morelli *et al*. (2004) New modified polyetheretherketone membrane for liver cell culture in biohybrid systems: adhesion and specific functions of isolated hepatocytes. Biomaterials 25(17):3621-9

de Graaf W., KP van Lienden *et al*. (2010) Assessment of future remnant liver function using hepatobiliary scintigraphy in patients undergoing major liver resection. J Gastrointest Surg 14(2): 369-78

De Loecker P., BJ. Fuller *et al*. (1993) Metabolic activity of freshly prepared and cryopreserved hepatocytes in monolayer culture. Cryobiology 30(1):12-8

De Loecker R., BJ. Fuller *et al*. (1990). The effects of cryopreservation on membrane integrity, membrane transport, and protein synthesis in rat hepatocytes. Cryobiology 27(2):143-52

De Loecker W., VA. Koptelov *et al*. (1998) Effects of cell concentration on viability and metabolic activity during cryopreservation. Cryobiology 37(2):103-9

de Sousa G., M. Dou *et al*. (1991) Freshly isolated or cryopreserved human hepatocytes in primary culture: Influence of drug metabolism on hepatotoxicity. Toxicology in Vitro 5(5-6):483-486

de Sousa G., S. Langouet *et al*. (1996) Increase of cytochrome P-450 1A and glutathione transferase transcripts in cultured hepatocytes from dogs, monkeys, and humans after cryopreservation. Cell Biol Toxicol 12(4-6):351-8

de Sousa G., F. Nicolas *et al*. (1999) A multi-laboratory evaluation of cryopreserved monkey hepatocyte functions for use in pharmaco-toxicology. Chem Biol Interact 121(1):77-97

Demetriou AA., RS. Brown Jr *et al*. (2004) Prospective, randomized, multicenter, controlled trial of a bioartificial liver in treating acute liver failure. Ann Surg 239(5):660-7

Demetriou AA., F. Watanabe *et al*. (1995) Artificial hepatic support systems. Prog Liver Dis 13:331-48

Desille M., B. Fremond *et al*. (2001) Improvement of the neurological status of pigs with acute liver failure by hepatocytes immobilized in alginate gel beads inoculated in an extracorporeal bioartificial liver. Transplant Proc 33(1-2):1932-4

Desille M., S. Mahler *et al*. (2002) Reduced encephalopathy in pigs with ischemia-induced acute hepatic failure treated with a bioartificial liver containing alginate-entrapped hepatocytes. Crit Care Med 30(3):658-63

Dethloff T., F. Tofteng *et al*. (2008) Effect of Prometheus liver assist system on systemic hemodynamics in patients with cirrhosis: a randomized controlled study. World J Gastroenterol 14(13):2065-71

Detry O., N. Arkadopoulos *et al*. (1999) Clinical use of a bioartificial liver in the treatment of acetaminophen-induced fulminant hepatic failure. Am Surg 65(10):934-8

Di Masi JA., Hansen RW., Grabowski HG. (2003) The Price of Innovation: New Estimates of Drug Development Costs. Journal of Health Economics 22(2):151–185

Dickson D. (2002) Human tissue samples more difficult to obtain for academics. Nat Med 8(6):543

Diener B., D. Utesch *et al*. (1993) A method for the cryopreservation of liver parenchymal cells for studies of xenobiotics. Cryobiology 30(2):116-27

Ding YT., YD. Qiu *et al*. (2003) The development of a new bioartificial liver and its

application in 12 acute liver failure patients. World J Gastroenterol 9(4):829-32

Dixit V. (1994) Development of a bioartificial liver using isolated hepatocytes. Artif Organs 18(5):371-84

DoH (2001) A code of practice for tissue banks providing tissues of human origin for therapeutic purposes. London, Department of Health:1-26

DoH (2003) Families and post-mortems. London, Department of Health:1-44

DoH (2003) The import and export of human body parts and tissue for non-therapeutic uses: a code of practice. London, Department of Health:1-10

DoH (2003) Isaacs Report Response. London

Donato MT., JV. Castell *et al*. (1999) Characterization of drug metabolizing activities in pig hepatocytes for use in bioartificial liver devices: comparison with other hepatic cellular models. Journal of Hepatology 31(3 SU):542-549

Donini A., U. Baccarani *et al*. (2001) Hepatocyte isolation using human livers discarded from transplantation: analysis of cell yield and function. Transplant Proc 33(1-2):654-5

Donini A., V. Corno *et al*. (1997) *Ex vivo* preparation of porcine hepatocytes for use in bioartificial hepatic support systems. Transplant Proc 29(4):1948-9

Dorko K., P. Freeswick *et al*. (1994) A new technique for isolating and culturing human hepatocytes from whole or split livers not used for transplantation. Cell Transpl 3(5):387-395

Dou M., G. de Sousa *et al*. (1992) Thawed human hepatocytes in primary culture. Cryobiology 29(4):454-69.

Dowling DJ, Mutimer DJ. (1999) Artificial liver support in acute liver failure. Eur J Gastroenterol Hepatol 11(9):991-6.

Duval DL., Sieg DJ., Billings RE. (1995) Regulation of hepatic nitric oxide synthase by reactive oxygen intermediates and glutathione. Arch Biochem Biophys 316;699-706

ECVAM. http://ecvam.jrc.it/ (Accessed 08.04.09)

Eiseman B., DS. Liem *et al*. (1965) Heterologous liver perfusion in treatment of hepatic failure. Ann Surg 162(3):329-45

Elaut G., T. Vanhaecke *et al*. (2005) Spontaneous apoptosis, necrosis, energy status, glutathione levels and biotransformation capacities of isolated rat hepatocytes in suspension: effect of the incubation medium. Biochem Pharmacol 69(12):1829-38

El Banayosy A., L. Kizner *et al*. (2004) First use of the Molecular Adsorbent Recirculating System technique on patients with hypoxic liver failure after cardiogenic shock. Asaio J 50(4):332-7

Ellis AJ., RD. Hughes *et al*. (1996) Pilot-controlled trial of the extracorporeal liver assist device in acute liver failure. Hepatology 24:1446-51

Encyclopedia Brittanica. Anatomy of the liver. http://www.britannica.com/ (Accessed 12.02.11)

Evans PJ. (1995) Maintenance of cytochrome P-450 levels in hepatocytes preserved on gelatin gels. Cell Biol Int 19(10):855-60

Everhart JE. ed. (1994) Digestive Diseases in the United States: Epidemiology and Impact. US Department of Health and Human Services, Public Health Service, National Institutes of Health, Diabetes and Digestive and Kidney Diseases. Washington DC publication 94-1447

Falasca E., V. Adami *et al*. (2001) Porcine endogenous retrovirus does not infect human cells using a bioartificial liver model system*1. Transplantation Proceedings 33(1-2 SU):1780-1781

Fautrel A., B. Joly *et al*. (1997).Long-term maintenance of drug-metabolizing enzyme activities in rat hepatocytes after cryopreservation. Toxicol Appl Pharmacol 147(1):110-4

Fernandez-Cordero X., L. Lorente *et al*. (1992) A study of the functional anatomy of the

pig liver. Research in Surgery 4(3):178-181

Ferrigno A., V. Rizzo *et al*. (2011) Machine perfusion at 20°C reduces preservation damage to livers from non-heart beating donors. Cryobiology 62(2):152-8

