

1. INTRODUCTION

“The map is not the territory”

Alfred Korzybski, circa 1930

1.1 Background

The liver is the largest internal organ in the body, weighing approximately 1.5 kg and accounting for 2% of the weight of an adult. It is of great anatomical and physiological complexity, second only to the brain, and is perfused by 25 % of cardiac output consuming 20-30% of the body's oxygen (O₂) supply. The functional units of the liver are polyhedral hepatic lobules whose corners have portal tracts containing venules, arterioles and bile ductules while the centre is drained by a central vein. The ‘hepatocytes’ are the parenchymal cell population of the liver, they compose 70-80 % of its mass and are arranged in unicellular plates in the lobule. Each lobule is interspersed with anatomically and functionally separate bile canaliculi and sinusoids. Along the periportal to perivenous axis of the sinusoid there is a polar zonation of metabolic functions which are determined by gradients of oxygen, growth factors and hormones. The remaining non parenchymal cell population includes Kupffer, endothelial, stellate, pit, Ito and bile duct cells [1,2].

At least 500 liver functions have been identified thus far. Examples include the endocrine secretion of albumin and urea into the blood, the exocrine secretion of bile into the intestine and the storage of fuel in the form of glycogen. The liver plays an integral role in maintaining the body's metabolic homeostasis by regulating carbohydrate, fatty acid, amino acid and cholesterol metabolism. It plays an important role in systemic responses to injury through its ability to switch to a hyper-metabolic state and the production of acute phase proteins. Additional to metabolism, the liver has a critical defense function by inactivating toxins and xenobiotics absorbed in the intestines and clearing foreign bodies from the blood [2].

The liver is unique in its ability to regenerate and may restore its original mass even if only as little as 15-20 % of the cells remain undamaged [2]. On a molecular level, liver regeneration is associated with hepatocyte hyperplasia [3]. However, the hepatocyte population may be restored through trans-differentiation from a variety of other cell types, including oval cells, multipotent stem cells and fetal liver cells [4].

Following damage, the liver has an initial orientation to protein synthesis, to restore its mass and manage the increased metabolic load, followed by an increase in lipid and fatty acid synthesis. Under the synchronized expression and control of large scale genetic pathways, many factors impact regeneration. These include for example, insulin and glucagon production by the pancreas, glucose metabolism, portal blood flow, plasma amino acid composition, bile acid concentration, cytokines such as tumor necrosis factor (TNF), interleukin-6 (IL-6), transcription factors such as AP-1 complex and STAT-3, growth factors such as hepatocyte growth factor (HGF), epidermal growth factor (EGF) and transforming growth factor- β (TGF- β). Regeneration also requires complex signaling interactions between hepatocytes and other cell types, particularly Kupffer, endothelial, stellate cells and blood platelets, in view of their secreting growth and transcription factors critical to the process [3].

Acute liver failure (ALF) is a severe form of liver injury. It is associated with an abrupt loss of hepatic cellular function in a patient without pre-existing liver disease with the subsequent development of coagulopathy, jaundice and encephalopathy. It is a rapid and devastating clinical syndrome with significant mortality (60 % - 90 %) despite advances in its clinical management [5-7]. ALF is complex and life threatening, often due to brainstem compression and herniation secondary to intracranial hypertension. Additional to the neurologic complications, multi-organ failure often follows [8].

Presently, orthotopic liver transplantation (OLT) is the only treatment of proven benefit in ALF. However, there is a global shortage of donors and patients are often forced to wait for organs to become available. Thus, a 'bridging' treatment to support the ALF patient prior to OLT is highly desirable. Such a treatment might also allow the patient's innate liver to spontaneously regenerate, in so doing avoiding the need for transplantation [7].

One such bridging treatment is a bio-artificial liver support system (BALSS). This is a ‘hybrid’ technology composed of an artificial extracorporeal circulation system with an integrated biological component in the form of a hepatocyte-seeded bioreactor. The operation of a BAL device relies on replacing/augmenting as many of the functions of an *in vivo* liver as possible. The most important functions are particularly the detoxifying, transformatory and synthetic properties since these are not reproducible by artificial chemical or mechanical means [9].

The University of Pretoria (UP) and the Council for Scientific and Industrial Research (CSIR) have developed a BALSS. This device has novel characteristics, for example, it contains a radial-flow, plasma-perfused polyurethane foam [PUF] matrix bioreactor loaded with primary hepatocytes and stellate cells. The system is perfused with plasma as opposed to whole blood, to prevent immunological reactions, and a perfluorocarbon (PFC) oxygen carrier is included in the bioreactor sub-circulation to improve O₂ mass transfer to hepatocytes [10]. The UP-CSIR BALSS is currently in a preclinical phase of testing.

Developing a BAL unavoidably necessitates implementing a variety of studies aimed at demonstrating or improving various aspects of the metabolic functionality and clinical efficacy of the system. These inherently employ ‘models’, which are necessarily abstractions of the *in vivo* human situation. Emulating multitudinous liver functionality and the practicalities of the required experimental and clinical systems present particular challenges. Testament to this fact is that despite approximately four decades of research, BAL technology (globally) remains in a pre-commercial stage. Significant scientific and financial commitments are required. As part of such an endeavor, this thesis aims to present, evaluate and provide thoughts and recommendations for progress in models and technology of this type.

1.2 Defining *Models*

To quote Wikipedia (<http://en.wikipedia.org/wiki/Model>):

“A model is a pattern, plan, representation (especially in miniature), or description designed to show the main object or workings of an object, system, or concept.”

In this thesis the term *model* is used in the generic sense of an ‘abstraction’, however, the investigations that follow include specifically three types:

1. *In vitro* models, which are miniature or *scaled-down* laboratory versions of the BAL and are designed to demonstrate and/or improve the metabolic functionality of the bioreactor component,
2. *In vivo* animal models, which are used to demonstrate particular aspects of the clinical scenario being studied (i.e. severely liver-compromised circumstances), and,
3. Mathematical mass-balance and bio-process models that are used to demonstrate and/or predict aspects of the combined BAL-patient system’s clinical functionality.

The means of evaluation of each type is as per the methods that are normal with respect to the field in which they would be published.

1.3 Problem statement

Demonstrating the metabolic functionality and/or clinical efficacy of BAL technology requires the implementation of a variety of in vitro, in vivo and mathematical studies or systems. As a formal necessity these are ‘models’, or experimentally controlled configurations of the in vivo circumstance. Bearing this in mind, how can these models be used to facilitate the development of a BAL that meets the human clinical need and what research is underway or necessary in this regard?

1.4 Thesis structure

The thesis is composed of a set of logically progressive studies arranged in technically-related sections. Each section is preceded by an overview introducing the included studies. All the studies and underlying models are evaluated individually, as per the methods that are normal to their respective fields, followed by thoughts and recommendations regarding progress in BAL technology particular to and succeeding each section. In general, all the studies have a composition as required for publication in a scientific journal i.e. an introduction, materials and methods, results and discussion/conclusion. For purposes of efficiency, certain methods and/or verification

results that are only incidental to model definition/evaluation have been truncated in the text and detailed in the appendices.

