TOXIC MOLECULES IN LIVER FAILURE PLASMA

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I Rebecca Saich, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

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ABSTRACT

Liver failure remains a disease with a high mortality and with the exception of transplantation therapeutic options are limited. The liver however has regenerative potential, and strategies based not only at supporting the failing liver, but promoting its recovery would be a significant evolution. Plasma from patients with liver failure contains toxic molecules that have many effects on the liver including loss of cell viability. These factors represent a significant barrier to stem cell transplantation, bioreactor function and autologous liver recovery, suggesting removal or antagonism of these factors may be appropriate therapeutic strategies. Since apoptosis has been implicated in the pathogenesis of a number of liver diseases including liver failure we proposed that it may be one of the mechanisms by which plasma is toxic to hepatocytes. We developed and validated a model using primary human hepatocytes to investigate if plasma from patients with acute and acute-on-chronic liver disease was pro-apoptotic. Compared with normal plasma, acute liver failure plasma induced apoptosis whereas plasma from patients with acutely-decompensated chronic liver disease did not.

Having identified that acute-liver failure plasma was pro-apoptotic we investigated the pathway via which the apoptosis was mediated by using specific inhibitors of caspases, key components of the death receptor and mitochondrial pathways. We found that apoptosis was induced via a pathway involving caspase 8 and caspase 3, suggesting involvement of the death-receptor pathway. We investigated the effects of Caspase inhibition as a therapeutic option in acute liver failure by using an established animal model but did not find an improved outcome in treated animals.

We also investigated the effects of treatment with molecular adsorbent dialysis (MARS) on the pro-apoptotic effects of plasma and found MARS dialysis improved biochemical parameters, indicating effective removal of albumin-bound molecules, but the apoptotic effects of the patients' plasma were unchanged.

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ABBREVIATIONS

Alb	Albumin
AHF	Acute Hepatic Failure
ALF	Acute Liver Failure
ALFP	Acute liver failure plasma
ALP	Alkaline Phosphatase
ALT	Alanine Transaminase
APTT	Activated partial thromboplastin time
AST	Aspartate transaminase
ATP	Adenosine-5'-triphosphate
A-MEM	alpha Minimum Essential Medium
Bili	Total Bilirubin
BSA	Bovine serum albumin
C	Centigrade
CI	Confidence Interval
CO_2	Carbon Dioxide
Cr	Creatinine
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DR	Death Receptor
EGF	Epidermal Growth Factor
FADD	Fas associated death domain
FFP	Fresh Frozen Plasma
FCS	Fetal Calf Serum
Fig	Figure
Fas-L	Fas ligand
GABA	γ-Aminobutyric acid
GFR	Glomerular filtration rate
γ-GT	Gamma glutamyl transferase
Hb	Haemoglobin
HBSS	Hank's Balanced Salt Solution
HCV	Hepatitis C Virus
HE	Hepatic Encephalopathy
HF	Hepatic Failure
HGF	Hepatocyte Growth Factor
Hrs	Hours
HRS	Hepato-renal Syndrome
IL	Interleukin
INR	International Normalised Ratio
LDH	Lactate Dehydrogenase
LFP	Liver Failure Plasma
LPS	Lipolysaccharide
LU	Luminescence units
MARS	Molecular Adsorbents Recirculating System
Min	Minutes
MMP	Mitochondrial Membrane Permeablisation
MPT	Mitochondrial Permeablisation Transition
MOPS	3-(N-morpholino) propanesulfonic acid
MTT	(3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

MW	Molecular Weight
NASH	Non-alcohol Steatohepatitis
NFκβ	Nuclear Factor kappa beta
PBS	Phosphate Buffered Saline
POD	Paracetamol overdose
PT	Prothrombin Time
RNA	Ribonucleic Acid
RPM	Revolutions per minute
SDS-PAGE,	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Sig	Significant
SIRS	Systemic Inflammatory Response Syndrome
SiRNA	Silent interfering RNA
TAA	Thioacetamide
TGF-ß	Transforming Growth Factor-beta
TPr	Total Protein
TRIAL	Tumour Necrosis Factor related apoptosis inducing ligand
TNF-α	Tumour Necrosis Factor – alpha
TNFR1	Tumour Necrosis Factor receptor 1
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
Ur	Urea
UV	Ultra-violet
Vols	Volumes

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SYNOPSIS

This project has developed from the need to identify techniques to provide artificial liver support to patients with liver failure, a condition in which the pathogenesis is poorly understood and mortality remains high. The ultimate aim of the liver group is to develop a bio-artificial liver which can maintain life until recovery of the native liver or availability of a donor organ. There are multiple problems which must be overcome to develop such a device, particularly identifying cells that are available in sufficient numbers at short notice; that are safe; and can provide all the essential and complex functions that endogenous hepatocytes and cholangiocytes perform. These functions must continue in an efficient manner when the cells are exposed to the patient's plasma, an environment that may be substantially hostile both as consequence and cause of the patient's original pathology. The starting point for the project was therefore to investigate the observation that plasma from patients with acute liver failure was deleterious to the function and viability of hepatocytes derived from tumour cell lines.

The first chapter is a general introduction and describes the clinical features and pathophysiology of liver failure. It summarises the evidence that liver failure plasma is toxic to multiple organ systems and cell types, describes the two mechanisms of cell death, apoptosis and necrosis, and their role in liver disease and identifies some candidate molecules which have been identified as hepatotoxic. Finally it sets out the aims and objectives of the project which were to develop a model by which the cytopathic effect of liver failure plasma could be studied, to attempt to isolate and identify molecules within plasma that are

toxic, to elucidate their mechanism of action and to find a method of removing or antagonising this toxicity.

There follows preliminary work using a model developed previously within the department, and utilised plasma samples for which large volumes were available (samples taken from patients with Acute Liver Failure collected at the onset of plasmapheresis). It identified that there was variability in toxicity in acute liver failure plasma but that not all samples were cytotoxic. Using the plasma sample that induced the greatest loss in cell viability (using MTT activity in HepG2 cells as a marker of cell number) a number of experiments were performed to identify some of the physico-chemical properties of the toxic molecule/s within Acute Liver Failure Plasma. By doing this we hoped to find a method to purify the substances so that they could subsequently be identified using high pressure liquid chromatography or magnetic resonance spectroscopy.

We identified a toxic component of acute liver failure plasma that passed through a centrifugation device with a molecular weight cut off of 100kDa, but was excluded by a 30kDa device, suggesting the substance/s had a molecular weight of between 30kDa and 100kDa or was bound to a substance in that molecular weight range for example albumin. The substance was water soluble and heat labile. The removal of the toxic substance by removal of albumin from the plasma suggested that the toxic substance was albumin bound and the molecule itself was not dialyzable across a 10kDa cut off membrane with either phosphate buffered saline solution or 20% bovine serum albumin solution, nor was it removed by adsorption with activated charcoal, inferring that therapeutic devices using charcoal haemadsorption, or dialysis would not be beneficial in removing the toxin, and would be unlikely to show benefit in clinical studies.

During these experiments we found that some hepatocytes lost adhesion when exposed to acute liver failure plasma and further study revealed this effect occurred over a time course of 6-24 hours. Reviewing the original earlier studies of cytopathic effects we noted the experimental methods used for quantifying cell death relied on an assumption that cells were only lost by cell death, i.e. did not account for cell loss by loss of adhesion (even though changes in cell morphology and adhesion were noted in one study) . The time course of these earlier experiments exposed cells to liver failure plasma at concentrations from 10% to 25% vol:vol, for time periods ranging from overnight to 48 hours, and would therefore have been subject to this loss of adhesion effect.

At the end of this preliminary work we concluded that the toxic molecule/s that had been physically characterised and others had previously described, was responsible for loss of cellular adhesion and not cell death. Having eliminated this adhesion effect, there was little toxicity that could be identified with the current model and certainly not enough of a toxic effect to be traced through separation and purification.

The second chapter therefore focused on the development of a robust in-vitro model in which to investigate the effects of liver failure plasma on hepatocytes. We defined the ideal characteristics of such a system and using primary human hepatocytes established a quantitative system for assessment of the effects of substances on the process of apoptosis, a key biological process in the pathogenesis of a number of liver diseases including acute liver failure. By the end of the chapter we had identified a positive control, established two reliable techniques for the measurement of apoptosis and then validated the model by

investigating the protective effects of the growth factors EGF and HGF against Fas-Ligand induced apoptosis.