Fitzpatrick DB., M. Karmazyn. (1984) Comparative effects of calcium channel blocking agents and varying extracellular calcium concentration on hypoxia/ reoxygenation and ischemia/reperfusion-induced cardiac injury. J Pharmacol Exp Ther 228(3): 761-8

Freeman JG., K. Matthewson *et al*. (1986) Plasmapheresis in acute liver failure. Int J Artif Organs 9(6):433-8

Fremond B., C. Malandain *et al*. (1993) Correction of bilirubin conjugation in the Gunn rat using hepatocytes immobilized in alginate gel beads as an extracorporeal bioartificial liver. Cell Transplant 2(6):453-60

Fu T., D. Guo *et al*. (2001) Apoptosis occurs in isolated and banked primary mouse hepatocytes. Cell Transpl 10(1):59-66

Fujii Y., ME. Johnson *et al*. (1994) Mitochondrial dysfunction during anoxia/reoxygenation injury of liver sinusoidal endothelial cells. Hepatology 20(1):177-85

Funaki N., J. Tanaka *et al*. (2002) Successive cultures of mature hepatocytes for hepatocyte autotransplantation to assist liver function after liver resection for cancer. Oncol Rep 9(4):713-21

Gavrieli Y., Y. Sherman *et al*. (1992) Identification of programmed cell death *in situ* via specific labeling of nuclear DNA fragmentation. J Cell Biol 119(3):493-501

Gazzard BG., MJ. Weston *et al*. (1974) Charcoal haemoperfusion in the treatment of fulminant hepatic failure. Lancet 1(7870):1301-7

Gerlach JC., J. Brombacher *et al*. (1994) Comparison of four methods for mass hepatocyte isolation from pig and human livers. Transplantation 57(9):1318-22

Gerlach JC., J. Encke *et al*. (1994) Bioreactor for a larger scale hepatocyte *in vitro*

perfusion. Transplantation 58(9):984-8

Gerlach JC., K. Mutig *et al*. (2003) Use of primary human liver cells originating from discarded grafts in a bioreactor for liver support therapy and the prospects of culturing adult liver stem cells in bioreactors: a morphologic study. Transplantation 76: 781-786

Gibson GG., Skett P. (1994) Introduction to drug metabolism. 2nd ed. Chapman & Hall, London and New York

Gimson AE., S. Braude *et al*. (1982) Earlier charcoal haemoperfusion in fulminant hepatic failure. Lancet 2(8300):681-3

Goll V., E. Alexandre *et al*. (1999) Comparison of the effects of various peroxisome proliferators on peroxisomal enzyme activities, DNA synthesis, and apoptosis in rat and human hepatocyte cultures. Toxicol Appl Pharmacol 160(1):21-32

Gomez-Lechon MJ., P. Lopez *et al*. (1990) Culture of human hepatocytes from small surgical liver biopsies. Biochemical characterization and comparison with *in vivo*. In Vitro Cell Dev Biol 26(1):67-74

Goodman ZD. (2007) Grading and staging systems for inflammation and fibrosis in chronic liver diseases. J Hepatol 47(4):598-607

Grace PA. (1994) Ischaemia-reperfusion injury. Br J Surg. 81(5):637-47

Grant MH., EH. Rodgers *et al*. (2001) The effect of serum from liver cancer patients on the growth and function of primary and immortalised hepatocytes. Int J Artif Organs 24(11):807-13

Green D., Reed J. (1998) Mitochondria and apoptosis. Science 281:1309-1312

Griffiths BJ., PJ. Evans (2000) Ultrastructural changes in hypothermically preserved hepatocytes. Cryobiology 40(2):176-81

Grosse-Siestrup C., S. Nagel *et al*. (2002) The isolated perfused liver: a new model using autologous blood and porcine slaughterhouse organs. J of Pharm Tox Meth 46:163-168

Gugun-Guillouzo C., B. Clement *et al*. (1983) Maintenance and reversibility of active albumin secretion by adult rat hepatocytes co-cultured with another liver epithelial cell type. Exp Cell Res 143(1):47-54

Guillouzo A. (1998) Liver cell models in *in vitro* toxicology. Environ Health Perspect 106 S2:511-32

Guillouzo A., L. Rialland *et al*. (1999) Survival and function of isolated hepatocytes after cryopreservation. Chem-Biol Interactions 121(1 SU):7-16

Gujral JS., TJ. Bucci *et al*. (2001) Mechanism of cell death during warm hepatic ischemia-reperfusion in rats: apoptosis or necrosis? Hepatology 33(2):397-405

Guyomard C., L. Rialland *et al*. (1996) Influence of alginate gel entrapment and cryopreservation on survival and xenobiotic metabolism capacity of rat hepatocytes. Toxicol Appl Pharmacol 141(2):349-56

Haghighi KS., WW. Woon *et al*. (2004) A new source of hepatocytes for transplantation. Trans Proc 36(8):2466-8

Halliwell B. (1989) Free radicals, reactive oxygen species and human disease: a critical evaluation with special reference to atherosclerosis. Br J Exp Pathol 70(6):737-57

Hamilton GA., C. Westmorel *et al*. (2001) Effects of medium composition on the morphology and function of rat hepatocytes cultured as spheroids and monolayers. In Vitro Cell Dev Biol Anim 37(10):656-67

Harada K., T. Mitaka *et al*. (2003) Rapid formation of hepatic organoid in collagen sponge by rat small hepatocytes and hepatic nonparenchymal cells. J Hepatology 39(5 SU):716-723

Hassanein TI., F. Tofteng *et al*. (2007) Randomized controlled study of extracorporeal albumin dialysis for hepatic encephalopathy in advanced cirrhosis. Hepatology 46(6): 1853-62

Hawksworth G. (1994). Advantages and disadvantages of using human cells for pharmacological and toxicological studies. Human Exp Tox 13:568-573

Heemann U., U. Treichel *et al*. (2002) Albumin dialysis in cirrhosis with superimposed acute liver injury: a prospective, controlled study. Hepatology 36(4): 949-58

Heneine W., A. Tibell *et al*. (1998) No evidence of infection with porcine endogenous retrovirus in recipients of porcine islet-cell xenografts. Lancet 352(9129):695-9

Hengartner M. (1998) Death by crowd control. Science 281:5398-5399

Hengstler JG., D. Utesch *et al*. (2000) Cryopreserved primary hepatocytes as a constantly available *in vitro* model for the evaluation of human and animal drug metabolism and enzyme induction. Drug Metab Rev 32(1):81-118

Higashiyama S., M. Noda *et al*. (2004) Maintenance of hepatocyte functions in co-culture with hepatic stellate cells. Biochemical Engineering Journal 20:113-118

Holcik M., Korneluk RG. (2001) XIAP, the guardian angel. Nature Rev Mol Cell Biol 2;550-556

Hong JT., Glauert HP. (2000) Effect of ECM on expression of peroxisome proliferation associated genes in cultured rat hepatocytes. Tox in vitro. 14:177-184

Hong SH., EJ. Gang *et al*. (2005) *In vitro* differentiation of human umbilical cord blood-derived mesenchymal stem cells into hepatocyte-like cells. Biochem Biophys Res Commun 330(4):1153-61

Hoofnagle JH., RL. Carithers *et al*. (1995) Fulminant hepatic failure: summary of a workshop. Hepatology 21(1):240-52

Horslen S., HJ. Fristoe *et al*. (2000) Extra-corporeal liver perfusion using human and pig livers for acute liver failure. Transplantation 70:1472-1478