Briefly,

1. Section 1 (this one) introduces the thesis and provides technical information regarding the manner of its presentation.
2. Section 2 is a literature review that defines the clinical context of ALF and the biological factors involved in the development of BAL devices.
3. Section 3 briefly details the UP-CSIR BAL technology which, together with section 2, lays a foundation for what follows.
4. Section 4 includes 3 *in vitro* cell biology studies: The first details a model for isolating large quantities of liver cells, a formal requirement for creating metabolically functional BAL bioreactors. The second investigates the functionality of these bioreactors and the effect of including a PFC oxygen (O₂) carrier in the system. The third, in response to earlier difficulties, uses improved methods to further investigate bioreactor functionality under conditions simulating the treatment of an ALF patient. In the thoughts and recommendations section concerns regarding cell source models and recent progress in the field are mentioned.
5. Section 5 includes 2 *in vivo* (animal) studies: The first employs a model of liver injury and regeneration in rats to investigate the potential toxicity of the PFC O₂ carrier if leaked intravenously in a BAL treatment. The second describes the standardization of an *in vivo* surgically-induced large animal model of acute liver failure in preparation to clinically testing the BAL device. The thoughts and recommendations detail the impact of the latter model on such tests, the prognostically important metabolism and reduction of blood ammonia and the impact of this on BAL device design.
6. Section 6 includes 2 mathematical modeling studies: The first is a mass-balance pharmacokinetic compartment model of the BAL-patient system using actual clinical and metabolic data to provide useful insights regarding BAL design. The second is a conceptual and numerical model whose purpose is the development of an *on-line* ‘in theatre’ ALF-bioprocess monitoring system that provides *real-time* prognosis indications [11,12]. Thoughts and recommendations follow regarding

- refining biomarker/prognostic models, additional mathematical experiments, ‘fusing’ model types and the implementation of bioprocess monitoring systems.
7. The thesis is concluded with an evaluation of success, and consensus on what is required for progress in the technology.
 8. The references are followed by,
 9. The appendices, which provide additional details on methods and verifications incidental to defining the models.

Please note, the University of Pretoria Animal Use and Care Ethics committee (or the Animal Ethics Committee of the Tshwane University of Technology) first granted approval prior to commencement of any animal experiments described in this thesis.

1.5 Copyright and authorship issues

Permission was requested and received from journals for articles in which copyright had been assigned for publication. These include the following:

Nieuwoudt MJ, Kreft E, Olivier B, Malfeld S, Vosloo J, Stegman F, Kunneke R, Van Wyk AJ, Van der Merwe SW. A Large Scale Automated Method for Hepatocyte Isolation: Effects on Proliferation in Culture. *Cell Transplantation* 2005;14(5): 291-299.

Nieuwoudt M, Moolman S, Van Wyk AJ, Kreft E, Olivier B, Laurens JB, Stegman F, Vosloo J, Bond R, van der Merwe SW. Hepatocyte Function in a Radial-flow Bioreactor Using a Perfluorocarbon Oxygen Carrier. *Artif Organs* 2005;29(11): 915-918.

Nieuwoudt M, Kunnike R, Smuts M, Becker J, Stegmann GF, Van der Walt C, Naser J, Van der Merwe S. Standardization criteria for an ischemic surgical model of acute hepatic failure in pigs. *Biomaterials* 2006;27(20):3836-45.

Nieuwoudt MJ, Engelbrecht GHC, Sentle L, Auer R, Kahn D, van der Merwe SW. Non-toxicity of IV injected perfluorocarbon oxygen carrier in an animal model of liver regeneration following injury. *Artificial Cells, Blood substitutes, and Biotechnology* 2009;37(3):117-24.

Nieuwoudt MJ, Wiggett WS, Malfeld S, van der Merwe SW. Imaging glucose metabolism in perfluorocarbon-perfused hepatocyte bioreactors using positron emission tomography. *Journal of Artificial Organs* 2009;12:247-57.

Publications in progress include:

Moolman SF, Nieuwoudt MJ, Shatalov M. A pharmacokinetic compartment model for demonstrating Bioartificial liver performance.

Nieuwoudt MJ, Cilliers P, van der Merwe SW. The development of an *on-line* predictive clinical monitoring system for acute liver failure patients.

2. THE CLINICAL AND BIOLOGICAL BACKGROUND OF ACUTE LIVER FAILURE (ALF) AND LIVER SUPPORT TECHNOLOGY

2.1 Introduction

Acute liver failure (ALF) is a devastating clinical syndrome associated with the sudden loss of a patient's liver function. The course of ALF is rapid, multi-systemic, and variable and the mortality rate is high. Liver support devices are being developed with the intent of 'bridging' ALF patients to the only treatment that has shown statistically significant improvements in survival, namely orthotopic liver transplantation (OLT). The shortage of donor organs and the time taken to OLT validate the use of the technology. This is particularly relevant in Africa in that the only centres conducting transplants are the Groote Schuur and Red Cross hospitals in Cape Town, the Wits Donald Gordon hospital in Johannesburg and an Egyptian Transplant Centre in Cairo. The University of Pretoria (UP) and the Council for Scientific Investigation and Research (CSIR), has collaborated in the development of a Bio-artificial liver support system (BALSS), with which to treat ALF.

Understanding the clinical aspects of ALF and the biological principles underlying BAL devices requires knowledge of several scientific disciplines. For this reason the following section reviews the relevant issues to provide a foundation for the studies and/or models subsequently presented and evaluated.

2.2 Defining ALF

The original definition of fulminant hepatic failure (FHF) by Trey and Davidson (1959) [13] was based on the occurrence of hepatic encephalopathy (HE) as the consequence of severe liver injury developing within 8 weeks of the onset of non-specific symptoms in patients without pre-existing liver disease.

Bernau *et al* (1986) [14] discriminated between fulminant hepatic failure (FHF) and subfulminant hepatic failure (SFHF). In FHF, hepatic encephalopathy (HE) develops within 2 weeks of the onset of jaundice, while in SFHF the HE is delayed beyond 2 weeks, taking up to several months. FHF and SFHF were similar in etiology, i.e. viral hepatitis (A, B, D and E), acetaminophen overdose, idiosyncratic drug reactions, ingestion of toxins (e.g. amanita mushrooms) and metabolic disorders (e.g. Reye's syndrome). However, the prognoses were different and the classification was therefore important. FHF resulted in better long-term prognosis than SFHF.

Subsequently, O'Grady *et al* (2005) [15], introduced the terms hyperacute, acute and subacute to differentiate between ALF in which the onset of HE occurs within 7 days of the onset of jaundice (survival 36%), 7-28 days (survival 7%) and 28 days or more (survival 14%).

ALF is currently defined by the sudden loss of hepatic function in a person without preexisting liver disease. In contrast to the original definition the later classifications allow for the inclusion of cases with previously asymptomatic chronic liver conditions, such as Wilson's disease and the reactivation of an underlying hepatitis B infection [16]. ALF is considered a syndrome rather than a disease due to multiple causes that give rise to variations in course and outcome. Severe acute liver failure is characterized by the presence of coagulopathy resulting in spontaneous bleeding (e.g. epistaxis with international normalized ratio (INR) ≥ 1.5), any degree of HE and the duration of illness anywhere ≤ 24 weeks. Many patients develop coma within ≤ 1 week [17].