Chapter 3 then used the model to investigate the effects of liver failure plasma on apoptosis. We established that plasma from patients with acute liver failure induced increased apoptosis in primary human hepatocytes, but that plasma from patients with acutely-decompensated chronic liver disease did not. There were no clinic-pathological correlates between a sample's pro-apoptotic tendency and other markers of liver function or renal function. We used specific Caspase inhibitors to investigate the mechanism by which acute liver failure plasma was inducing apoptosis and established that the effects were inhibited by Caspase 3 (a general effector caspase) and caspase 8 a caspase through which death receptors execute apoptosis. We concluded that acute liver failure plasma increased apoptosis via a death receptor pathway. Further studies suggested that the factor inducing apoptosis via a death receptor pathway was heat labile.

In chapter 3 we used the Thioacetamide toxic liver injury in the rat as a model to investigate if caspase inhibitors could be of therapeutic value. We demonstrated that apoptosis was implicated in the patho-physiology of liver injury in this model but could not demonstrate any therapeutic benefit in our primary endpoint, which was decreased mortality. This may have been in-part to a lower than expected mortality in the control population, which was significantly lower than described previously in this model.

In chapter 5 we discussed the development of bio-artificial liver support devices and in particular the mechanism of action of the Molecular Adsorbents recirculating system, we proposed that the removal of albumin bound toxins could remove the pro-apoptotic factor/s and therefore post treatment plasma

would be less pro-apoptotic than pre-treatment. We described the clinical application and outcomes of MARS treatment in our unit. We showed improvement in several clinical parameters but treatment did not have a significant effect on the pro-apoptotic effect of liver failure plasma.

We summarise our findings, critique our work and describe future experiments to further this area of research in the final chapter.

CHAPTER 1: INTRODUCTION

1.0 BACKGROUND

The liver is one of the largest organs in the body and plays a crucial role in metabolism, it consists of several cell types, 60% of which are hepatocytes (parenchymal cells); non-parenchymal cells include endothelial cells, Kupffer cells, Stellate cells, pit cells and biliary epithelial cells (Cholangiocytes). The major synthetic, metabolic and detoxifying functions of the liver reside in hepatocytes, and although it has huge functional reserve and regenerative capacity, allowing transplantation and resection of large volumes of liver and complete recovery from severe liver insults, a minimum amount of hepatocyte function must always be retained or death will inevitably follow.

1.1 LIVER FAILURE – CLINICAL FEATURES

Hepatic Failure (HF) is the clinical syndrome which results from loss of liver function usually due to sudden hepatocyte cell death, which despite advances in critical care medicine and transplant medicine, remains a condition with a high mortality (Auzinger *et al.*, 2008, Craig *et al.*, 2010, O'Grady, 2007).

It can be caused by a wide variety of insults including drugs, toxins, viruses, alcohol, ischeamia, metabolic disorder, septic shock, massive malignant infiltration and autoimmune conditions (Lee et al, 2008). Globally the commonest cause remains viral hepatitis but the commonest cause in the western world is drug induced liver injury with the vast majority being as a result of Paracetamol ingestion (Lee, 2004, Lee & Lee, 2008b, Polson & Lee, 2007, Williams, 2003). This continues to be the case in the United Kingdom; despite changes in packaging and sales of over the counter Paracetamol (Bateman & Bateman, 2009), Paracetamol poisoning still accounts for 50-60% of admissions

to specialist liver units (Marudanayagam *et al.*, 2009) (O'Grady, 1997). Despite the diverse nature of the aetiologies of this disease a fundamental end point of them all is death of hepatoctytes and loss of the minimum requirement of functioning hepatocytes to maintain normal function, this loss of liver cell mass can only occur by two basic mechanisms, apoptosis or necrosis (Alison & Sarraf, 1994, Malhi *et al.*, 2006).

The original definition of Fulminant Liver failure by Trey & Davidson was that of a potentially reversible failure of liver function resulting in encephalopathy within 8 weeks of the first appearance of any signs or symptoms of liver disease (Trey *et al.*, 1970). However more recent definitions further classify liver failure depending on the time from onset of jaundice to encephalopathy into hyperacute (onset of encephalopathy within seven days of the onset of jaundice), acute (time from jaundice to encephalopathy eight to twenty-eight days) and sub-acute (time from jaundice to encephalopathy between four and twelve weeks).

These definitions have been particularly useful in the management of patients enabling risk stratification of patients in terms of prognosis, with patients with hyperacute liver failure having the best prognosis (36% survival) compared to those with sub-acute liver failure (14% survival) (O'Grady *et al.*, 1993, O'Grady, 2007)

The syndrome of liver failure is characterised by a number of symptoms and signs.

Jaundice

Jaundice occurs due to the inability of the liver to conjugate and excrete bilirubin. There is also a small contribution from increased production of bilirubin due to increased destruction of red blood cells (Brunner G & Mito M, 1992).

Neurological Features

Neurological disturbance is a common manifestation of liver failure which is categorized by the West Haven criteria as grades I-IV (Ferenci *et al.*, 2002). It actually represents a continuous spectrum of abnormalities from subclinical electrophysiological changes, through subtle changes in personality, changes in sleep wake pattern and fine motor disturbance, to changes in conscious level and flapping tremor through to coma.

The exact cause of hepatic encephalopathy is unknown; but increased ammonia, the presence of products of bacterial metabolism, increased quantities of aromatic amino-acids and other toxins are all postulated mechanisms which contribute to disturbances in several neurotransmitter systems including glutamate, GABA, Dopamine, and Serotonin systems (Ash, 1991, Butterworth, 2003, Haussinger *et al.*, 2008, Lemberg *et al.*, 2009).

As well as hepatic encephalopathy, patients with acute liver failure develop cerebral oedema and intra-cranial hypertension (Vaquero *et al.*, 2003). Mechanisms suggested for the development of this phenomenon include the cytotoxic hypothesis which suggests that the accumulation of osmolytes (osmotically active substances) result in glial cell swelling and the vasogenic hypothesis which suggests that changes in blood flow and permeability of the

blood-brain barrier are responsible. It appears that proinflammatory cytokines derived from microglial cells and oxidative/nitrosative stress are key components in the generation and perpetuation of astrocyte swelling and changes to the permeability of the blood brain barrier (Jiang *et al.*, 2009).

Raised intra-cranial pressure can result in brainstem herniation and death (Larsen & Wendon, 2002).

Coagulopathy

With the exception of factor VIII all clotting factors, as well as components of the fibrinolytic system and inhibitors of coagulation are produced by the liver. Failure of hepatocyte protein synthesis results in decreased production of these factors and prolongation of the prothrombin time. Disseminated vascular coagulation is also common further disturbing clotting and consuming platelets. Life threatening bleeding is thus a frequent complication of liver failure.

Renal failure

Renal failure is a common complication of acute liver failure affecting 55% of patients referred to specialist liver units. It is often due to Acute Tubular Necrosis which results from factors such as sepsis, hypotension, hypoxia and changes in renal perfusion or due to direct toxicity in the case of paracetamol toxicity.

Liver failure itself can also directly give rise to renal failure, the so-called hepatorenal syndrome (HRS). The pathogenesis of HRS is a consequence of circulatory changes which result in a hyperdynamic circulation, decreased renal perfusion pressures, activation of the sympathetic nervous system and release of

vaso-active mediators, which result in renal vasoconstriction and direct changes in the glomerular ultrafiltration coefficient resulting in a decrease in glomerular filtration rate beyond that caused by changes in renal perfusion alone. HRS is reversible with the recovery of the native liver or liver transplantation (Moore, 1999)

Metabolic changes

There are multiple metabolic derangements in liver failure. Decreased insulin uptake and decreased gluconeogenesis by the liver results in hypoglycaemia.

Failure of conversion of ammonia to urea, changes in amino-acid metabolism resulting in increased aromatic and decreased branch chain amino acids all result from the liver's failure of nitrogen metabolism and may contribute to hepatic encephalopathy. Decreased synthetic function results in decreased albumin synthesis and a consequent fall in serum albumin concentration, contributing to the production of oedema and ascites.

Hyponatraemia, hypokalaemia, hypomagnesaemia, hypocalcaemia, hypophasphataemia, respiratory alkalosis and metabolic acidosis are all consequences of liver failure (Bernal & Wendon, 1999).