Howard R., A. Christensen *et al*. (1967) The enzymatic preparation of isolated intact parenchymal cells from rat liver. J Cell Biol 35:675-684

Howard TK., GB. Klintmalm *et al*. (1990) The influence of preservation injury on rejection in the hepatic transplant recipient. Transplantation 49(1):103-7

Hu MY., M. Cipolle *et al*. (1995) Effects of hepatocyte growth factor on viability and biotransformation functions of hepatocytes in gel entrapped and monolayer culture. Crit Care Med 23(7):1237-42

Hubel A., M. Conroy *et al*. (2000) Influence of preculture on the prefreeze and postthaw characteristics of hepatocytes. Biotechnol Bioeng 71(3):173-83

Hubel A., M. Toner *et al*. (1991) Intracellular ice formation during the freezing of hepatocytes cultured in a double collagen gel. Biotechnol Prog 7(6):554-9

Hughes RD. (2002). Review of methods to remove protein-bound substances in liver failure. Int J Artif Organs 25(10):911-7

Hui T., J. Rozga *et al*. (2001) Bioartificial liver support. J Hepatobiliary Pancreat Surg 8(1):1-15

Hwang YJ., YI. Kim *et al*. (2000) Development of bioartificial liver system using a fluidized-bed bioreactor. Trans Proc 32(7 SU):2349-2351

Ilan Y. (2002) Towards a bank of cryopreserved hepatocytes: which cell to freeze? J Hepatol 37(1):145-6

Illouz S., T. Nakamura *et al*. (2008) Comparison of University of Wisconsin and ET-Kyoto preservation solutions for the cryopreservation of primary human hepatocytes. Transplant

Iqbal, S., CR. Elcombe *et al*. (1991) Maintenance of mixed-function oxidase and conjugation enzyme activities in hepatocyte cultures prepared from normal and diseased human liver. J Hepatol 12(3):336-43

Irgang M., IM. Sauer *et al*. (2003) Porcine endogenous retroviruses: no infection in patients treated with a bioreactor based on porcine liver cells. J Clin Virology 28(2 SU):141-154

Ishak K., A. Baptista *et al*. (1995) Histological grading and staging of chronic hepatitis. J

Hepatol 22(6):696-9

Jack AL., C. Womack (2003) Why surgical patients do not donate tissue for commercial research: review of records. BMJ 327(7409):262

Jackson BA., JE. Davies *et al*. (1985) Cytochrome P-450 activity in hepatocytes following cryopreservation and monolayer culture. Biochem Pharmacol 34(18):3389-91

Jaeschke H. (1998) Mechanisms of reperfusion injury after warm ischemia of the liver. J Hepatobil Pancreat Surg 5(4):402-8

Jaeschke H. (2002) Reperfusion injury after warm ischemia or cold storage of the liver: role of apoptotic cell death. Trans Proc 34(7):2656-8

Jaeschke H., Farhood A. (1991) Neutrophil and Kupffer cell-induced oxidant stress and ischemia-reperfusion injury in rat liver. Am J Physiol 260(3 Pt 1):G355-62

Janssen H., PH. Janssen *et al*. (2003) Celsior solution compared with University of Wisconsin solution and histidine-tryptophan-ketoglutarate solution in the protection of human hepatocytes against ischemia-reperfusion injury. Transpl Int 16(7):515-22

Jimenez-Galanes S., MJ. Meneu-Diaz *et al*. (2009) Liver transplantation using uncontrolled non-heart-beating donors under normothermic extracorporeal membrane oxygenation. Liver Transpl 15(9):1110-8

Kahn D., R. Hickman *et al*. (1988) Partial hepatectomy and liver regeneration in pigs – the response to different resection sizes. J Surg Res 45(2):176-80

Kaner E., D. Newbury-Birch *et al*. (2007) A rapid review of liver disease epidemiology, treatment and service provision in England Dec. 2007: Institute of Health and Society, Newcastle University

Kasai S., Mito M. (1993) Large-scale cryopreservation of isolated dog hepatocytes. Cryobiology 30(1):1-11

Katsuki M., H. Kurosawa *et al*. (2004) Enhancement of albumin secretion by short-term

hyothermic incubation of primary rat hepatocytes. Biochem Eng J 20:137-141

Katz GG., NH. Shear *et al*. (2001) Signaling for ethanol-induced apoptosis and repair *in vitro*. Clin Biochem 34(3):219-27

Kaufmann PM., HC. Fiegel *et al*. (1999) Influence of pancreatic islets on growth and differentiation of hepatocytes in co-culture. Tissue Eng 5(6):583-96

Kerr JF. (1971) Shrinkage necrosis: a distinct mode of cellular death. J Pathol 105(1):13-20

Kerr JF., AH. Wyllie *et al*. (1972) Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer 26(4):239-57

Kiley J., J. Pander *et al*. (1957) Treatment of hepatic coma by hemodialysis. Am Soc Artif Int Organs 3(1):86-88

Kiley JE., HF. Welch *et al*. (1956) Removal of blood ammonia by hemodialysis. Proc Soc Exp Biol Med 91(3): 489-90

Kimmerle K., Strathmann H. (1990) Analysis of structure-determining process of phase inversion membranes. Desalination 79:283-302

Kobayashi N., H. Noguchi *et al*. (2001) A tightly regulated immortalized human fetal hepatocyte cell line to develop a bioartificial liver. Trans Proc 33(1-2):1948-9

Kobayashi N., H. Noguchi *et al*. (2000) Establishment of a tightly regulated human cell line for the development of hepatocyte transplantation. Hum Cell 13(1):7-13

Kobayashi O., M. Sakaguchi *et al*. (2002) Improvement in the differentiated hepatic phenotype of immortalized human hepatocytes by adenovirus mediated p21 gene transfer. Asaio J 48(4):355-9

Koebe HG., C. Dahnhardt *et al*. (1996) Cryopreservation of porcine hepatocyte cultures. Cryobiology 33(1):127-41

Koebe HG., JC. Dunn *et al*. (1990) A new approach to the cryopreservation of hepatocytes

in a sandwich culture configuration. Cryobiology 27(5):576-84

Koebe HG., SA. Pahernik *et al*. (1995) Porcine hepatocytes from slaughterhouse organs. An unlimited resource for bioartificial liver devices. Asaio J 41(2):189-93

Koebe HG., SA. Pahernik *et al*. (1996) Porcine hepatocytes for biohybrid artificial liver devices: a comparison of hypothermic storage techniques. Artif Organs 20(11):1181-90

Kohli V., M. Selzner *et al*. (1999) Endothelial cell and hepatocyte deaths occur by apoptosis after ischemia-reperfusion injury in the rat liver. Transplantation 67(8):1099-105

Koide N., K. Sakaguchi *et al*. (1990) Formation of multicellular spheroids composed of adult rat hepatocytes in dishes with positively charged surfaces and under other non-adherent environments, Exp Cell Res 186:227-235

Kono Y., S. Yang *et al*. (1995) Establishment of a human hepatocyte line derived from primary culture in a collagen gel sandwich culture system. Exp Cell Res 221(2):478-85

Kono Y., Yang S., Roberts EA. (1997) Extended primary culture of human hepatocytes in a collagen gel sandwich system. In vitro Cell Dev Biol Anim 33:467-472

Kooby DA., Y. Fong *et al*. (2003) Impact of steatosis on perioperative outcome following hepatic resection. J Gastrointest Surg 7(8): 1034-44