2.3 Epidemiology and etiology

ALF is rare and figures are not readily available for global incidence possibly as a result of its multi-systemic presentation leading to spurious diagnoses. ALF has an incidence of about 1 per 150 000 inhabitants yearly. There are approximately 2000 cases per year in the USA (<http://www.unos.org> 2006/7) with a similar incidence in Western Europe. Patients suffering from chronic liver diseases have been estimated at 250 000 per year with 25 000 deaths [18]. Current South African estimates are approximately 300 ALF, with 30 000 chronic liver disease patients per year [19]. Due

to the shortage of donor livers, a large fraction of patients suffering from FHF will die while on a waiting list for OLT. In the Eurotransplant zone there was a need for 2249 liver transplants in 2006, while only 1277 liver transplantations were actually conducted (<http://www.eurotransplant.nl>). In the US at the end of 2001, 18 500 patients were awaiting OLT. In 2004, 5250 of 25 750 patients (20 %) received a donor liver, whereas 1978 (8 %) of ALF patients died while on a waiting list [20]. The waiting times for donor livers and consequent deaths on waiting lists have been increasing in recent years [21].

The distribution of ALF etiologies varies geographically. In the US and UK acetaminophen overdose is the most common cause of death in ALF patients. In Africa and Asia, hepatitis A and B followed by E, are the leading causes [12,13]. ALF due to hepatitis C has been described but is very rare [22,23].

Since the liver metabolizes the majority of drugs almost any drug can cause acute hepatitis. Drug toxicities may be dose-dependent and predictable, but are more often idiosyncratic, in that the toxicity results from an immunological reaction triggered by the drug or its metabolite. 50% of all pediatric cases are due to indeterminate causes. Less common causes of ALF include various cancers, lymphoma, Wilson's disease, acute ischemic liver injury, autoimmune hepatitis and Budd-Chiari syndrome [24,25].

The outcomes of ALF have been changing in recent years owing to improvements in intensive care and patient management. Acetaminophen, shock and hepatitis A are more likely to demonstrate spontaneous recovery than drug-induced, autoimmune and indeterminate-cause ALF. However, ALF remains a challenging system with high mortality [17].

2.4 Pathogenesis and the clinical syndrome

ALF develops from either or both of cytotoxic and cytopathic injury to hepatocytes. The two processes of cell death, necrosis and apoptosis form the basis of the liver injury. Apoptosis, due to tumor necrosis factor (TNF α) and Fas ligand activation of the caspase cascade in ischemic liver injury, Wilson's disease and hepatitis B. Necrosis, due to ATP depletion from mitochondrial damage is more common in

acetaminophen overdose patients. Lipid accumulations associated with abnormalities in mitochondrial fatty acid and ammonia metabolism may be present. The degree of the injury is dependent on the balance of activated pro- and anti-inflammatory cascades, on the modulation of the adaptive immune responses and on factors related to the aetiology, such as viral factors. In a significant number of cases (15-30 % in the West) the aetiology is not identifiable (e.g. whether it is an idiosyncratic drug reaction, or autoimmune reaction, without autoimmune markers, or an unrecognized paracetamol hepatotoxicity).

The most common clinical features of ALF are abnormal liver chemistries, jaundice, systemic inflammatory response syndrome (SIRS) and hepatic encephalopathy. Patients normally present with icterus and elevations in liver aminotransferase levels. In patients that are likely to recover, serum bilirubin levels and prothrombin times (PT) normalize, whereas patients in whom the disease progresses have rising bilirubin levels and increasing PTs, even if the aminotransferase levels drop. The mortality of ALF is mainly due to the associated complications, including, cerebral edema, renal failure, sepsis and cardiopulmonary collapse resulting in multi-organ failure [24-26]. The severity and complexity of the disease have resulted in ongoing demands for improvement in treatment methods.

2.4.1 Hepatic encephalopathy (HE)

The presence of HE is a distinguishing prognostic feature of ALF. It is a spectrum of reversible neuropsychiatric abnormalities characterized by a rapid deterioration in consciousness level and increased intracranial pressure that may result in brain herniation and death. It is graded on a scale of 1 to 4 (table 2.1). Generally HE will fluctuate in the early stages but will progress with the severity of the ALF. Prognosis decreases as the grade increases. In acetaminophen overdose HE usually occurs on the 3rd to 4th day after ingestion and will rapidly progress to stage 4 within 24-48 hours. The prognosis will be poor as a result of the alterations in mental status leading to additional complications such as the inability to maintain respiratory functions, including secretion, which results in an increased risk of infections. Raised plasma ammonia levels above 150 $\mu\text{mol/L}$, the presence of SIRS and infection (with *especially* gram-negative bacteria) are important indicators of HE.

Table 2.1 Grade scale for hepatic encephalopathy [24]

Grade	Clinical features	Asterixis	EEG	Pupillary changes
I	slow mentation, disturbed sleep, reversal of diurnal/nocturnal rhythm	mild	normal	none
II	increasing drowsiness and inappropriate behaviour (agitation and aggression)	present	abnormal with slowing	none to hyperactive
III	stupor	present	abnormal	hyperactive to hippus to slow reactive
IV	coma	lacking	abnormal	slow reactive to fixed and dilated as coma worsens

Although extensive efforts have focused on elucidating the pathophysiology of HE in recent years, it is still only partially understood. For present purposes this complex clinical entity is only briefly summarized, please refer to the recent review of Haussinger *et al* (2009) [27] for additional information. HE originates in the failure of the biotransformation and excretion of toxins normally processed by the liver and is produced by the interplay of ammonia, inflammatory responses and cerebral haemodynamic autoregulation. It is accompanied by large scale pathogenetic changes in brain astrocytes.

Due to the liver injury a disruption in endogenous toxin clearance and the urea cycle occurs, leading to an increase in plasma ammonia and alterations in the ratio of circulating branch-chain to aromatic amino acids (Fischer's ratio). Brain blood-barrier permeability is directly increased by the ammonia, leading to an elevated cerebral metabolic rate, an increase in the production of reactive oxygen and nitrogen species (ROS/NOS), which trigger multiple protein and RNA modifications, and the build-up of glutamine in the astrocytes following ammonia detoxification by means of glutamine synthetase. The actions of ammonia, inflammatory cytokines, benzodiazepines and hyponatremia integrate at the level of astrocyte swelling and oxidative stress.

Mitochondrial dysfunction leads to a build-up in brain lactate in neurons and astrocytes, electrolyte imbalances and alterations in receptor concentrations. An

increase in ammonia (and possibly also glutamine) leads to an increase in osmotic water movement into the brain and a consequent predisposition to cerebral herniation. There is a positive feed-forward regulatory loop between astrocyte swelling and oxidative stress (stress leads to swelling and swelling leads to stress).

A consequence of the production of ROS/NOS is also the induction of protein tyrosine nitration, which may increase circulating inflammatory cytokine levels (TNF α and the interleukins (IL-1 β or IL-6)). This leads to the inactivation of glutamine synthetase in both the brain and the liver, further impairing ammonia detoxification. In addition to electrolyte imbalances and receptor concentrations, RNA oxidation also leads to alterations in neurotransmission as a result of the inhibition of synaptic protein synthesis. This plays a role in memory formation, providing a link between oxidative stress and the cognitive defects seen in HE.