Haemodynamic changes

Changes in systemic vascular resistance cause peripheral vasodilatation and result in an increase in cardiac output, by increasing both heart rate and stroke volume. Despite this high cardiac output state patients remain hypotensive. On examination they may be found to be peripherally warm with a bounding pulse, tachycardic, prominent apex beat and ejection systolic flow murmur. This

hypotension results in decreased perfusion of organs such as kidneys and the liver, resulting in renal failure and further liver injury. Renal hypoperfusion results in activation of the renin-angiotensin system and consequently retention of salt and water contributing to oedema and ascites formation.

The aetiology of this systemic vasodilation is currently unknown, and is most likely multifactorial. Some vasoactive substances may be produced by 'sick' cells within the liver, other factors may be due to the presence of vasoactive substances from the bowel (which may have increased permeability) which are usually inactivated/removed by hepatocytes, appearing in the circulation or being shunted through intra- or extra-hepatic shunts by-passing the liver (Ellis & Wendon, 1996).

Systemic Inflammatory Response and Sepsis

Septicaemia is a frequent terminal complication of liver failure. Impaired function of cells of the immune system such as Kupffer cells and polymorphs, impaired production of opsonins and factors of the complement cascade decrease resistance to infection. With increased bacterial translocation through the bowel and instrumentation of patients by cannulae and catheters possible sources of infection are increased. Diagnosis can be difficult with many patients being apyrexial in the presence of sepsis. A generalised systemic inflammatory response can occur in the absence of infection due to increased cytokines such as TNF-alpha resulting in a low grade temperature, acute lung injury and other end-organ damage.

Non-specific symptoms and signs

Lethargy, malaise and poor appetite are common non-specific features of liver failure, often associated with generalised weakness and muscle wasting due to a combination of malnutrition, poor protein synthetic function and a generalised catabolic state.

1.2 PATHOPHYSIOLOGY OF FULMINANT HEPATIC FAILURE

The clinical syndrome of acute liver failure is thought to be accounted for by three major pathophysiological processes.

- The first is the failure of normal hepatocyte metabolic function to reach the critical minimum threshold required to meet the basic metabolic needs of the body. This failure results in other organs becoming damaged; examples of this are coagulopathy due to lack of production of clotting factors and hypoglycaemia. This theory is called 'the metabolic mass theory' (Atillasoy *et al.*, 1995).
- The second process of major importance is the detoxifying ability of the liver. All blood from the portal circulation passes directly to the liver; here a variety of toxins are normally processed by the healthy liver being completely removed or inactivated before they reach the systemic circulation and thus other organs. The 'toxin hypothesis' proposes that failure of this detoxification function results in elevated levels of toxins such as ammonia, phenols, mercaptans, aromatic amino acids, fatty acids, benzodiazepine like substances, endotoxin, nitric oxide and cytokines in the systemic circulation thus allowing them to damage other organs e.g. causing encephalopathy.

- A third factor in the development of the clinical syndrome of acute liver failure is the contribution of the liver itself. The liver consists of several different cell types in addition to hepatocytes, including Kupffer cells, vascular endothelial cells, and stellate cells; these cells as well as hepatocytes may be partly responsible for the perpetuation of liver injury by producing substances that may cause end-organ damage. Thus the injured hepatocyte may itself aggravate and exacerbate liver injury ultimately leading to hepatocyte loss by a variety of mechanisms. These include loss of plasma membrane integrity, loss of intracellular homeostasis, oxidative stress, mitochondrial dysfunction, ATP depletion and activation of degradative hydrolysis ultimately resulting in cell death by apoptosis or necrosis (Rosser & Gores, 1995). The suggestion that the liver is itself responsible for end organ damage rather than simply lack of metabolic or detoxifying functions is supported by the short-term clinical improvement in patients with acute liver failure when the failing liver is removed, temporarily rendering the patient anhepatic (Butterworth, 2003). Many of these substances released from the liver result in a marked systemic inflammatory immune response (SIRS) which is a dominant feature of acute liver failure.

Whilst these three mechanisms are suggested as possible alternative mechanisms in the pathogenesis of acute liver failure it is likely that all three are involved to a greater or lesser degree in the development and perpetuation of liver injury after the initial insult. A complex interplay between these mechanisms with the liver releasing substances, or allowing substances it normally removes to build

up to such a degree that hepatocyte death occurs, may result in further hepatocyte death and decreased functional liver cell mass, which results in the release of further toxins thus resulting in a vicious circle that ultimately results in the patient's demise.

Countering this effect is the ability of the liver to regenerate and thus the balance between the rate of hepatocyte death and hepatocyte regeneration will ultimately determine the patient's survival.

1.3 TOXIC MOLECULES IN LIVER FAILURE PLASMA

Early studies in which cross circulation between baboons and men with hepatic coma occurred, improved the condition of the patient but led to a deterioration in the health of the baboon (Abouna, 1968). This suggested that toxins accumulating within the blood were responsible for other end-organs being damaged. Some of these factors may be due to changes in the cellular component of blood, but many of these deleterious effects are due to changes in the humoral component of circulating blood. These toxins may arise either as a consequence of a failure of normal hepatic clearance, or because those substances are generated within the liver or elsewhere in the body as a consequence of severe liver disease (Bradham *et al.*, 1998, Cain & Freathy, 2001, Spengler *et al.*, 1996). These toxic factors in the blood affect the function of many organ systems, such as the systemic and portal vasculature and the brain, as well as the liver itself. The exact nature of these toxins is unknown and may be different and multiple for each organ system damaged. Ammonia, aromatic amino-acids, tryptophan, indoles, mercaptans and endogenous benzodiazepines are implicated in the development of hepatic encephalopathy.

Whereas, prostanoids, inflammatory cytokines, nitric oxide and oxidative stress, are all considered to be important factors in the development of the haemodynamic and renal changes seen in liver failure. It is, however, the substances that are directly hepatotoxic that are particularly important in terms of recovery, as they may perpetuate liver injury invoking a downward spiral with further reduction in functional liver mass and increased toxin load, persisting long after the withdrawal of the original insult precipitating liver failure (Williams *et al.*, 1977). It is notable that many of the suggested toxins are insoluble in water and exist in the circulation bound to albumin.

Since Abouna's original experiment it has been demonstrated that liver failure plasma contains increased levels of a vast array of substances, for example bile salts, which in-vitro are toxic to hepatocytes. More significantly direct evidence for cytotoxicity has been shown by the application of plasma from patients with acute liver failure to both primary rabbit hepatocytes (Hughes *et al.*, 1976) and immortalised cell lines (Anderson *et al.*, 1999, McCloskey *et al.*, 2002) both of which suffered from increased cell death compared to exposure to normal control plasma. The measurement of cell death has usually been by demonstrating decreased viable cells after exposure to liver failure plasma, although few studies have labelled dead cells using substances necrotic cells are permeable to, for example, propidium iodide and trypan blue and thus the mechanism of cytotoxicity remains unidentified. Whilst these results provide interesting data, problems with using immortalised cell lines, which by definition have dysregulated cell death and proliferation pathways and the use of primary non-human hepatocytes in experimental models has lead to concerns over the applicability of these results to human disease processes.

The previously described toxic effects are not limited to simply inducing cell death but also have inhibitory effects on metabolic function, adhesion and hepatocyte proliferation. These effects on regeneration have been demonstrated in-vitro using radiolabelled thymidine incorporation as a measure of DNA synthesis in hepatocyte cell lines (Williams, Hughes, Cochrane, Ellis, & Murray-Lyon, 1977) and in primary rodent hepatocytes (Yamada *et al.*, 1994) and in-vivo using either human plasma injected into partially hepatectomised rats (Hughes *et al.*, 1991) or plasma from a rodent model of acute liver failure exchanged with plasma from normal control rats (Anilkumar *et al.*, 1997). In addition to cytotoxicity these inhibitory effects on regeneration are particularly important since it is the balance between cell death and proliferation which determines final recovery.

In addition to inducing endogenous hepatocyte injury these effects remain a significant barrier to hepatocyte/stem cell transplantation and maintenance of function of bioartificial liver support devices when exposed to patient plasma.

Although many candidate molecules, particularly cytokines, with the above effects have already been identified at increased levels in acute liver failure plasma their exact roles have yet to fully elucidated in human disease, particularly in the presence of increased levels of other protective factors found in acute liver failure.

Also there are likely to be many factors as yet unidentified which may have a role to play. It remains a huge challenge to identify which factors are of key importance in the pathophysiology of acute live failure.

The problem can either be approached by trying to identify the physico-chemical properties of these substances, purify them and identify them using mass

spectrometry or by identifying the molecular mechanisms of liver injury, identifying ligands and identifying their role in acute liver failure. Both methods have their proponents but a significant barrier to them both is the lack of a good model to test the effects of proposed toxins on model systems applicable to human liver disease.