Koti RS., AM. Seifalian *et al*. (2003) Protection of the liver by ischemic preconditioning: a review of mechanisms and clinical applications. Dig Surg 20(5):383-96

Kramer L., E. Bauer *et al*. (2003) Successful treatment of refractory cerebral oedema in ecstasy/cocaine-induced fulminant hepatic failure using a new high-efficacy liver detoxification device (FPSA-Prometheus). Wien Klin Wochenschr 115(15-16):599-603

Kreamer BL., JL. Staecker *et al*. (1986) Use of a low-speed, iso-density Percoll® centrifugation method to increase the viability of isolated rat hepatocyte preparations. In Vitro Cell Dev Biol 22(4):201-11

Kroemer G., P. Petit *et al*. (1995) The biochemistry of programmed cell death. Faseb J

9(13):1277-87

Kuddus R., JF. Patzer *et al*. (2002) Clinical and laboratory evaluation of the safety of a bioartificial liver assist device for potential transmission of porcine endogenous retrovirus. Transplantation 73(3):420-9

Kumamoto Y., M. Suematsu *et al*. (1999) Kupffer cell-independent acute hepatocellular oxidative stress and decreased bile formation in post-cold-ischemic rat liver. Hepatology 30(6):1454-63

Laleman W., A. Wilmer *et al*. (2006) Effect of the molecular adsorbent recirculating system and Prometheus devices on systemic haemodynamics and vasoactive agents in patients with acute-on-chronic alcoholic liver failure. Crit Care 10(4):R108

Landry J., D. Bernier *et al*. (1985) Spheroidal aggregate culture of rat liver cells: histotypic reorganisation, biomatrix deposition and maintenance of functional activities. J Cell Biol 101:914-923

Laurson J., C. Selden *et al*. (2005) Hepatocyte progenitors in man and in rodents—multiple pathways, multiple candidates. Int J Exp Pathol 86(1):1-18

Lawrence J., Benford D. (1991) Development of an optimal method for the cryopreservation of hepatocytes and their subsequent monolayer culture. Toxic in Vitro 2(1):39-50

Le Tissier P., JP. Stoye *et al*. (1997) Two sets of human-tropic pig retrovirus. Nature 389(6652):681-2

Leaf A. (1973) Cell swelling. A factor in ischemic tissue injury. Circulation 48(3):455-8

Leavitt FJ. (2003) A volunteer to be killed for his organs. J Med Ethics 29(3):175

Lecluyse EL., E. Alexandre *et al*. (2005) Isolation and culture of primary human hepatocytes. In: Methods in Cell Biology: Basic Cell Culture Protocols (Helgason CD. and Miller CL. Ed.) Chapter 15:207-229, Humana Press, Inc. Totowa, NJ

LeCluyse E., PL. Bullock *et al*. (1996) Strategies for restoration and maintenance of normal hepatic structure and function in long-term cultures of rat hepatocytes. Adv Drug Disc Rev 22:133-186

LeCluyse EL. (2001) Human hepatocyte culture systems for the *in vitro* evaluation of cytochrome P450 expression and regulation. Eur J Pharm Sci 13(4):343-68

Lee C., Tink A. (1958). Exchange transfusion in hepatic coma: report of a case. Med J Aust 45(2): 40-2

Lee WM. (1993) Acute liver failure. N Engl J Med 329(25):1862-72

Lemasters JJ., AL. Nieminen *et al*. (1998) The mitochondrial permeability transition in cell death: a common mechanism in necrosis, apoptosis and autophagy. Biochim Biophys Acta 1366(1-2):177-96.

Lepore MJ., LJ. Stutman *et al*. (1972) Plasmapheresis with plasma exchange in hepatic coma. II. Fulminant viral hepatitis as a systemic disease. Arch Intern Med 129(6):900-7

Li AE., H. Ito *et al*. (1999) A role for reactive oxygen species in endothelial cell anoikis. Circ Res 85(4):304-10

Li AP. (1999) Overview: hepatocytes and cryopreservation—a personal historical perspective. Chemico-Biological Interactions 121(1 SU):1-5

Li AP., C. Lu *et al*. (1999) Cryopreserved human hepatocytes: characterization of drug-metabolizing activities and applications in higher throughput screening assays for hepatotoxicity, metabolic stability, and drug-drug interaction potential. Chem-Biol Interact 121(S1):17-35

Li J., LJ. Li *et al*. (2005) Isolation and short term cultivation of swine hepatocytes for bioartificial liver support system. Hepatobiliary Pancreat Dis Int 4(2):249-53

Liu X., C. Kim *et al*. (1996) Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. Cell 86 (1):147–57

Liu J., LL. Kjaergard *et al*. (2002) Artificial and bioartificial support systems for liver failure: a Cochrane Hepato-Biliary Group Protocol. Liver 22(5):433-8

Liu Q., Z. Liu *et al*. (2005) Prevalence of porcine endogenous retrovirus in Chinese pig breeds and in patients treated with a porcine liver cell-based bioreactor. World J Gastroenterol 11(30): 4727-30

Lloyd T., S. Orr *et al*. (2003) A survey of consumer attitudes to the supply and use of human hepatocytes in the United Kingdom. Altern Lab Anim 31(5):483-8

Lloyd T., S. Orr *et al*. (2004) Effect of patient, operative and isolation factors on subsequent yield and viability of human hepatocytes for research use. Cell Tiss Bank 5(2):81-7

Loretz LJ., AP. Li *et al*. (1989) Optimization of cryopreservation procedures for rat and human hepatocytes. Xenobiotica 19(5):489-98

Lovelock JE. and MW. Bishop (1959) Prevention of freezing damage to living cells by dimethyl sulphoxide. Nature 183(4672):1394-5

Lu HF., WS. Lim *et al*. (2003) Galactosylated PVDF membrane promotes hepatocyte attachment and functional maintenance. Biomaterials 24(27):4893-4903

Ludwig B., FB. Kraus *et al*. (2003) Viral zoonoses - a threat under control? Intervirology 46(2):71-8

Luyt BJ. (1937) The vitrification of organic colloids and of protoplasm. Biodynamica 1: 1-14

Lysaght MJ., Aebischer P. (1999) Encapsulated cells as therapy. Sci Am 4:76-82

Madan A., RA. Graham *et al*. (2003) Effects of prototypical microsomal enzyme inducers on cytochrome P450 expression in cultured human hepatocytes. Drug Metab Dispos 31:421-431

Madan A., R. DeHaan *et al*. (1999) Effect of cryopreservation on cytochrome P-450 enzyme induction in cultured rat hepatocytes. Drug Metab Dispos 27(3):327-35

Maeda S., H. Yoshida *et al*. (2002) Analysis of apoptotic and antiapoptotic signalling pathways induced by Helicobacter pylori. Gut 50(6):771-8

Matshushita T., Ijima H., Koide N. (1991) High albumin production of multicellular spheroids of adult rat hepatocytes formed in the pores of polyurethane foam. Appl Microbiol Biotechnol 36:324-6b

Matsumura KN, GR. Guevara *et al*. (1987) Hybrid bioartificial liver in hepatic failure: preliminary clinical report. Surgery 101(1):99-103

Matsushita T., T. Yagi *et al*. (2003) Apoptotic cell death and function of cryopreserved porcine hepatocytes in a bioartificial liver. Cell Transplant 12(2):109-21

Mazariegos GV., JF. Patzer *et al*. (2002) First clinical use of a novel bioartificial liver support system (BLSS). Am J Transplant 2(3):260-6