Infection, which is detectable in 80 % of ALF patients, gives rise to a proinflammatory cytokine cascade with a synergistic worsening of ammonia-HE as important as the effects of the pathogen itself. The activation of the cytokine cascade subsequently contributes to the development of SIRS in which increased glycolysis leads to an increase in circulating lactate. Other circulating bowel-produced toxins that may also affect the severity of HE include glutamate, zinc, mercaptans, short-chain fatty acids, benzodiazepine-like substances, γ -amino butyric acid and toxic metals [27-42].

2.4.2 Cerebral edema (CE)

Disruptions in blood-brain-barrier permeability, neurotransmission and cerebral circulatory auto-regulation all contribute to CE and an increasing intracranial pressure (ICP) [19]. Patients with grade IV HE have an 80 % chance of developing CE, which is the primary cause of death in paracetamol-induced ALF [43]. The clinical signs include systemic hypertension, bradycardia, papillary abnormalities, decerebrate posturing, epileptiform activity, seizures and brainstem respiratory patterns [25]. Diagnosis of CE is performed by jugular oxymetry or intracranial epidural pressure monitoring [26]. A prolonged cerebral perfusion pressure (CPP) below 50 mmHg or an ICP above 40 mmHg is associated with poor neurological recovery. ICP may

initially be treated with mannitol or thiopentone which reduce brain water. Phenytoin may subsequently be given with mechanical hyperventilation and moderate (32-33 °C) hypothermia [25,44].

2.4.3 Coagulopathy

ALF is associated with a profound decrease in the synthesis and an increase in the consumption of clotting factors involved in coagulation. This manifests in an increase in the prothrombin time (PT). The disturbance of the coagulation profile in ALF may resemble a disseminated intravascular coagulopathy (DIC), which may make the distinction between the two difficult [25,26].

2.4.4 Metabolic abnormalities

There are several metabolic abnormalities associated with ALF, including, hyponatremia, hyperkalemia, hypophosphatemia, acidosis (with acetaminophen), alkalosis (metabolic and respiratory), lactic acidosis, hypoglycemia and acute pancreatitis. Hypoglycemia is seen in 40 % of patients and is due to the depletion of glycogen stores and impaired gluconeogenesis [24]. With acetaminophen overdose a pH of less than 7.3 carries a poor prognosis. Lactic acidosis, associated with SIRS, causes increasing tissue hypoxia. The management of the abnormalities is achieved through treating the consequent alterations in the systemic circulation [25,26].

Circulatory failure is most likely due to the high levels of circulating endotoxin and tumor necrosis factor. Hypovolemia with a decreased systemic vascular resistance and cardiac arrhythmias may present as a result of the metabolic abnormalities. Hyperventilation, hypercapnia and respiratory alkalosis occur which exacerbate HE resulting in respiratory depression and apnea. Intrapulmonary shunting occurs with sepsis resulting in respiratory distress and pulmonary edema [24]. Management of the systemic circulation relies on maintaining adequate central venous pressure (CVP), maintaining plasma volume and increasing sodium levels. Hypotension (mean arterial pressure (MAP) below 60 mmHg) is treated with inotropics paired with continuous arterial pressure monitoring. Epinephrine and norepinephrine are commonly provided to increase vasoconstriction. In the US dopexamine (a dopamine analogue) is often

used to increase splanchnic and renal blood flow and to improve oxygen delivery to tissues. It also has an anti-inflammatory effect, which aids in attenuating leucocyte adherence to the splanchnic microvasculature [25].

2.4.5 Renal Failure

Renal failure develops in approximately 55 % of ALF patients. If it is secondary to the liver failure it is known as hepatorenal syndrome (HRS). The renal failure may also be due to an insult that affects both the kidneys and liver (e.g. paracetamol overdose). HRS is characterized by a hyperdynamic circulation and is caused by intense renal vasoconstriction [45]. The MAP is generally low, the cardiac output is high and the patients are hypotensive. There is severe arterial underfilling in the systemic circulation due to pronounced arterial vasodilation in the splanchnic circulation, which is related to portal hypertension.

In the kidney, on the other hand, there is intense vasoconstriction activated by the sympathetic nervous system and the renin-angiotensin and arginine vasopressin systems, as a homeostatic response to improve the under-filling of the arterial circulation. As a result of the increased vasoconstriction, renal perfusion and glomerular filtration are greatly reduced and tubular function is preserved [46]. Data suggests that the arterial under-filling is due to vasodilatation of the splanchnic circulation related to increased splanchnic production of vasodilator substances, particularly nitric oxide (NO) [47].

The renal failure is functional and will always recover when there is a return of liver function, thus, the kidneys are histologically normal in the early stages [48]. In the absence of spontaneous hepatic recovery, OLT will reverse the HRS [45]. There are two types of HRS: Type I is characterized by rapidly progressive renal failure, associated with the ALF, with a serum creatinine above 2.5 mg/dl or a glomerular filtration rate (GFR) below 20 ml/min and is followed by death. Type II is a chronic form, associated with chronic liver disease, characterized by moderate renal failure with a GFR below 40 ml/min or a serum creatinine above 1.5 mg/dl.

HRS may also be precipitated by spontaneous bacterial peritonitis and increased endotoxin levels, or by large volume paracentesis without plasma volume expansion. The increased endotoxin levels are associated with bacterial overgrowth and this correlates with increased serum NO and TNF- α levels [48]. These observations explain the association of HRS with SIRS. The treatment of HRS aims at improving renal perfusion and the GFR. In general systemic vasoconstrictors (e.g. vasopressin analogues) in combination with plasma volume expanders (e.g. colloids) are used to reduce the splanchnic vasodilatation, increase the MAP and to suppress the vasoconstrictors activated in the HRS. Antibiotics are usually also given [25,26,46,48].

2.4.6 Multi-organ failure

A wide range (10 % - 80 %) of ALF patients develop bacterial infection and sepsis as a result of the failure of the hepatic reticuloendothelial system. Staphylococcus and Streptococcus are the common invading organisms. SIRS usually precipitates multi-organ failure and it is the end point of the activation of multiple inflammatory pathways mediated by the chemokine-cytokine responses. It can be measured through the pulse rate, respiratory rate, the leucocyte count and temperature [42].

SIRS is linked to respiratory distress and sepsis. Prolonged hospitalization may also predispose patients to bacterial and fungal infection [49,50]. Once multi-organ failure is present the prognosis is poor. The liver, however, has a unique capacity for regeneration following an acute self-limited injury. Since there is no specific therapy for ALF, treatment generally focuses on supportive measures for the anticipated complications, allowing the liver time to heal [51]. N-acetylcysteine (NAC) is given in all cases of acetaminophen overdose to help in replenishing hepatic glutathione stores, but may also be useful in non-acetaminophen cases [24].