1.4 MECHANISMS OF HEPATOCYTE INJURY

Liver cell death is the fundamental cause of the clinical syndrome of liver failure. Liver cells can only die by two distinct pathways, necrosis or apoptosis. Apoptosis or programmed cell death, first defined by Kerr et al in 1965 "a process in which cells die in a controlled manner in response to specific stimuli, following an intrinsic program" (Kerr JF & Wyllie AH, 1972, Kerr JF, 1965). Distinct from necrosis, cells which execute their apoptotic programme undergo a variety of characteristic morphological and biochemical changes which result in the formation of small packages of eosinophillic intracellular material called "apoptotic bodies" or "Councilman bodies" which can be removed by phagocytosis. The controlled manor in which the cell dies, and the lack of spillage of potentially noxious intracellular substances into the surrounding microenvironment, allows removal of a single cell in the absence of inflammation or disturbance to its neighbours.

However, in the majority of diseases causing acute liver failure, inflammation and hepatocyte necrosis are prominent. Cellular swelling is the predominant feature of necrosis, associated with small protrusions of the cell wall called blebs. Lysosomal breakdown, mitochondrial depolarisation and anionic ion flux add to cell swelling which ultimately results in rupture of one of the blebs

resulting in leakage of intracellular contents. Distinct from apoptosis, necrosis is often seen in contiguous cells.

Since necrotic cells vastly outnumber apoptotic ones it has only been with advances in our understanding of the mechanisms of apoptosis and molecular biology that adequate sensitive and specific techniques for identifying apoptotic cells have been identified. These have shown that there are increased numbers of apoptotic hepatocytes in a number of human liver diseases compared to being exceedingly rare in normal liver tissue. Although the total numbers at first glance seem inconsequentially small, the apoptotic process is very quick being complete in 4-6 hours and therefore apoptosis alone could account for substantial hepatocyte loss. Schulte-Hermann et al have suggested that a 4% rate of apoptosis would result in a 25% loss in liver cell mass in 72 hours (Schulte-Hermann *et al.*, 1999). In addition a number of animal models of liver failure have shown apoptotic cells appear very early in the course of liver damage, prior to the appearance of necrosis or significant symptoms, since most of the human tissue examined for apoptosis has occurred after the onset of liver failure it may be that this underestimates the degree of apoptosis earlier in the disease process. Finally, and most surprisingly, the inhibition of apoptosis in some animal models of acute liver failure by specific apoptosis inhibitors results in the absence of both apoptosis and necrosis on liver histology as well as the attenuation of liver failure and mortality (Bajt *et al.*, 2001, Hoglen *et al.*, 2001, Rouquet *et al.*, 1996). It may therefore be that apoptosis is an essential early event in the initiation of liver damage and that by overwhelming the processes that remove apoptotic bodies and protect surrounding cells secondary necrosis occurs.

The polarised view of cell death occurring by either apoptosis or necrosis with discrete initiating factors is however being replaced by an emerging view that both types of cell death can be initiated by common factors and that both represent extremes on a continuum of cell death. Fundamental to the type of cell death that occurs is the fact that apoptosis is an energy requiring process requiring ATP. Thus the key feature that differentiates the choice of cell death is the ability of that cell to generate enough ATP via its mitochondria to execute the apoptotic pathway.

Cytochrome c release from mitochondria represents the point of no return for initiation of cellular apoptosis, but it also is noteworthy that it is the point of destruction for the mitochondria and thus the cessation of ATP production and commitment of the cell to death via necrosis.

Noxious insults, ligand-receptor pairs and signalling pathways may thus be common to both types of cell death; it is therefore not surprising that both types of cell death are seen concomitantly in the same disease processes. It is likely that different types of liver injury have a predilection for inducing different types of liver cell death and that different cell types and differing energy status within same cell types have differing predilections for the manner of cell death. For example the early phase of ischaemia/reperfusion injury induces predominantly necrosis with hepatocytes in the pericentral (perilobular) areas most vulnerable, with apoptotic cells being scarce (only about 2% of cells), and caspase inhibitors offering no protection. The later phase of ischaemic injury results from activation of innate cellular immunity triggered by ischaemia of Kupffer cells inducing them to release reactive oxygen species, cytokines, chemokines and other factors which lead to infiltration by neutrophils and CD4+ lymphocytes.

These cells may induce apoptosis, and therefore both types of cell death co-exist in the same disease, the balance of which type of cell death is determined by the severity of the original injury. Inhibition of apoptosis by various mechanisms has been shown to attenuate this later phase of liver injury.

It could be argued that necrosis is the default pathway by which cells undergo cell death in the absence of adequate ATP; the contrary can also be argued, in that cellular damage may be insufficient to induce necrosis but sufficient to induce apoptosis ensuring removal of damaged cells. It is however fascinating that evolution has devised a "belt and braces" approach to so many fundament cellular mechanisms including cell death.

In the long-term the production of apoptotic bodies leads to fibrosis (Murphy *et al.*, 2002). Stellate cells are activated by engulfment of apoptotic bodies in culture. Activated stellate cells produce collagen, inhibition of hepatocyte apoptosis decreases fibrosis in murine models of liver injury (Canbay *et al.*, 2003).

1.5 APOPTOSIS BASIC SCIENCE

The current widely accepted model is that apoptosis can be initiated by two basic converging pathways - receptor mediated apoptosis and the mitochondrial pathway. In addition there are several minor pathways, but these are less well characterised. Receptor mediated apoptosis is initiated by the binding of ligand to a cell surface receptor. There are a number of these so called "death receptors" including TNF-alpha, TGF-beta, and Fas (CD95). These are transmembrane proteins with three domains, an extra-cellular ligand binding domain, a transmembrane domain and an intracellular death domain. Death

receptors of importance in liver disease include Fas (CD95/Apo-1), tumour necrosis factor receptor 1 (TNFR-1), tumour necrosis factor related apoptosis inducing ligand (TRAIL) receptors 1&2, death receptor (DR) 5 & 6, and various other death receptors and combinations of receptors. Fas is extensively distributed through a wide range of tissues including cholangiocytes, sinusoidal endothelial cells, stellate and Kupffer cells, and is constitutively expressed by hepatocytes. It is the best characterised of the death receptors and is thought to play a central role in initiating apoptosis within the liver. Binding of ligand, Fas-Ligand, to its receptor results in trimerisation of the receptor and brings their intracellular N-terminal domains into close proximity; these bind to other intracellular proteins and form an active "death domain". In the example of Fas, this is the fas associated death domain or FADD. In hepatocytes Fas localises predominantly to the Golgi complex and trans-Golgi network with only small amounts expressed in the plasma membrane. This allows rapid translocation of receptors to the plasma membrane in response to noxious stimuli (Feldmann *et al.*, 1998). Fas activation classically occurs by ligand binding, but if the density of Fas receptors becomes sufficiently dense trimerisation occurs spontaneously and thus activation of receptors can occur in the absence of ligand binding representing a second means of activation. Fas-L itself increases localisation of Fas to the cell membrane.

TNFR1 is distinct from other death receptors in that it also activates survival pathways. Proteins which form components of its death receptor domain (TRADD) include RIP (receptor-interacting protein) and TRAF-2 (TNF associated factor 2). TRAF-2 first activates NFkB and c-jun N terminal kinase (JNK) before internalisation of the ligand disassociated complex forming the

death inducing signal complex (DISC) which recruits FADD via interactions between conserved death domains.

These death effector domains (DED) activate pro-caspase 8, a member of the caspase family. Caspase 8 cleaves BID (BCL-2 Interacting Domain, a pro-apoptotic member of the Bcl-2 family of proteins) to tBID and with sufficient stimulation directly activates Caspase 3.