Mazur P. (1970) Cryobiology: the freezing of biological systems. Science 168(934):939-49

McGinnity DF., MG. Soars *et al*. (2004) Evaluation of fresh and cryopreserved hepatocytes as *in vitro* drug metabolism tools for the prediction of metabolic clearance. Drug Metab Dispos 32(11):1247-53

McKay GC., C. Henderson *et al*. (2002) Cryopreservation of rat hepatocyte monolayers: cell viability and cytochrome P450 content in post-thaw cultures. Toxicol In Vitro 16(1):71-9

Millis JM., DJ. Kramer *et al*. (2001) Results of phase I trial of the extracorporeal liver assist device for patients with fulminant hepatic failure. Am J Transplantation 1(S1):391

Millis JM., DC. Cronin *et al*. (2002) Initial experience with the modified extracorporeal liver-assist device for patients with fulminant hepatic failure: system modifications and clinical impact. Transplantation 74(12):1735-46

Mitaka T., T. Kojima *et al*. (1995) Growth and maturation of small hepatocytes isolated

from adult rat liver. Biochem Biophys Res Commun 214:310-317

Mitry RR., A. Dhawan *et al*. (2004) One liver, three recipients: segment IV from split-liver procedures as a source of hepatocytes for cell transplantation. Transplantation 77(10):1614-6

Mitry RR., RD. Hughes *et al*. (2003) Human hepatocyte isolation and relationship of cell viability to early graft function. Cell Transplant 12(1):69-74

Mitzner SR., J. Stange *et al*. (2000) Improvement of hepatorenal syndrome with extracorporeal albumin dialysis MARS: results of a prospective, randomized, controlled clinical trial. Liver Transpl 6(3):277-86

Mito M., Kusano M., Kawaura Y. (1992) Hepatocyte transplantation in man. Trans Proc. 24:3052–3053

Morgan ET. (1997) Regulation of cytochromes P450 during inflammation and infection. Drug Metab Rev 29(4):1129-88

Mori T., M. Okumura *et al*. (1997) Effects of chitin and its derivatives on the proliferation and cytokine production of fibroblasts *in vitro*. Biomaterials 18:947-51

Morin D., S. Barthelemy *et al*. (2001) Curcumin induces the mitochondrial permeability transition pore mediated by membrane protein thiol oxidation. FEBS Lett 495(1-2):131-6

Morin O., Normand C. (1986) Long-term maintenance of hepatocyte functional activity in co-culture: requirements for sinusoidal endothelial cells and dexamethasone. J Cell Physiol 129(1):103-10

Morsiani E., P. Pazzi *et al*. (2002) Early experiences with a porcine hepatocyte-based bioartificial liver in acute hepatic failure patients. Int J Artif Organs 25(3):192-202

MRC (2001) Human tissue and biological samples for use in research:operational and ethical guidelines. London, Medical Research Council:1-34

Mullin EJ., MS. Metcalfe *et al*. (2004) Artificial liver support: potential to retard regeneration? Arch Surg 139(6):670-7

Muraca M. (2011) Evolving concepts in cell therapy of liver disease and current clinical perspectives. Dig Liver Dis 43(3):180-7

Murakami S., H. Ijima *et al*. (2004) Development of co-culture system of hepatocytes with bone marrow cells for expression and maintenance of hepatic functions. Int J Artif Org 27(2):118-26

Nagamori S., S. Hasumura *et al*. (2000) Developments in bioartificial liver research: concepts, performance, and applications. J Gastroenterol 35(7):493-503

Nanji AA., K. Jokelainen *et al*. (2003) Curcumin prevents alcohol-induced liver disease in rats by inhibiting the expression of NF-kappa B-dependent genes. Am J Physiol Gastrointest Liver Physiol 284(2):G321-7

Natori S., M. Selzner *et al*. (1999) Apoptosis of sinusoidal endothelial cells occurs during liver preservation injury by a caspase-dependent mechanism. Transplantation 68(1):89-96

Neuman MG., NH. Shear *et al*. (1999) Ethanol-induced apoptosis *in vitro*. Clin Biochem 32(7):547-55

NHS Blood and Transplant (2011) Transplant Activity in the UK 2010-11. NHSBT, Bristol UK

Noormohamed MS., DE. Cowie *et al*. (2010) Extracorporeal membraneous oxygenation in a porcine non-heat beating liver model of organ preservation – A tool for improving hepatocyte isolation. Toxicology 278:369-370

Nuffield Council on Bioethics (1995). Human tissue: ethical and legal issues. London, Nuffield Council on Bioethics:1-153

Nyberg SL., RA. Shatford *et al*. (1993) Evaluation of a hepatocyte-entrapment hollow

fiber bioreactor: a potential bioartificial liver. Biotechnol Bioeng 41(2):194-203

Nyberg SL., RP. Remmel *et al*. (1994) Primary hepatocytes outperform Hep G2 cells as the source of biotransformation functions in a bioartificial liver. Ann Surg 220(1):59-67

Nyberg SL, JR. Hibbs JR, *et al*. (1999) Transfer of porcine endogenous retrovirus across hollow fiber membranes: significance to a bioartificial liver. Transplantation 67:1251–5

Nyberg SL., JA. Hardin *et al*. (2000) Cytoprotective influence of ZVAD-fmk and glycine on gel-entrapped rat hepatocytes in a bioartificial liver. Surgery 127(4):447-55

Office of National Statistics (2007) Mortality statistics 2005. London: The Stationery Office

O'Grady JG., SW. Schalm *et al*. (1993). Acute liver failure: redefining the syndromes. Lancet 342(8866):273-5

Opolon P., JR. Rapin *et al*. (1976) Hepatic failure coma (HFC) treated by polyacrylonitrile membrane (PAN) hemodialysis (HD). Trans Am Soc Artif Intern Organs 22:701–710

Oosterlee A., Rahmel A. (2009). Eurotransplant Annual Report. Leiden

Orr S., Trafford J. (2004) The establishment of the UK Human Tissue Bank. ATLA 32(S1):51-55

Ostapowicz G., Lee WM. (2000) Acute hepatic failure: a Western perspective. J Gastroenterol Hepatol 15(5): 480-8

Ostrowska A., DC. Bode *et al*. (2000) Investigation of functional and morphological integrity of freshly isolated and cryopreserved human hepatocytes. Cell Tissue Bank 1(1):55-68

Otto JJ., JC. Pender *et al*. (1958) The use of a donor liver in experimental animals with elevated blood ammonia. Surgery 43(2):301

Pahernik SA., WE. Thasler *et al*. (1996) Hypothermic storage of pig hepatocytes: influence of different storage solutions and cell density. Cryobiology 33(5):552-66

Paradis K., G. Langford *et al*. (1999) Search for cross-species transmission of porcine endogenous retrovirus in patients treated with living pig tissue. The XEN 111 Study Group. Science 285(5431):1236-41

Park JK., Lee DH. (2005). Bioartificial liver systems: current status and future perspective. J Biosci Bioeng 99(4):311-9

Pascher A., IM. Sauer *et al*. (2002) Extracorporeal liver perfusion as hepatic assist in acute liver failure: a review of world experience. Xenotransplantation 9(5):309-24

Pascher A., IM. Sauer *et al*. (2002) Analysis of allogeneic versus xenogeneic auxiliary organ perfusion in liver failure reveals superior efficacy of human livers. Int J Artif Organs 25(10):1006-12