2.5 Prognostic scoring systems

Prognostic criteria aid in determining the likelihood of spontaneous recovery from ALF and therefore aid in decision making regarding OLT. These have been defined using the multivariate analysis of patient data at discrete time points, normally at

admission to the clinical institution. Prognostic criteria are dealt with in greater detail in section 6.2.

Briefly, fulfilling for example the King's criteria carries a poor prognosis for spontaneous recovery. What is apparent is that survival depends on several factors, such as etiology, patient age, severity of hepatic dysfunction, degree of liver necrosis, the number and nature of the complications and the duration of the illness. There is also a strong correlation between the grade of HE and mortality: At grade II mortality is 30 %, at grade III it is 50 % and at grade IV it is over 80 %. Generally survival is better in cases of acute hepatitis A and acetaminophen overdose (40 %) than in idiopathic, toxin related, HBV-hepatitis D co-infection and idiosyncratic drug reactions (80%) (table 2.2) [24].

Table 2.2 Some historically commonly employed prognostic criteria for ALF

System	Criteria	Reference/s
King's College criteria		[52,53]
in acetaminophen overdose	arterial pH < 7.3 despite normal intravascular filling pressures (irrespective of grade of HE), or all three of the following: <ul style="list-style-type: none"> • PT > 100 secs, • serum creatinine >300 µmol/l, • grade III- IV HE. 	
in all other cases of ALF	PT > 100 secs (irrespective of grade of HE), INR > 3.5, or any three of the following (irrespective of grade of HE): <ul style="list-style-type: none"> • non-A, non-B reaction (cryptogenic), • halothane hepatitis, • or other drug toxicity • jaundice > 7 days before the onset of HE, • age < 10 or > 40 years, • serum bilirubin > 300 µmol/l. • PT > 50 secs 	
APACHE II	acute physiology and chronic health evaluation score	[54]
Cliché Criteria	clotting factor V < 20% of normal in a person of < 30 years, or both of the following: <ul style="list-style-type: none"> • Factor V < 30% and • grade III-IV HE in patients of any age. 	[55,56]
serum-globulin [Gc protein] level	scavenger protein bound to actin and released into the circulation by dying hepatocytes	[57]
serum alpha-fetoprotein [AFP] level	an increase from day 1 to day 3 correlates with survival	[58]
severity of SIRS	using various indices of the inflammatory response	[59-61]

liver biopsy	when necrosis > 70% mortality is > 90% without transplantation	[62]
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2.6 Orthotopic Liver Transplantation (OLT) for ALF

The first OLT procedure was performed by Starlz *et al* (1963) [63] and it has remained the definitive treatment for ALF patients who meet the criteria for transplantation. Practically speaking, the majority of transplants are full organ grafts from cadaveric donors. Coagulation factors and platelets are replaced prior to surgery and this is usually adequate to reverse clinical coagulopathy and keep blood losses low. Cerebral edema may be problematic during the dissection and reperfusion phases but often dramatically improves during the anhepatic period. Cerebral autoregulation usually returns to normal within 48 hours of successful transplantation. All cases require immunosuppression. The risk of sepsis, including fungal infection, extends into the post-transplant period and is aggravated by immunosuppression. Renal support is often required for several weeks after the procedure. This is due to the use of nephrotoxic immunosuppressives, antimicrobial drugs and potentially hepatorenal syndrome.

Survival rates vary between 60 % and 90 % depending on the centre. The best transplant results are those for Wilson's disease while the worst are for idiosyncratic drug reactions. For paracetamol overdoses the survival is favored if the transplant occurs within four days of the ingestion. Survival also decreases with progression in the grades of HE at the time of transplantation: 90% for grade I, 77% for grade II, 79% for grade III, and 54% for grade IV. Renal function also correlates with outcome, a serum-creatinine > 200 $\mu\text{mol/l}$ is associated with a poorer outcome [15].

In South Africa, OLTs have historically mostly been done at the Groote Schuur and Red Cross children's hospitals. However, since 2005 the Wits Donald Gordon transplant unit in Johannesburg has also been conducting these surgeries. The problem facing all institutions is donor organ shortage (the global waiting list mortality is in the region of 20 %). In the Eurotransplant zone (<http://www.eurotransplant.nl>) in 2006 there were 2249 patients on the waiting list and only 1277 liver transplantations were conducted. In Africa there is also a reluctance on the part of physicians to refer

patients and the use of ‘marginal’ (non-ideal) donors, along with the presence of HIV and HBV virus and tuberculosis with associated isoniazid and rifampicin toxicity [64,65].

2.7 Liver support systems

Ideally, complete hepatic support should prevent or halt the acceleration of the cytokine cascade seen in ALF, provide metabolic, synthetic and detoxifying functions and allow time for organ regeneration [25]. This is clearly a tall order. Minimal support lies in ‘bridging’ patients to transplantation. There are a variety of design configurations for extracorporeal liver support systems, including dialysis-like artificial (non-biological), hybrid (bio-artificial) support systems and purely biological, (hepatocyte transplantation and liver-to-liver, cross-dialytic systems) respectively (table 2.3):

Table 2.3 Summary of non-biological and biological liver support systems (adapted from van de Kerkhove *et al* (2005) [20] with permission from the author)

Liver support	Technique	Basic outcomes
Artificial or dialysis-like:		
Hemodialysis	Exchange diffusion across a semipermeable membrane between blood and a dialysis fluid	Improved coma, no improvement in survival
Hemofiltration	Continuous convective solute removal across a permeable membrane	Limited outcome
High volume plasmapheresis	Exchange of high plasma volumes	Improvement in biochemical parameters and clinical status
Hemodiafiltration	Convection (large molecules) and diffusion (small molecules) removal across a membrane	Case reports, improved biochemical parameters and neurological status
Hemoperfusion	Perfusion of blood/plasma over charcoal, synthetic neutral resins, or anion exchange resins	Removal of toxins, improvement of mental status, no survival benefit
Hemodiabsorption	Dialysis against a combination of charcoal and cation-exchanger	Biochemical improvement and clinical status, no improvement in survival
Molecular Adsorbent Recirculating System (MARS)	Removal of protein-bound and water soluble substances across a specialized albumin impregnated membrane against albumin rich recirculating dialysate	Improvement in biochemical parameters and clinical status, significant survival benefit for subgroup of patients

Albumin dialysis system	Hemodiafiltration using albumin dialysate without recirculation	Improvement in biochemical parameters and clinical status
Artificial liver support system	Combination of plasma exchange, charcoal hemoperfusion, plasma bilirubin absorption, charcoal plasma perfusion, hemofiltration and hemodialysis	Improvement in biochemical parameters and clinical status
PF-Liver Dialysis	Combines hemodiabsorption with push-pull sorbent-based pheresis	Improvement in biochemical parameters and clinical status
Biological and Bio-artificial:		
Blood xeno cross-hemodialysis	Patient's blood dialyzed against blood of a living animal	Beneficial to patient, not suitable for further clinical application
Tissue xeno cross-hemodialysis	Patient's blood dialyzed against animal liver tissue preparations	Beneficial to patient, not suitable for further clinical application
Xenogeneic liver perfusion	Patient's blood perfused through an animal liver	Safe and provides metabolic support to the comatose AHF patient
Human cross-circulation	Shunt between patient's blood and blood of healthy human	Beneficial to patient, but harmful for donor
Exchange transfusion	Replace patient's plasma with healthy human plasma	Reversal of hepatic coma, large amount of normal plasma needed
Hepatocyte transplantation	Transplantation of isolated human hepatocytes in the patient's spleen or peritoneal cavity	Not much known in AHF patients, beneficial to patients with inborn metabolic errors, survival improvement in animal studies
BAL	Patient's blood or plasma perfused through an extracorporeal bioreactor filled with hepatocytes	Significant survival improvement in animals Safe in humans, improvement in clinical and biochemical parameters. Improved survival ALF subpopulations