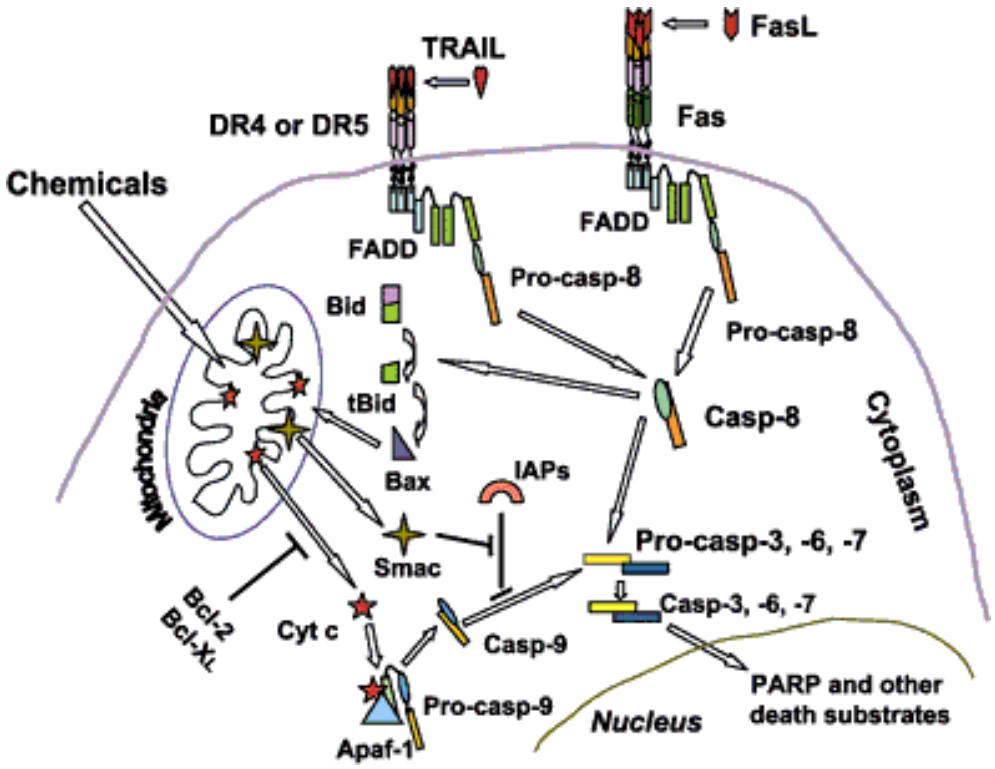


Figure 1. Schematic of pathways utilised by death receptors in induction of apoptosis *(courtesy of S.Sun)*

Cells can be classified into discrete subtypes; Type 1 cells which utilise the pathway of Caspase 8 directly activating Caspase 3 and type 2 cells which rely on Caspase 8 cleaving BID to tBID. This tBID translocates to mitochondria resulting in mitochondrial permeablisation and release of cytochrome c. Hepatocytes are type 2 cells dependent on this pathway to execute apoptosis.

This is demonstrated by the fact that the BID knockout mouse is significantly protected against Fas-L induced liver injury. (El Hassan *et al.*, 2003). In type 2 cells cytochrome c released from mitochondria associates with Apaf-1 to form haptomeric apoptosomes which proteolytically activate Caspase 9 which in turn activates Caspase 3.

Caspases are a family of cysteine-aspartate proteases which characteristically possess an active site cysteine and cleave substrates after Aspartic acid residues. The specificity of each Caspase is determined by the four amino acid residues to the amino-terminal side of the cleavage site. They exist in the cytoplasm in an inactive pro-caspase form and limited proteolysis causes conversion to their active form. All pro-caspases are activated by cleavage of their prodomain after an Aspartic acid residue. This makes them candidates for autocatalytic activation. In this way activation of caspases lower down the cascade can catalyse those upstream, as well as themselves, resulting in a positive feedback loop allowing rapid activation and massive amplification of this signal. There are currently 13 caspase members some of which are involved in initiating the cascade - "so called initiator caspases" - e.g. Caspase 8 and 9 which seem to have more limited substrates and be responsible for propagation of the apoptotic signal, and some more abundant "effector caspases" which are considered the workhorses of the caspase family. These have a wide variety of substrates including endonucleases, cytoskeletal proteins and transcription factors which are largely responsible for the controlled dismantling of intracellular machinery and the development of the morphological changes characteristic of apoptosis culminating in the formation of an apoptotic body (Cohen, 1997). For example, Caspase 3 causes activation of transcription factors that modulate the expression

of various pro- and anti-apoptotic factors among which are the Bcl-2 family of mitochondrial proteins, cytochrome c, and various tumour suppressor genes, as well as endonucleases. Caspase 3 also cleaves anti-apoptotic proteins e.g. Bcl-2 and Bcl-xl that normally protect the mitochondrial membrane from permeablisation. Activation of specific serine/threonine phosphatases results in changes in phosphorylation status and activation of pro-apoptotic factors e.g. BAX, BAD, BID and BIK in addition to caspases cleaving them to more potent pro-apoptotic forms. It should also be noted that some caspases (1,4 & 5) are involved in the mediation of the inflammatory response (Martinon & Tschopp, 2004).

Changes such as these to the protein composition of the mitochondrial membrane and the state of various ion channels and receptors within it result in a sudden change in its permeability, so called mitochondrial membrane permeablisation (MMP) and this causes rapid release of cytochrome c (the electron transfer protein) from the mitochondrial inner membrane into the cytoplasm.

The exact mechanism by which cytochrome c release occurs remains controversial and multiple mechanisms may exist. Mechanisms suggested include the formation of specific channels by pro-apoptotic members of the Bcl-2 family proteins (e.g. tBID, BAX, BAD) in the mitochondrial outer membrane, the opening of non-specific solute conducting channels called Mitochondrial Permeability Transition Pores in the mitochondrial inner membrane which cause mitochondrial swelling and outer membrane rupture, and the formation of pores by aggregation of misfolded membrane proteins associated with high mitochondrial Ca^{2+} levels and a lack of chaperoning proteins which normally

bind and close pores. Whatever the mechanism, the sudden release of cytochrome c represents the point of no return for the cell. The released cytoplasmic cytochrome c (associated with Caspase 9 and Apaf 1) forms apoptosome, this further activates Caspase 3 which in turn activates further release of cytochrome c and thus a positive feedback loop is created (Li *et al.*, 1998). In addition there is activation of CAD, a caspase activated DNAse, an endonuclease which is responsible for the fragmentation of genomic DNA into 50kb fragments one of the hallmarks of apoptotic cell death. Other mitochondrial membrane proteins e.g. Apoptosis inducing factor are transported to the nucleus where they induce the characteristic morphological changes of apoptosis as seen on light microscopy (see table 1.). With the use of special stains differences in membrane expression (e.g.Annexin V), DNA fragmentation (e.g.TUNEL) and caspase activation (e.g. Caspatag) can be utilised to differentiate between apoptotic and necrotic cells.

Table 1. Morphological changes in Apoptosis and Necrosis

APOPTOSIS	**NECROSIS**
Deletion of single cells	Death of groups of cells
Membrane blebbing, but no loss of integrity	Loss of membrane integrity
Cells shrink, ultimately forming apoptotic bodies	Cells swell and lyse
No inflammatory response	Significant inflammatory response
Phagocytosis by adjacent normal cells and some macrophages	Phagocytosis by macrophages
Lysosomes intact	Lysosomal leakage
Compaction of chromatin into uniformly dense masses	Clumpy, ill-defined aggregation of chromatin

The mitochondrial pathway is less well characterised, it does not require activation of cell surface receptors or Caspase 8, is mediated by Caspase 9, and can be instigated by a number of physical events particularly those that generate oxidative stress, typically by DNA damage, p53 activation and PUMA expression. It still relies on changes in MMP, cytochrome c release and Caspase 3 as the final common pathway of cell death.

Much is still to be learnt about the control of apoptosis and its pathways and the current model is an oversimplification. Alternative pathways must exist as the Caspase3 knockout mouse still undergoes Fas-L binding mediated liver injury and death, albeit delayed (Woo *et al.*, 1999). However there is at least an outline which provides a framework in which to develop strategies to inhibit apoptosis.

1.6 APOPTOSIS IN LIVER DISEASE

The following is a brief résumé of evidence suggesting mechanisms by which apoptosis is implicated in various forms of liver disease:

Cholestatic liver disease

There are increased numbers of hepatocytes undergoing apoptosis in some cholestatic liver diseases including Primary Biliary Cirrhosis (Papakyriakou *et al.*, 2002).

This may be due to direct toxicity by accumulated bile salts since it has been demonstrated in cell culture that toxic hydrophobic bile acids can initiate apoptosis both via Fas translocation and Fas-ligation (Guicciardi & Gores, 2000) and via activation of TRAIL (Kahraman *et al.*, 2008). Bile acid levels in cholestatic patients are increased to sufficient levels to activate death receptor pathways and Fas deficient lpr mice are protected against hepatocyte apoptosis

and subsequent liver fibrosis seen in normal mice induced by bile duct ligation (Canbay *et al.*, 2002), demonstrating a mechanistic link between cholestasis and fibrosis. Acute cholestasis also induces oxidative stress via activation of NADPH oxidase, which increases superoxide which, increases translocation of Fas to the plasma membrane and also activates TRAIL-R2.