Patience C., Y. Takeuchi *et al*. (1997) Infection of human cells by an endogenous retrovirus of pigs. Nat Med 3(3):282-6

Patzer JF., GV. Mazariegos *et al*. (2002) Preclinical evaluation of the Excorp Medical, Inc, bioartificial liver support system*1. J Am Coll Surg 195(S3):299-31

Patzer JF., GV. Mazariegos *et al*. (1999) Novel bioartificial liver support system: preclinical evaluation. Ann N Y Acad Sci 875:340-52

Pazzi P., E. Morsiani *et al*. (2002) Serum bile acids in patients with liver failure supported with a bioartificial liver. Aliment Pharmacol Ther 16(8):1547-54

Pichard-Garcia L., S. Gerbal-Chaloin *et al*. (2002) Use of long-term cultures of human hepatocytes to study cytochrome P450 gene expression. Methods Enzymol 357:311-21

Polge C., A. Smith *et al*. (1949) Revival of spermatozoa after vitrification and dehydration at low temperatures. Nature 164(4172):666

Ponsoda X., E. Pareja *et al*. (2001) Drug biotransformation by human hepatocytes. *In vitro*/*in vivo* metabolism by cells from the same donor. J Hepatol 34(1):19-25

Poropat G., V. Giljaca *et al*. (2011) Bile acids for primary sclerosing cholangitis. Cochrane

Database Syst Rev 19;(1):CD003626

Qiao L., Farrell GC. (1999) The effects of cell density, attachment of substratum and dexamethasone on spontaneous apoptosis of rat hepatocytes in primary culture. In vitro Cell Dev Biol Animal 35:417-424

Quinones-Baldrich WJ., A. Chervu *et al*. (1991) Skeletal muscle function after ischemia: no reflow versus reperfusion injury. J Surg Res 51(1):5-12

Raffray M., Cohen GM. (1997) Apoptosis and necrosis in toxicology: a continuum or distinct modes of cell death? Pharmacol Ther. 75(3):153-77

Rao CV., A. Rivenson *et al*. (1995) Chemoprevention of colon carcinogenesis by dietary curcumin, a naturally occurring plant phenolic compound. Cancer Res 55(2):259-66

RCP (2007). Guidelines on the practice of ethics committees in medical research with human participants. London, RCP

Reddy SP., J. Brockmann *et al*. (2009) Normothermic perfusion: a mini-review. Transplantation 87(5):631-2

Rees MA., AJ. Butler *et al*. (2002) Porcine livers perfused with human blood mount a graft-versus-host reaction. Transplantation 73(9):1460-7

Rhodes RS., DePalma RG (1980) Mitochondrial dysfunction of the liver and hypoglycemia in hemorrhagic shock. Surg Gynecol Obstet 150(3):347-52

Richert L., D. Binda *et al*. (2002) Evaluation of the effect of culture configuration on morphology, survival time, antioxidant status and metabolic capacities of cultured rat hepatocytes. Toxicol In Vitro 16(1):89-99

Richert L., E. Alexandre *et al*. (2004) Tissue collection, transport and isolation procedures required to optimize human hepatocyte isolation from waste liver surgical resections. A multilaboratory study. Liver Int 24(4):371-8

Rifai K., T. Ernst *et al*. (2003) Prometheus-a new extracorporeal system for the treatment

of liver failure. J Hepatol 39(6): 984-90.

Rifai K. *et al*. (2010) Extracorporeal liver support by fractionated plasma separation and adsorption (Prometheus) in patients with acute-on-chronic liver failure (HELIOS study): a prospective randomised controlled multi-centre study. Presented at the International Liver Congress 2010

Riordan SM., Williams R. (1999). Extracorporeal support and hepatocyte transplantation in acute liver failure and cirrhosis. J Gastroenterol Hepatol 14(8):757-70

Riordan SM., Williams R. (2000). Acute liver failure: targeted artificial and hepatocyte-based support of liver regeneration and reversal of multiorgan failure. J Hepatol 32(S1):63-76

Rivera DJ., GJ. Gores *et al*. (1999) Apoptosis by gel-entrapped hepatocytes in a bioartificial liver. Transplant Proc 31(1-2):671-3

Rodrigues CMP., S. Sola *et al*. (2000) Apoptosis induced by deoxycholic acid, unconjugated bilirubin andamyloid [beta]-peptide reflects mitochondrial perturbation which may beinhibited by ursodeoxycholic acid. Journal of Hepatology 32(S2): 40

Rodrigues CM., X. Ma, *et al*. (1999) Ursodeoxycholic acid prevents cytochrome c release in apoptosis by inhibiting mitochondrial membrane depolarization and channel formation. Cell Death Differ 6(9): 842-54

Rosenthal P. (2000) Is there a future for liver-assist devices? Curr Gastroenterol Rep 2(1):55-60

Rosser BG, Gores GJ. (1995) Liver cell necrosis: cellular mechanisms and clinical implications. Gastroenterology 108(1):252-75

Roux W. (1885) *Beitrage zur Entwicklungsmechanik des Embryos*. Z. Biol 21:411-526

Rozga J. (2002) Hepatocyte proliferation in health and in liver failure. Med Sci Monit 8(2):RA32-8

Rozga J., L. Podesta *et al*. (1993) Control of cerebral oedema by total hepatectomy and extracorporeal liver support in fulminant hepatic failure. Lancet 342(8876):898-9

Rozga J., L. Podesta *et al*. (1994) A bioartificial liver to treat severe acute liver failure. Ann Surg 219(5):538-44

Rubbia-Brandt L., V. Audard *et al*. (2004). Severe hepatic sinusoidal obstruction associated with oxaliplatin-based chemotherapy in patients with metastatic colorectal cancer. Ann Oncol 15(3): 460-6

Rubio CA., Porwit A. (1988) Quantitation of fibrosis in liver biopsies. Anal Quant Cytol Histol 10:107-9

Samuel D., P. Ichai *et al*. (2002) Neurological improvement during bioartificial liver sessions in patients with acute liver failure awaiting transplantation. Transplantation 73(2):257-64

Sandritter WA., Reid UN. (1975) Morphology of liver cell necrosis. Pathogenesis and Mechanisms of Liver Cell Necrosis. D. Keppler. Lancaster, MTP Press:1-14

Sauer IM., Gerlach JC. (2002) Modular extracorporeal liver support. Artif Organs 26(8):703-6

Sauer IM., N. Obermeyer *et al*. (2001) Development of a hybrid liver support system. Ann N Y Acad Sci 944:308-19

Sauer IM., K. Zeilinger *et al*. (2003) Extracorporeal liver support based on primary human liver cells and albumin dialysis—treatment of a patient with primary graft non-function. J Hepatol 39(4):649-53

Savulescu J. (2002) No consent should be needed for using leftover body material for scientific purposes. Against. BMJ 325(7365):648-51

Schauer RJ., AL. Gerbes *et al*. (2003) Induction of cellular resistance against Kupffer cell-derived oxidant stress: a novel concept of hepatoprotection by ischemic preconditioning.