2.7.1 Non-biological liver support

Renal support technology has been adapted for treating ALF. Water-soluble and protein-bound, low and middleweight toxic substances are thought to cause multiple organ failure, HE and consequently coma and death. It was therefore thought that dialytic filtration systems for detoxifying the patient's blood would be successful. To date no single system has demonstrated statistically significant improvements in patient survival in prospective, randomized controlled clinical trials. Non-biological therapies have routinely demonstrated improvements in HE and patient biochemistry

(such as with the MARS system [66]). However, their limited benefit is thought to be due to their inability to synthesize liver proteins and hepatotropic factors and the non-specific removal of toxins and mitogens from the blood.

2.7.2 Biological liver support

The biological approach relies on the ability of the parenchymal cells of the liver, i.e. the hepatocytes, to support ALF patients. This is owing to their ability to perform detoxification, metabolism functions and the synthesis of proteins and mitogens critical for liver regeneration.

The two most promising approaches include the transplantation of isolated human hepatocytes into ALF patients and extracorporeal bio-artificial liver circulation systems. Purely biological, animal-to-human and human-to-human, liver to liver or blood, cross-dialytic systems have been discarded due to xenozoonotic or immunological concerns. Portal hepatocyte transplantation has shown some promise in animals but in human patients long-term efficacy has not been demonstrated [67]. This may be due to the susceptibility of hepatocytes to viral infection in hepatitis-virus positive patients, or exposure to toxins and drugs in drug-induced ALF. Research has also been conducted on implanted hepatocytes encapsulated in biocompatible matrices. The two basic designs include vascularized or micro-encapsulated implants, the former involving a biodegradable matrix with direct exposure to the host immune system and the latter a protected but diffusible non-biodegradable matrix. Stimuli responsive and bio-active matrices that (for example) stimulate tissue healing have also been experimented with [68].

Bio-artificial liver support systems (BALSS) are designed for temporary extracorporeal dialysis and contain a bioreactor housing hepatocytes. A large variety of bioartificial liver systems have historically been developed, with the differences mainly in the design of the bioreactor. The most common design has been the hollow-fibre bioreactor in which the cells are incorporated into either the internal or external blood perfusion surfaces. Other designs include for example packed-bed alginate encapsulated or direct-plasma contact radial-flow bioreactors. In all cases, there is a complex trade-off between metabolite and gas mass-transfer gradients, contact with

the host immune system and the degree to which the cellular environment is *in vivo*-like [69-73]. A selection of systems that have been employed in the human clinical setting demonstrated results as follows (table 2.4):

Of the tested devices only two (ELAD [74] and the HepatAssist [75] systems) have progressed to Phase II/III, i.e. clinical efficacy, while all of the others have only been evaluated in Phase I trials, i.e. clinical safety. In the Phase I group an improvement in survival was not viewed as the primary end-point. In the Phase II group only the HepatAssist system showed statistically significant improvements in survival. Specifically, when survival was analyzed accounting for confounding factors, in the entire patient population which included primary (OLT) graft non-function patients there was no difference between the treated and un-treated control patients. However, survival in the fulminant/subfulminant hepatic failure sub-group of patients was significantly higher in the BAL versus control group (risk ratio = 0.56, $P = 0.048$). Unfortunately, the trial was terminated by the FDA prior to completion as it was concluded that demonstrating a significant survival benefit using the particular analytical methods was unlikely. This study in particular demonstrated the challenges associated with undertaking multi-centre randomized controlled trials [75].

The majority of the tested devices were well-tolerated and safe, displayed improvements in the patient's neurological status, improvements in blood biochemistry, tested negative for porcine endogenous retrovirus (PERV) (when the cell type in the bioreactor was of porcine origin), no clinical complications as a result of the treatments and several patients could be bridged to OLT. These trials demonstrated that the utility of BAL devices resides in their ability to bridge ALF patients to OLT rather than in completely replacing liver functions. There remains considerable positive expectation that a BAL device will eventually demonstrate significant improvements in survival in all patient sub-groups in the future.

Table 2.4 System characteristics and outcomes of clinical trials (adapted from van de Kerkhove *et al* (2005) [16] and Chamuleau *et al* (2006) [70] with permission from the authors)

System	ELAD	HepatAssist	TECA	BLSS	RFB	LSS-MELS	AMC-BAL	HBAL
Reference	[74]	[75]	[76]	[77]	[78]	[79]	[80]	[81]
Cell type	C3A-Human tumor	Porcine	Porcine	Porcine	Porcine	Human primary	Porcine	Porcine
Cell source	cultured	cryopreserved	isolated	isolated	isolated	isolated	isolated	isolated
Amount of cells	200-400g	5-7*10 ⁹	10-20*10 ⁹	70-120g	200-300g	600g	1*10 ¹⁰	1*10 ¹⁰
Membrane pore size	70 kD	0.2 µm		100 kD	1 µm	400 kD	Direct contact	100 kD
Perfusion medium	blood	plasma	plasma	blood	plasma	plasma	plasma	plasma
Exchange rate	150-200 ml/min	50 m l/min		100-250 ml/min	22 ml/min	31 ml/min	50 ml/min	
Bioreactor flow rate	200 ml/min	400 ml/min		250 ml/min	1.5 ml/min/g hepatocytes	200 ml/min	150 ml/min	
Added detox device	no	Activated charcoal	Activated charcoal	no	no	albumin dialysis	no	Activated charcoal
Trial type	clinical	clinical	safety	safety	safety	safety	safety	safety
Total of patients	24	171	6	4	7	8	12	12
Outcomes	6 bridged, 7 died no OLT		atleast 2 survived no OLT		6 bridged, 1 died	6 bridged, 1 survived no OLT 1 died no OLT	11 bridged, 1 died no OLT	9 survived no OLT, 1 died post BAL
Survival improvement	no	only 33% of acetaminophen group	N/A	N/A	N/A	N/A	N/A	N/A
Complications	hypotension, bleeding	hypotension	no	hypotension	no	no	hypotension	no
Neurological improvement	possibly	yes	yes	no	yes	yes	yes	unclear
ammonia elimination	-8%	18%		33%	33%		44%	unclear
bilirubin elimination	-20%	18%		6%	11%		31%	unclear
PERV	N/A	negative		negative	negative	N/A	negative	

Notes: N/A=not applicable. Where data has been omitted none was provided

Subsequent to the above, an additional Phase I trial has been conducted by the US company Vital Therapies with the ELAD system in Beijing, China. The results have not been published as yet, but according to the website (as at June 2009, <http://www.vitaltherapies.com>) demonstrate significant improvements in transplant-free survival for acute-on-chronic liver failure (in mostly hepatitis-B patients) that has been sufficient to justify a Phase II trial currently underway in the US (<http://www.clinicaltrials.gov>).