However not all bile salts are harmful to hepatocytes; some delay apoptosis by induction and translocation of Bax, a pro-apoptotic protein to mitochondria, and Ursodeoxycholic acid protects against bile salt induced apoptosis by preventing mitochondrial membrane permeability as well as decreasing stellate cell activation and fibrosis (Paumgartner & Beuers, 2004).

Apoptosis in cholangiocytes may also be involved in the pathogenesis of conditions such as Primary Sclerosing Cholangitis as demonstrated by the induction of apoptosis and a similar liver injury by an agonistic Death Receptor 5 antibody (Takeda *et al.*, 2008).

Viral hepatitis

Cytotoxic T lymphocytes and natural killer cells secrete Fas-L and it is thought to be one of the mechanism that virally infected hepatocytes are killed (Kagi *et al.*, 1994). Increased numbers of Hepatocytes undergoing apoptosis have been demonstrated via TUNEL in chronic viral liver disease (Papakyriakou *et al.*, 2002b) Hepatitis B (Mochizuki *et al.*, 1996) and C virus (Hiramatsu *et al.*, 1994) increase both soluble Fas (Iio *et al.*, 1998) and Fas expression whose levels correlate with histological disease activity (not biochemical) and response to therapy (Lee *et al.*, 2004, Luo *et al.*, 1997, Mita *et al.*, 1994). In the case of Hepatitis C an increase in activated Caspase 3 and 7 is in liver tissue which

correlates with inflammatory activity (Bantel *et al.*, 2001) TRAIL is also increased in the serum of patients with viral hepatitis (Mundt *et al.*, 2003).

When HCV specific CD8+ lymphocytes are transplanted into mice expressing HCV proteins liver damage and increase in ALT occurs (suggesting necrosis). This effect is also seen when lymphocytes are transplanted into transgenic mice expressing HBsAg and the flare in hepatitis is antagonised by soluble Fas which acts as a decoy receptor (Kondo *et al.*, 1997) , suggesting that the necrosis is secondary to apoptosis.

Alcoholic Liver Disease

In liver biopsies of patients with Alcoholic Hepatitis there is increased hepatocyte apoptosis measured both by TUNEL (Zhao *et al.*, 1997) and increased activation of Caspase 3 (Ziol *et al.*, 2001). In Ziol's study there was correlation between apoptotic index and Maddrey's discriminant function, bilirubin levels and the presence of ascites, whilst Natori's study showed a correlation to Bilirubin and histological severity (Natori *et al.*, 2001). Also liver biopsies of heavy drinkers show apoptotic cells in an identical distribution to hepatocytes containing intracellular Mallory bodies suggesting hepatocytes damaged by alcohol might be eliminated by apoptosis (Kawahara *et al.*, 1994). Soluble Fas, hepatic Fas and Fas-L expression are increased in Acute Alcoholic Hepatitis compared with those with alcoholic liver disease with no hepatitis (Tagami *et al.*, 2003) and levels correlate with liver injury, and in patients with alcoholic hepatitis TNFalpha and TNFR1 levels are elevated and correlate with mortality. These data suggest that apoptosis induced by the death receptor pathway is a key factor in the pathogenesis of Alcoholic Hepatitis. For this

reason there was hope that neutralising anti-TNF antibodies would be beneficial in the treatment of Alcoholic Hepatitis, unfortunately however this treatment did not result in a survival advantage (Blendis & Dotan, 2004), as there was an increase in death secondary to infection due to the immunosupressive effects of anti-TNF drugs.

In animal models of chronic alcohol related liver damage hepatocyte apoptosis has been demonstrated from acinar zone 3 to zone 2 following ethanol feeding of rats (Benedetti *et al.*, 1988, Rust *et al.*, 2000) . The exact mechanism by which alcohol has this effect is not fully elucidated but alcohol is known to induce oxidative stress which, in turn, can induce apoptosis both by direct and indirect mechanisms, thus oxidative stress has been implicated in both acute and chronic alcohol induced liver injury (Ishii *et al.*, 1997, Kurose *et al.*, 1997) (Higuchi *et al.*, 2001, Minana *et al.*, 2002). Again it appears death receptor mediated pathways are important in chronic liver disease, particularly the TNFR1 receptor as studies in TNFR1 knockout mice show attenuated ethanol induced liver injury induced by chronic alcohol ingestion (Yin *et al.*, 1999).

Nonalcoholic Steatohepatitis (NASH)

There are increased numbers of apoptotic cells demonstrated both by TUNEL and activation of Caspase 3 and 7 in liver biopsy specimens in patients with steato-hepatitis compared to both healthy controls and patients with steatosis only. The quantity of apoptosis being associated with biochemical markers of liver injury and histological severity (Feldstein *et al.*, 2003). Both Fas & TNFR1 expression are increased in steatohepatitis again compared to normal

controls suggesting that death receptor pathways may be implicated in this process (Ribeiro *et al.,* 2004).

The role of steatosis may be to increase hepatocyte sensitivity to Fas induced apoptosis as demonstrated in a murine model of liver injury induced by Jo-2, a Fas binding antibody that induces oligerization and activation of Fas; there is increased apoptosis in animals with diet-induced steatohepatitis compared to sham fed animals.

This has led to the concept of the "two hits" in the pathogenesis of NASH, the first being steatosis and the second being inflammation, apoptosis mediated by death receptor pathway activation being a key component of this second hit.

Liver transplantation

Apoptosis has been implicated in ischaemia-reperfusion injury post transplantation (Sasaki *et al.*, 1997), as well as the Seventh Day Syndrome (Memon *et al.*, 2001), and the long-term complication of Vanishing Bile Duct Syndrome (Nawaz *et al.,* 1994). The role of apoptosis in acute rejection may however only be a minor component (Tox *et al.*, 2001) In the immediate post transplant setting apoptosis is mediated at least in part by death receptor pathways as demonstrated by the attenuation of ischaemic induced apoptosis by silent interfering Fas RNA (Li *et al.*, 2007), silent interfering Caspase 8 and 3 (Contreras *et al.*, 2004) and by direct inhibition of Caspase 3 by direct Caspase inhibitors (Mueller *et al.*, 2004), Expression of soluble Fas but not soluble Fas Ligand is seen in serum post liver transplantation and may represent part of the immunological response post transplant (Seino *et al.,* 1999). TNFR1 knockout

mice have shown that graft TNFR1 deficiency increases graft injury whereas recipient TNFR1 deficiency results in decreased liver injury (Conzelmann *et al.*, 2002).

Manipulation of apoptotic pathways may present opportunities to decrease graft rejection and may allow progress in isolated hepatocyte transplantation, for example TGF-α over expression protects against apoptosis and increases liver repopulation by hepatocytes (Kosone *et al.*, 2008).

This is however a complex area as many of the pathways by which apoptosis is mediated are also essential for liver regeneration. TNFR1 expression is low in normal liver but is increased in a number of liver diseases suggesting a pathogenic role. Partial hepatectomy has a protective effect against Fas-ligand induced apoptosis and this effect surprisingly appears to be mediated by TNF-α (Takehara *et al.*, 1998). Global inhibition of apoptotic pathways may therefore not always be advantageous.

Acute Liver Failure

Increased hepatocyte apoptosis (Kasahara *et al.*, 2000), Fas expression, infiltration with Fas-L expressing cytotoxic lymphocytes and increased soluble Fas occur in FHF due to various aetiologies (Ryo *et al.*, 2000) Serum cytochrome c is elevated and correlates to serum ALT in ALF.

Various animal models of acute liver failure induce injury via death receptor pathways and many of these models have been utilised to investigate ways of limiting liver injury and will be discussed in subsequent chapters.

Acetaminophen toxicity

Acetominophen is metabolised to the toxic metabolite N-acetyl-p-benzoquinone imine (NAPQI) by the cytochrome p450 system. NAPQI forms covalent adducts and initiates mitochondrial oxidative stress and mitichondrial permeability transition(MPT) (Cohen & Khairallah, 1997, Nelson, 1990).

Cyclosporin A protects against acetaminophen toxicity both in vitro and vivo by blocking the MPT (Masubuchi *et al.,* 2005).