Hepatology 37(2):286-95

Schoemaker MH., Moshage H. (2004) Defying death: the hepatocyte's survival kit. Clin Sci (Lond) 107(1):13-25

SEC(91)1794, Communication of the European Commission to Council and the European Parliament

Seglen P. (1976) Preparation of isolated rat liver cells. Methods in Cell Biology 13: 29-83

Selden C., R. Johnstone *et al*. (1986) Human serum does contain a high molecular weight hepatocyte growth factor: studies pre- and post-hepatic resection. Biochem Biophys Res Commun 139(1):361-6

Selden C., A. Shariat *et al*. (1999) Three-dimensional *in vitro* cell culture leads to a marked upregulation of cell function in human hepatocyte cell lines—an important tool for the development of a bioartificial liver machine. Ann N Y Acad Sci 875:353-63

Selden C., Hodgson H. (2004) Cellular therapies for liver replacement. Transpl Immunol. 12(3-4):273-88

Sen S., NA. Davies *et al*. (2004) Pathophysiological effects of albumin dialysis in acute-on-chronic liver failure: a randomized controlled study. Liver Transpl 10(9):1109-19

Sen S., Jalan R. (2005). Ideal hepatocyte: quest for the Holy Grail. J Gastroenterol Hepatol 20(1):1-4.

Serralta A., MT. Donato *et al*. (2003) Functionality of cultured human hepatocytes from elective samples, cadaveric grafts and hepatectomies. Toxicol In Vitro 17(5-6):769-74

Sheil AG., J. Sun *et al*. (1996) Preclinical trial of a bioartificial liver support system in a porcine fulminant hepatic failure model. Aust N Z J Surg 66(8):547-52

Shi XL., YD. Qiu *et al*. (2005) *In vitro* differentiation of mouse bone marrow mononuclear cells into hepatocyte-like cells. Hepatol Res 31(4):223-31

Shimaoka S., T. Nakamura *et al*. (1986) Growth of adult-rat hepatocytes is stimulated

without any growth-factors by co-culturing with nonparenchymal liver-cells in primary culture. Cell Struct Funct 11:473

Shirabe K., M. Shimada *et al*. (1999) Postoperative liver failure after major hepatic resection for hepatocellular carcinoma in the modern era with special reference to remnant liver volume. J Am Coll Surg 188(3):304-9

Sielaff TD., MY. Hu *et al*. (1995) A technique for porcine hepatocyte harvest and description of differentiated metabolic functions in static culture. Transplantation 59(10):1459-63

Sielaff TD., SL. Nyberg *et al*. (1997) Characterization of the three-compartment gel-entrapment porcine hepatocyte bioartificial liver. Cell Biol Toxicol 13(4-5):357-64

Silk DB., Williams R. (1978) Experiences in the treatment of fulminant hepatic failure by conservative therapy, charcoal haemoperfusion, and polyacrylonitrile haemodialysis. Int J Artif Organs 1(1):29-33

Silva JM., SH. Day *et al*. (1999) Induction of cytochrome-P450 in cryopreserved rat and human hepatocytes. Chem Biol Interact 121(1):49-63.

Sindram D., RJ. Porte *et al*. (2000) Platelets induce sinusoidal endothelial cell apoptosis upon reperfusion of the cold ischemic rat liver. Gastroenterology 118(1):183-91.

Sinnatamby CS. (1999) Last's Anatomy, 10th ed. Churchill Livingstone: Edinburgh

Skjaek-Break G. (1992) Biosynthesis and structure function relationships relevant to biomedical and biotechnological applications. Biochem Soc Trans 20(1):27-33

Skett P., Bayliss M. (1996) Time for a consistent approach to preparing and culturing hepatocytes? Xenobiotica 26(1):1-7

Smrzova J., Z. Dvorak *et al*. (2001) Optimisation of porcine hepatocyte cryopreservation by comparison of viability and enzymatic activity of fresh and cryopreserved cells. Acta Vet Brno 70:141-147

Sorrentino F. *Prime ricerche per la realizzazione di un fegato artificiale*. Chir Patol Sperim. 1956;4:1401–1404

Soltys KA., A. Soto-Gutiérrez *et al*. (2010) Barriers to the successful treatment of liver disease by hepatocyte transplantation. Journal of Hepatology 53(4):769-774

Sosef MN., LS. Abrahamse *et al*. (2002) Assessment of the AMC-bioartificial liver in the anhepatic pig. Transplantation 73(2):204-9

St Peter SD., CJ. Imber *et al*. (2002) Liver and kidney preservation by perfusion. Lancet 359(9306):604-13

Stange J., TI. Hassanein *et al*. (2002) The molecular adsorbents recycling system as a liver support system based on albumin dialysis: a summary of preclinical investigations, prospective, randomized, controlled clinical trial, and clinical experience from 19 centers. Artif Organs 26(2):103-10

Starzl T., T. Marchioro *et al*. (1963). Homotransplantation of the Liver in Humans. Surg Gynecol Obstet 117: 659–76

Steen S., R. Ingemansson *et al*. (2007) First human transplantation of a nonacceptable donor lung after reconditioning *ex vivo*. Ann Thorac Surg 83(6):2191-4

Stevens AC., R. Busuttil *et al*. (2001) An interim analysis of a phase II/III prospective, randomized, multicener, controlled trial of the Hepatassist bioartificial liver support system for the treatment of fulminant hepatic failure (abstract). Hepatology 34:299A

Strain AJ., Neuberger JM. (2002). A bioartificial liver-state of the art. Science 295(5557):1005-9

Stroh C., U. Cassens *et al*. (2002) The role of caspases in cryoinjury: caspase inhibition strongly improves the recovery of cryopreserved hematopoietic and other cells. FASEB 16:1651-1653

Strom SC., RA. Fisher *et al*. (1997) Hepatocyte transplantation as a bridge to orthotopic

liver transplantation in terminal liver failure. Transplantation 63(4):559-69

Strom SC., K. Dorko *et al*. (1998) Large scale isolation and culture of human hepatocytes. In Ilots de Langerhands et Hepatocytes. pp195-205. Les Editions INSERM, Paris

Strong RW. (2001) Liver transplantation: current status and future prospects. J R Coll Surg Edinb 46(1):1-8

Studzinski G. (1999) Overview of apoptosis. Apoptosis. G. Studzinski. New York, Oxford University Press:1-18

Stutchfield BM., K. Simpson *et al*. (2011) Systematic review and meta-analysis of survival following extracorporeal liver support. Br J Surg 98(5): 623-31

Sun, E. L., D. G. Aspar, *et al*. (1990). Cryopreservation of cynomolgus monkey (Macaca fascicularis) hepatocytes for subsequent culture and protein synthesis studies. In Vitro Cell Dev Biol 26(2): 147-50

Susick R., N. Moss *et al*. (2001) Hepatic progenitors and strategies for liver cell therapies. Ann N Y Acad Sci 944:398-419

Sussman NL., Chong MG., Koussayir T. (1992) Reversal of FHF using an extracorporeal liver assist device. Hepatol 16:60-65

Sussman NL, G. Gislason *et al*. (1993) The Hepatix extracorporeal liver assist device: Initial Clinical Experience. Artif Organs 18:390-6

Takahashi M., H. Matsue *et al*. (1993) Isolation and culture of human hepatocytes from resected liver tissue as a bioreactor for a hybrid artificial liver. Artif Organs 17(7):653-9

Takeda Y., S. Arii *et al*. (2003) The impairment of hepatocytes and sinusoidal endothelial cells during cold preservation in rat fatty liver induced by alcohol and the beneficial effect of hepatocyte growth factor. Transpl Int 16(4):241-9

Talamini MA., B. Kappus *et al*. (1997) Repolarization of hepatocytes in culture. Hepatology 25(1):167-72