2.7.3 Biological principles in the design of BAL devices

As alluded to above, a variety of design aspects of BAL systems impact their clinical efficacy. Important choices include, the cell type and source, the cell culturing method, the amount of cells in the bioreactor, the means of cellular oxygenation, the type of cell-adhesion matrix in the bioreactor, the bioreactor sub-circulation flow rate (i.e. mass transfer characteristics), the exchange rate between the BAL device and the patient and the presence of an additional (artificial) detoxification device in the BAL circuit [79,82,83]. These factors are briefly reviewed below:

2.7.3.1 Cell type, source and mass

Cell type and source remains an important issue in the design of BAL devices and this subject is consequently returned to subsequently (section 4.4). In the beginning the following questions must be asked: Should the cell type be human or animal and should the cell type be cultured or primary in origin? The advantages and disadvantages of the various BAL appropriate cell types are as follows (table 2.5):

The choice of cell type has an important practical aspect, in that it determines the development of techniques for routinely sourcing large amounts of cells in a sterile manner. The expenses and difficulties faced in establishing these models are significant. For example, transformed and immortalized cells require sterile *in vitro* culturing in large quantities, which is expensive, time consuming and requires extreme vigilance on a technical level. Primary xenogenic (pig) cells, on the other hand, require the establishment of a sterile, numerically large scale isolation method which is usually more feasible than the above (section 4.1). The easy availability,

inexpensiveness, metabolic efficacy of the cells and the physiological and anatomical similarity of pigs to humans has led to many research groups initially working with primary porcine cells. Porcine endogenous retro-virus (PERV) contaminations have also not been demonstrated in clinical tests of BAL systems utilizing these cells. However, concerns regarding zoonoses limit the international applicability of these cells.

Table 2.5 The advantages and disadvantages of cell sources for BAL's [83-86]

Cell Source	Advantages	Disadvantages
primary human hepatocyte-non-parenchymal co-cultures.	functionally active allo-compatible	low availability difficult to stimulate for <i>in vitro</i> growth
transformed human hepatocytes, hepatoma cells. e.g. C3A-(HepG2)	allo-compatible highly available	poor functionality potential tumorigenicity must be cultured in sufficient quantity for a BAL
human stem cells- e.g. embryonic stems (ECs)and multipotent adult progenitor cells (MAPCs)	allo-compatible moderate availability	differentiation into hepatocytes cannot be guaranteed in a BAL
reversibly immortalized human hepatocyte-non parenchymal co-cultures. e.g. NKNT3 + TWNT3 cells, OUMS-29 cells	allo-compatible highly available	potential tumorigenicity lower functionality than primary cells logistically difficult to culture in sufficient quantity for a BAL
primary xenogenic hepatocyte-non parenchymal co-cultures. e.g. porcine cells	highly functional physiological similarity to human cells highly available	potential immunogenicity potential transmission of zoonoses [PERVs] questionable biocompatibility

The *in vitro* cell culture model in the bioreactor impacts BAL metabolic functionality; Liver hepatocytes have an epithelial polarity and junctional cell-cell communication structures. These structures are lost in *in vitro* culturing and several studies have established that *in vitro* hepatocytes do not independently perform as effectively as they do *in vivo*. For this reason, non-parenchymal liver cells such as stellate or fibroblast cells may be co-cultured with hepatocytes in a specific ratio, facilitating the phenotypic and functional stabilization of the hepatocyte aggregate structures. The cell culture media that is used to perfuse the bioreactor prior to BAL connection also determines subsequent functionality. Media composition must be carefully attended to and usually involves supplementation with mitogens and hormones.

Interestingly, in Africa many donor livers go to waste simply due to the insurmountable logistical difficulty of getting the livers to transplant facilities within acceptable time frames. This fact validates the development of a liver support system in this context but potentially also presents an opportunity to populate the bioreactor with primary human cells. Primary human cells are obviously preferable for use in a BAL system, for the reasons stated above, but are mostly only available following liver resections from oversize organs used in transplants.

Stem cells of human origin are an attractive potential source of liver cells in view of their greater replicative capacity and immunological *naïveté* relative to adult tissue. There are currently several techniques for *immortalizing* mammalian cells so that they will maintain primary-like properties for as long as possible. For example, simian virus 40 (SV40) T antigen, adenovirus E1A and E1B, human papillomavirus (HPV) E6 and E7 work by inactivating the tumor suppressor genes (such as p53 and Rb). Alternately, the use of recombinant telomerase adenovirus or retroviral vectors results in the expression of the human telomerase reverse transcriptase protein (hTERT) which causes the cells to maintain telomere lengths that will prevent replicative senescence. hTERT immortalized cells often indefinitely retain a stable genotype along with critical phenotypical markers. The Cre-lox system uses Cre recombinase (from the P1 bacteriophage of *S. cerevisiae*) which catalyzes the site-specific recombination between two 34-bp repeats called loxP. When Cre binds to loxP the intervening section is permanently excised. Using this it is possible to *reversibly* immortalize primary cells by transducing an oncogene flanked with loxP sequences, enabling subsequent site specific excision with Cre. Having said this, stem cell research is still in a relatively early stage [83-86]. This subject is returned to subsequently (section 4.4).

An early question in the history of BAL research was what minimal cell mass is required to maintain a patient in ALF? The relevance of this question is three-fold: First, an obvious concern is to source sufficient cells for a metabolically effective device, second, the size of the cell mass is a practical determinant of the employed methods [87] and third, the cellular metabolic requirements determine aspects of BAL design [88].

Early surgical studies attempted to answer this question through resections to determine the minimal liver mass required to maintain survival [89-92]. However, this methodology is misleading in that the resected liver has an existing blood supply and surgical complications may confuse the outcomes. Additionally, hepatocytes in a liver are not all simultaneously maximally functional, thus, the organ may have huge 'metabolic functional reserves' under stress.

Current estimates are that between 10 % and 30 % of the host liver mass is required for effective support of ALF [83,92]. Since an adult liver weighs approximately 1500 grams and is composed of approximately 80 % hepatocytes, the estimated minimal amount of hepatocytes required for a BAL is in the region of 150-300 grams. However, subsequent studies on rats in ALF have shown biochemical improvements with as little as 2 % of the normal liver mass [93] and adult humans are known to survive following even 90 % partial hepatectomy [75]. To maintain a cell mass of 150 g a bioreactor design is required that will facilitate sufficient mass transfer to all or as many of the cells as possible.

2.7.3.2 Cellular oxygenation

For effective hepatocyte function BAL design must focus on an adequate oxygen supply to maintain optimal metabolic properties. Due to the liver's intense metabolic activity, it consumes 20-30% of the body's oxygen (O_2) translating to 30-40 ml/min, in the maintenance of liver functions [94-98].