The role of apoptosis in acetominophen toxicity is controversial with some studies showing TUNEL cell positivity and DNA laddering in-vitro and in-vivo (Ray *et al.,* 1996) in mouse hepatocytes, and some studies showing only minimal apoptosis (Gujral *et al.,* 2002).

This demonstrates the fact that apoptosis and necrosis can be initiated by the same pathways but the final outcome is determined by the ATP status of the cell. Necrosis occurs if Acetaminophen causes mitochondrial dysfunction such that ATP is depleted; if this is prevented by fructose and glycine then apoptosis ensues (Kon *et al.,* 2004).

1.7 OPPORTUNITIES FOR DESIGN OF NOVEL THERAPEUTIC MODALITIES.

Every step of the pathways culminating in apoptosis is amenable to manipulation and is therefore a potential therapeutic target: For Example Bcl2 proteins are a family of mitochondrial proteins, some of which are pro-(Bid, Bax, Bak) and some of which are anti-apoptotic (Bcl_2, Bcl_{XL}). Manipulation of mitochondria to prevent MPT pore complex formation has been tried (Waldmeier *et al.,* 2003) or blockage of pores, using non-immunosupressive

analogues of Cyclosporin A (Halestrap *et al.*, 2004, Matsuki *et al.*, 2002, Waldmeier *et al.*, 2002). Caspase 8 inhibition using siRNA prevents acute liver failure in mice (Zender *et al.*, 2003), and ischaemia reperfusion injury in mice (Contreras, Vilatoba, Eckstein, Bilbao, Anthony, & Eckhoff, 2004). Caspase inhibitors decrease liver injury in many models of liver disease, including the bile duct ligated mouse (Canbay *et al.*, 2004), ischaemia reperfusion injury (Natori *et al.*, 2003), SiRNA targeting Fas protects mice against fulminant hepatitis (Song *et al.*, 2003). Fas siRNA protects transplanted hepatocytes in mouse spleen (Wang *et al.*, 2003). Soluble Fas gene therapy protects against Fas mediated apoptosis although does not prevent the lethal effects of Fas induced TNF-alpha production by kupffer cells.

1.8 ARTIFICIAL LIVER

Liver transplantation cures approximately 90% of patients with acute liver failure, however a shortage of donor organs combined with the fact that many patients will become too ill to undergo transplantation means mortality remains high.

Unlike many other organs the liver has a tremendous capacity for regeneration and repair even in adulthood. This ability is clearly demonstrated by the fact that after surgical resection the liver regenerates back to its original size and that some patients will make a complete recovery from fulminant hepatic failure with no long-term sequelae. Strategies that allow temporary liver support buying time for transplantation or endogenous liver recovery are therefore required. By performing the detoxifying function of the liver it is hoped an “artificial liver” would not only prevent other end-organs from damage but would make the

general environment of the patient's own hepatocytes less toxic, thus decreasing liver cell death and promoting regeneration and repair, breaking the vicious circle described above.

Early artificial liver support devices in acute and acute-on-chronic liver failure aimed at toxin removal such as whole blood exchange (Redeker *et al.*, 1973), haemoperfusion (Bartels, 1978), charcoal haemoperfusion (O'Grady *et al.*, 1988), haemodiabsorption (BioLogic-DT) (Hughes *et al.*, 1994) have failed to show any survival benefit. More recent detoxifying systems such as Extracorporeal albumin dialysis (MARS) (Stange *et al.*, 1999) have possibly been more successful, reducing mortality in a stratified meta-analysis in acutely decompensated chronic liver disease (Steiner & Mitzner, 2002). MARS allows removes albumin bound molecules. A special membrane allows transfer of water soluble and albumin bound toxins with molecular weight less than 50kDa from blood into a dialysate solution containing 20% human albumin. This albumin solution is then "cleaned" by passing it over a charcoal filter, resin adsorbents and a haemofilter before recirculation. MARS has shown a survival advantage in small trials in acute-on-chronic liver failure (Heemann *et al.*, 2002, Mitzner *et al.*, 2000), but, despite improvement in several parameters, particularly encephalopathy, has yet to be shown to produce any survival benefit in acute liver failure.

Plasmapheresis, another technique aimed at removing toxins in plasma has recently shown some improvement in survival in acute liver failure in case series (Larsen *et al.*, 1994).

Due to the complexities of liver function it is unlikely that any mechanical artificial liver will be able to reproduce the myriad of functions performed by the

liver. Bio-artificial liver support systems containing hepatocytes in a bio-reactor have therefore been developed in an attempt to replicate normal liver function. Several systems have reached clinical trial. The 'Extra-corporeal Assist Device' (ELAD) utilises a hepotoblastoma cell line in its bioreactor (Ellis *et al.*, 1996), whereas the Bio-artificial Liver (BAL), the 'Berlin Extra-corporeal Liver Support System' (BELS) and the HepatAssist devices contain primary porcine hepatocytes (Demetriou *et al.*, 2004). Whilst biological systems perform more of the liver's endogenous functions no bio-artificial device has been shown to reduce mortality in acute liver failure although trials are small and therefore often underpowered. There are also unique considerations with systems that contain live hepatocytes; animal sources of hepatocytes may contain transmissible infectious agents, cell lines whilst having the advantage of proliferative ability do not perform all the functions of primary hepatocytes and should any enter the patient circulation would have malignant potential, and primary human hepatocytes do not proliferate, are difficult to isolate with high viability and are in limited supply. The maintenance of good cell viability and function in the presence of toxins in liver failure plasma also remains an issue of ongoing concern.

Overall meta-analysis of all liver support devices compared with standard medical therapy has failed to show any significant reduction in mortality (RR 0.86, 95% CI 0.65-1.12). However stratified meta-analysis has shown that in acutely decompensated chronic liver disease mortality is reduced by 33%, whereas mortality in acute liver failure shows no reduction (Khuroo & Farahat, 2004, Kjaergard *et al.*, 2003, Liu *et al.*, 2002).

1.9 HYPOTHESIS

The core hypothesis of this project is that plasma from patients with liver failure contains substances that cause hepatocyte death.

1.10 AIMS AND OBJECTIVES

The initial aims of this project were:

1. To develop a model applicable to human disease by which the cytopathic effects of liver failure plasma could be studied.
2. To isolate and identify the toxic molecule/s present in liver failure plasma that are responsible for loss of viability and decreased function of hepatocytes.
3. To elucidate the mechanism of action of these toxins.
4. To investigate if cytotoxic substances are present in acutely decompensated chronic liver disease.
5. To determine if there are any clinico-pathological variables that correlate with acute liver failure plasma's cytopathic effect.
6. To assess ways of removing/antagonising this toxicity.

By doing so, we hope to be able to identify processes which can be applied to manipulate liver failure plasma, to make a more favourable environment for hepatocyte survival. This ability would have multiple applications including improving function/recovery of the patients' endogenous liver cells, improving the environment for transplanted cells e.g. stem cells and allowing better function/survival of cells in a bio-artificial liver. The ultimate aim of these strategies is to improve the survival of patients with liver failure.

PRELIMINARY WORK:

CONFIRMATION OF TOXICITY AND CHARACTERISATION OF PHYSICAL PROPERTIES OF TOXIC MOLECULES IN ACUTE LIVER FAILURE PLASMA.

1.11 INTRODUCTION

Previous work has suggested that plasma from patients with acute liver failure is toxic to hepatocytes affecting both their viability and function (McCloskey, Tootle, Selden, Larsen, Roberts, & Hodgson, 2002). Native hepatocytes in patients with acute liver failure plasma are continuously exposed to plasma and therefore any toxic substances within it are likely to have a deleterious effect on the patient's clinical outcome. In addition, hepatocytes are likely to be a key component of any bioartificial liver and they would also be exposed to possibly "toxic" plasma ultimately causing their demise or suboptimal function of the bioartificial liver. An understanding of the "toxic" nature of liver failure plasma is therefore essential in developing an effective bioartificial liver, and developing new therapies to optimise the patient's endogenous liver function and hepatocyte survival, thus improving the likelihood of liver recovery and ultimately patient survival.

The initial aim of this project was to identify these toxic molecules, to understand their mechanism of action and to develop strategies for their removal or antagonism.

1.12 CONFIRMATION OF PLASMA TOXICITY AND IDENTIFICATION OF VARIABILITY IN TOXICITY FROM DIFFERENT INDIVIDUALS WITH ACUTE LIVER FAILURE.