Teoh NC., Farrell GC. (2003) Hepatic ischemia reperfusion injury: Pathogenic mechanisms and basis for hepatoprotection. J Gastroenterol Hepatol 18(8):891-902

Terblanche J., Hickman R. (1991) Animal models of fulminant hepatic failure. Dig Dis Sci 36(6):770-4

Terry C., A Dhawan *et al*. (2005) Preincubation of rat and human hepatocytes with cytoprotectants prior to cryopreservation can improve viability and function upon thawing. Liver Transpl. 11(12):1533-40

Terry C., RR. Mitry *et al*. (2005) The effects of cryopreservation on human hepatocytes obtained from different sources of liver tissue. Cell Transplant. 14(8):585-94

Thapliyal R., Maru GB. (2001). Inhibition of cytochrome P450 isozymes by curcumins *in vitro* and *in vivo*. Food Chem Toxicol 39(6):541-7

Trey C., Davidson CS. (1970). The management of fulminant hepatic failure. Prog Liver Dis 3:282-98

Tsiaoussis J., Newsome PN. *et al*. (2001) Which hepatocyte will it be? Hepatocyte choice for bioartificial liver support systems. Liver Transpl 7(1):2-10

Utesch D., B. Diener *et al*. (1992) Characterization of cryopreserved rat liver parenchymal cells by metabolism of diagnostic substrates and activities of related enzymes. Biochem Pharmacol 44(2):309-15

van de Kerkhove MP., E. Di Florio *et al*. (2002) Phase I clinical trial with the AMC-bioartificial liver. Int J Artif Organs 25(10):950-9

van de Kerkhove MP., E. Di Florio *et al*. (2003) Bridging a patient with acute liver failure to liver transplantation by the AMC-bioartificial liver. Cell Transplant 12(6):563-8

van de Kerkhove MP., R. Hoekstra *et al*. (2004) Large animal models of fulminant hepatic failure in artificial and bioartificial liver support research. Biomaterials 25(9):1613-25

van Diest P. (2002) No consent should be needed for using leftover body material for

scientific purposes. For. BMJ 325:648-649

Vauthey JN., TM. Pawlik *et al*. (2004). Is extended hepatectomy for hepatobiliary malignancy justified? Ann Surg 239(5):722-30

Vauthey JN., TM. Pawlik *et al*. (2006) Chemotherapy regimen predicts steatohepatitis and an increase in 90-day mortality after surgery for hepatic colorectal metastases. J Clin Oncol 24(13): 2065-72

Vermes I., C Haanen *et al*. (1995) A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V. Journal of immunological methods 184(1):39-51

Vijayalakshmi V., B. Naseem *et al*. (2004) Comparison of biochemical and cytotoxic functions of hepatocytes from goat, pig and human fetuses. J Gastroenterol Hepatol 19(9):1029-35

Vilei MT., A. Granato *et al*. (2001) Comparison of pig, human and rat hepatocytes as a source of liver specific metabolic functions in culture systems—implications for use in bioartificial liver devices. Int J Artif Organs 24(6):392-6

Vondran FWR., E. Katenz, *et al*. (2008) Isolation of Primary Human Hepatocytes After Partial Hepatectomy: Criteria for Identification of the Most Promising Liver Specimen. Artificial Organs, 32:205–213

Wang L., S. Florman *et al*. (1998) Differential *in vivo* recovery of sinusoidal endothelial cells, hepatocytes, and Kupffer cells after cold preservation and liver transplantation in rats. Transplantation 66(5):573-8

Wang L., J. Sun *et al*. (2000) Analysis of multivariables during porcine liver digestion to improve hepatocyte yield and viability for use in bioartificial liver support systems. Cell Transplant 9(3):329-36

Wang XH., DP. Li *et al*. (2003) Crosslinked collagen/chitosan matrix for artificial livers.

Biomaterials 24(19SU):3213-3220

Watanabe F., CP. Mullon *et al*. (1997) Clinical experience with a bioartificial liver in the treatment of severe liver failure. Ann Surg 225(5):484-494

Watanabe F,. CJP. Mullon *et al*. (2001) Clinical experience with a bioartificial liver in the treatment of severe liver failure. Annals of Surgery 225(5):484-94

Watts P., Grant MH. (1996). Cryopreservation of rat hepatocyte monolayer cultures. Hum Exp Toxicol 15(1):30-37

Waxman DJ., JJ. Morrissey *et al*. (1990) Phenobarbitol induction of cytochromes p450; high level long term responsiveness of primary rat hepatocyte cultures to drug induction and glucocorticoid dependence of phenobarbitol response. Biochem J 271:113-119

Womack C., Jack AL. (2003) Family attitudes to research using samples taken at coroner's postmortem examinations: review of records. BMJ 327(7418):781-2

Wong H., Chang TMS. (1986) Bioartificial liver: implanted artificial cells microencapsulated living hepatocytes increases survival of liver failure rats. Int J Artif Organs 9:335-346

Wong H., Chang TMS. (1988) The viability and regeneration of artificial cell microencapsulated rat hepatocyte xenograft transplants in mice. Biomater Artif Cells Artif Organs 16:731-740

World Medical Association (1964). World Medical Association Declaration of Helsinki. WMA General Assembly. Helsinki

Wowk B., E. Leitl *et al*. (2000) Vitrification enhancement by synthetic ice blocking agents. Cryobiology 40(3):228-36

Wu FJ., JR. Friend *et al*. (1996) Hollow fibre BAL utilising collagen-entrapped porcine hepatocyte spheroid. Biotechnol Bioeng 52:34-44

Wu FJ., JR. Friend *et al*. (1999) Enhanced cytochrome P450 IA1 activity of self-assembled

rat hepatocyte spheroids. Cell Transplant 8(3): 233-46

Wyllie AH. (1980). Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. Nature 284(5756):555-6

Yagi T., JA. Hardin *et al*. (2001) Caspase inhibition reduces apoptotic death of cryopreserved porcine hepatocytes. Hepatology 33(6):1432-40

Yamada K., M. Kamihira *et al*. (2001) Self-organization of liver constitutive cells mediated by artificial matrix and improvement of liver functions in long-term culture. Biochemical Engineering Journal 8(2SU):135-143

Yang J., X. Liu *et al*. (1997) Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked. Science 275(5303):1129-32

Yang TH., H. Miyoshi *et al*. (2001) Novel cell immobilization method utilizing centrifugal force to achieve high-density hepatocyte culture in porous scaffold. J Biomed Mater Res 55(3):379-86

Yoon JH., Gores GJ. (2002) Death receptor-mediated apoptosis and the liver. J Hepatol 37(3):400-10

Zaleski J., J. Richburg *et al*. (1993) Preservation of the rate and profile of xenobiotic metabolism in rat hepatocytes stored in liquid nitrogen. Biochem Pharmacol 46(1):111-6

Zeilinger K., IM. Sauer *et al*. (2002) Three-dimensional co-culture of primary human liver cells in bioreactors for *in vitro* drug studies: effects of the initial cell quality on the long-term maintenance of hepatocyte-specific functions. Altern Lab Anim 30(5):525-38

Zheng TS., S. Hunot *et al*. (2000) Deficiency in caspase-9 or caspase-3 induces compensatory caspase activation. Nat Med 6(11):1241-7

Zinchenko YS., Coger RN. (2005). Engineering micropatterned surfaces for the coculture of hepatocytes and Kupffer cells. J Biomed Mater Res A 75(1):242-8