BAL systems are perfused either by whole blood or by plasma only. In the former case, adequate oxygenation relies on hemoglobin, but then the possibility for an immune foreign-surface-related reaction exists. In the latter case, the relatively low O_2 carrying capacity of plasma may become limiting at high cell densities in the BAL. In treating an ALF patient, in which the plasma may be toxic and/or hypoxic, this may be a real concern.

There are generally two means by which bioreactor oxygenation may be improved: Either the diffusion distance of O_2 to the hepatocytes may be decreased, or the concentration of O_2 entering the bioreactor may be increased [99]. Consequently,

several different whole blood and plasma perfused bioreactor designs have been described, including for example, stackable flat membrane bioreactors [100,101], direct contact bioreactors [102-103], and several different configurations of hollow-fiber bioreactors [104-106]. Each configuration has both advantages and disadvantages. However, an ongoing concern amongst investigators is the presence of domains of low O₂ tension and consequent hypometabolism in their bioreactors [107,108].

2.7.3.3 Cell support matrices

Since hepatocytes are aggregation dependant cells, maintaining normal cell polarity (phenotype) and thus function in a bioreactor requires a biocompatible cell-aggregation matrix to enable 3-D cell spheroid formation. The phenotype is also dependent on the extracellular matrix and cell-cell communication. Possibilities therefore exist to coat matrix surfaces with collagen (or similar) and non-parenchymal cells (such as stellates) may be included in a ‘co-culture’. Many culturing configurations have been experimented with, for example: bioresorbable matrices (e.g. hydrogels), non-resorbable open-cell sponge-type matrices (e.g. polyurethane), encapsulated or microcarrier packed-beds (e.g. alginate beads) and many different kinds of microfilament membrane matrices (e.g. polysulfone or cellulose acetate). Each has advantages and disadvantages. For good reviews on this see Tzanakis *et al* (2000)[1], Chan *et al* (2004)[68], Nahmias *et al* (2006)[2], Streetz *et al* (2008)[82].

The choice of matrix should ideally be preceded by modeling the bioreactor flow configuration and consequent gas and metabolite mass transfer characteristics to the cells. The availability of nutrients to a cell may be expressed quantitatively [108]. Assuming nutrients transfer by diffusion within the hepatocyte compartment with diffusivity D (cm/s). If the cell consumes at a rate R (mol/cm³/s), dimensional analysis gives a (unit-less) cell lifeline number (S).

$$S = R \cdot L^2 / D \cdot C_0 \quad (2.1)$$

where C_0 is the source solute concentration and L the distance from the source. In this model the source is the compartment from which the diffusion is taking place

(e.g. either whole blood or plasma depending on the design). For values of $S \approx 1$, L represents the greatest distance from the source of the nutrient. The same formula applies to hepatocyte synthesis, and then S correlates with the supply of hepatocyte synthetic products to the patient. A consequence of equation (2.1), is that the metabolic efficacy of the cellular compartment is determined by the distance (L) from the perfusate, thus, a design trade off must be made between the 3-dimensionality of the cell culture and the diffusion distance to the perfusing compartment. This fact has been borne out by the variable success of the many bioreactor designs proposed to date.

In principle, the more closely the configuration emulates *in vivo* liver structure, the better the metabolic function (table 2.6).

Table 2.6 The relative advantages and disadvantages of culture configurations

Culture Method	Duration and extent of hepatic functionality	Performance as BAL
<i>in vivo</i> liver structure*	long term and enhanced	excellent
3-D co-culture	↑	↑
2-D co-culture		
collagen sandwich		
spheroids		
microencapsulation		
porous matrices	↑	↑
extracellular matrices		
microcarriers	short term and poor	limited

* theoretical (not-yet existing) culture configuration.

2.7.3.4 Flow rates, exchange rates and the priming volume

A consequence of equation (2.1) is also that mass transfer to and from the hepatocyte compartment is determined by the rate of flow of the perfusate to that compartment. Similarly, mass transfer between the patient and the bioreactor is determined by the rate of exchange between the two compartments. In principle, the faster the flow rates the greater the mass transfer; however, the fluid shear rate over the cells may then become sufficiently great to have a deleterious effect on cell adhesion. In most BAL devices the bioreactor has a sub-circulation rate that is faster than the rate of exchange between the patient and the BAL. This is for practical reasons in that it is difficult to maintain exchange rates exceeding approximately 200 ml/min.

The priming volume is the amount of fluid (normally either plasma or blood) that is required to fill the BAL prior to enabling the recirculation circuit between the patient and the BAL. This volume creates a blood dilution effect that may temporarily benefit the patient through reducing circulating toxin levels. However, when excessive, the increase in blood volume may result in haemodynamic instability in the patient, which may impact the subsequent clinical course. For example, a loss of hematocrit is known to have deleterious effects on survival. ALF is also associated with haemodynamic instability. Consensus amongst research groups is that a system priming volume of 1 litre or less is ideal. However, achieving this in a complex recirculation system may present an engineering challenge.

2.7.3.5 Detoxification devices and the use of multiple, functionally optimized bioreactors

Despite all efforts it is unlikely that hepatocyte or co-culture bioreactors will replace all the functional abilities of a normal liver. Assuming these hepatocytes express the normal bile-conjugate transporters (such as MRP2 or BSEP) the cells still *physically* lack a biliary system. However, if the hepatocytes in the bioreactor form canaliculi during culturing they will be able to excrete conjugated bilirubin into the circulating blood plasma, which will subsequently be removed by the kidneys. Since the liver is compromised in BAL treated ALF patients, protein-bound bile acids and salts are likely to increase in the patient's blood. Bilirubin is toxic to both the patient and the cells in the bioreactor. Similarly, ammonia and other nitrogenous compounds accumulate in ALF patients due to the sub-optimal transformation of these substances into urea. Practically it is impossible to incorporate into a bioreactor the same amount of hepatocytes as found in an innate liver, nor maintain the same blood perfusion rate. Thus, the accumulation of blood borne toxins may be difficult or even impossible to prevent using BAL devices as defined to date.

It may therefore be desirable to include an artificial detoxification module in the BAL circuit since doing so aids both the patient and BAL bioreactor. Various adsorption columns exist and are often composed of a large surface area of activated charcoal. Albumin cross-dialytic renal technology may also be employed for blood protein-bound toxin removal. As stated, a potential disadvantage of artificial detoxification

lies in the non-selective removal of compounds including hepatotropic substances that may facilitate liver regeneration. The specification of the employed detoxification device is therefore important in terms of BAL clinical operation. This subject is returned to subsequently (in section 5.3.4).

In the *in vivo* liver there is metabolic functional ‘zonation’. This zonation is due to factors including, for example, hormone gradients, substrate concentrations and the perfusing O₂ gradient, the latter of which is an important modulator of cellular function [109]. Thus, ‘perivenous’ and ‘periportal’ regions exist in which enzymatic activity is different. Cytochrome P450 isoenzymes, which perform much of the biotransformations occurring in the liver, tend to be localized in the perivenous region while urea-synthesizing enzymes tend to occur in the periportal region. Both of these functions are important in terms of an effective BAL device. It has therefore been proposed [68], that potentially several bioreactors, each of which has been metabolically ‘primed’ for a particular function, may be included in a single BAL treatment. This is a principally sound concept, but would be difficult in practice to implement.