Measurement of toxicity of plasma was assessed by the effect of the addition of liver failure plasma to liver cells in-vitro. Due to the lack of an unlimited source of primary human hepatocytes, and due to the fact that liver tumour cell lines are the source of hepatocytes in some current versions of the bioartificial liver, HepG2 cells were used to assess cell toxicity using the MTT assay.

The MTT assay is a colorimetric assay used to estimate cell numbers. It relies on cleavage of the yellow tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide to give a purple coloured formazan product by the action of the mitochondrial enzyme succinate dehydrogenase, and is often considered to be proportional to the number of viable cells present (Mosmann, 1983).

Liver failure plasma (donated by Larsen et al, Coppenhagen) was taken at the onset of plasmapherisis treatment in patients with acute liver failure (as defined by O'Grady *et al* 1993). See table 1 for clinical details of liver failure plasma, see table 2 for biochemical details.

Table 2. Acute Liver Failure plasma donors clinical details.

Sample ID	Aetiology	Age	Gender	Encephalopathy Grade	Outcome
LFP 1	Paracetamol toxicity	32	Male	IV	Spontaneous Recovery
LFP 2	Paracetamol toxicity	51	Male	IV	Spontaneous Recovery
LFP 3	NonA-E Hepatitis	47	Female	IV	Spontaneous Recovery
LFP 4	Paracetamol toxicity	54	Female	IV	Spontaneous Recovery
LFP 5	Paracetamol toxicity	34	Male	IV	Died

Method

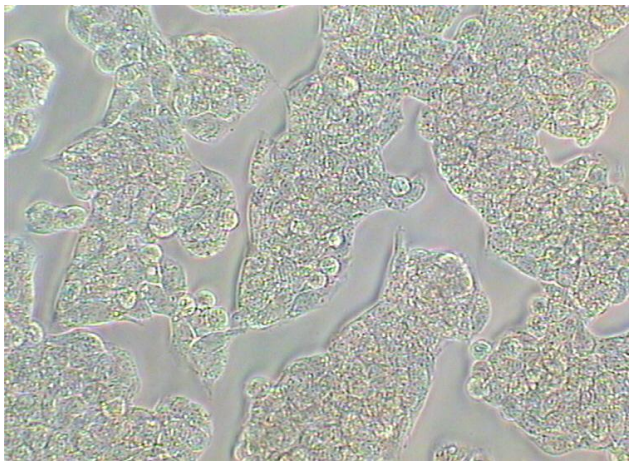
HepG2 cells from 90% confluent cell monolayers in tissue culture flasks were trypsinised (see general methods). Cells had over 95% viability measured by trypan blue exclusion and were plated into plastic 96 well plates at a density of 15 000 cells/well in 100uL of complete alpha-mem medium. Cells were incubated for 24 hours to allow cellular adhesion and proliferation. Cell monolayers at 40% confluence were washed twice with HBSS to remove dead cells and cellular debris. 100uL of plasma taken from patients with acute liver failure (see table 1 for clinical details), or normal controls was added to each well, each sample being repeated in triplicate. After 16 hours the plasma was removed and the cells washed thrice with complete alpha-mem medium. 50uL

of MTT (0.75mg/ml in PBS) was added to each well and then the plates were incubated at 37ºC for 3 hours in the dark. Excess MTT solution was then flicked off leaving the purple granules of formazan product in the bottom of the well. To solubilise the formazan product 100uL/well of 0.004M Hydrochloric acid in iso-propranol was added. The 96 well plate was wrapped in clingfilm to prevent evaporation and was agitated on a platform shaker for 30 minutes to ensure complete dissolution of the formazan product. The optical density at 550nm was then read by an automated plate reader.

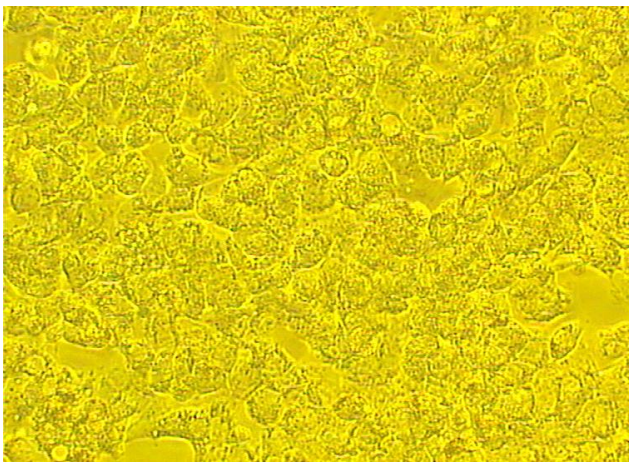
Toxicity in toxic samples was confirmed by exactly the above method but HepG2 cells were incubated with different percentages of liver failure plasma made up in complete alpha-mem forming a dose response curve.

Results

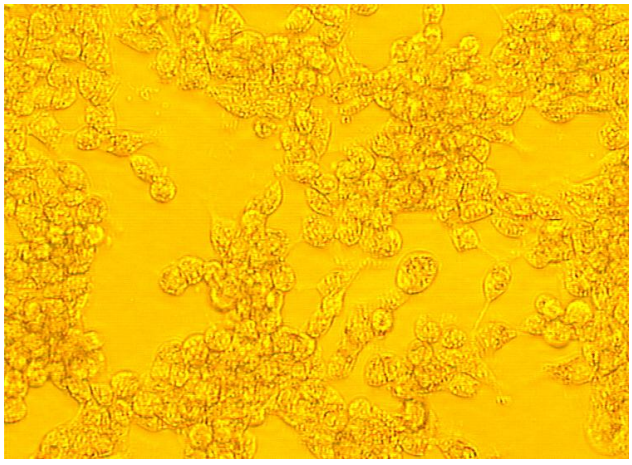
Viewing of HepG2cells under phase contrast microscopy after 16 hours exposure to 100% plasma from different individuals showed differences in cell density. This suggested differences in rates of cell death, proliferation, adhesion or combinations of those processes induced by different samples of plasma.



A. Normal Plasma



B. LFP 2



C. LFP 3

Figure 2. Phase contrast photographs, magnification x20 of HepG2 cells after 16 hours exposure to different samples of liver failure plasma. A. Normal Plasma. B. Liver failure plasma sample 2. C. Liver Failure Plasma Sample 3.

Liver failure plasma sample 2 shows increased cell density, whereas liver failure sample 3 shows reduced cell density compared with plasma from normal control.

This apparent difference in cell number was confirmed by the results of the MTT assay (Figure 2). A statistically significant decrease in mean MTT activity was observed in cells incubated in liver failure plasma sample 3 (LFP3) compared to normal plasma ($P<0.005$). Theses data were consistently reproducible on many occasions.

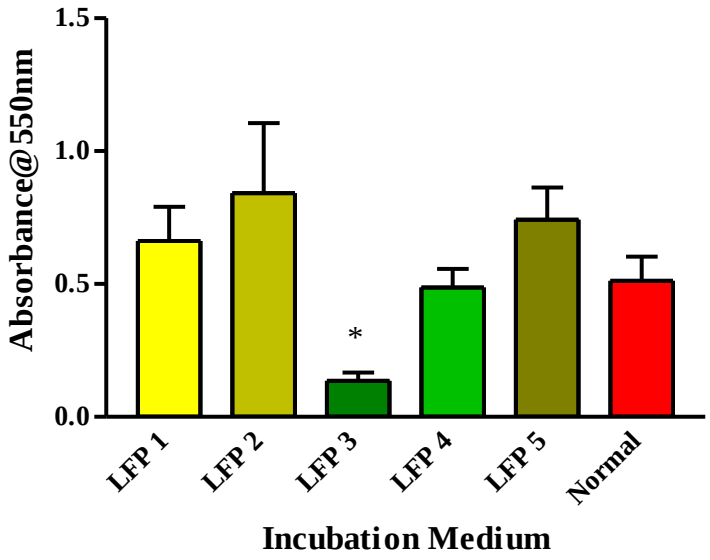


Figure 3. MTT activity, as an indirect measure of cell number, for HepG2 cells incubated in 100% plasma for 16 hours from patients with acute liver failure and normal control. Mean values ± SD. n=3 *= P value ≤ 0.05 cf Normal control.

Conclusion

Plasma from patients with acute liver failure and from normal patients varies in its effect on cell number. Liver failure plasma samples 1, 2 and 5 appear to

confer a survival or proliferative benefit on HepG2 cells compared with normal plasma. Liver failure plasma samples 4 and 6 appear to have no effect on HepG2 cell survival/proliferation compared to normal plasma. Whereas liver failure plasma sample 3 has a marked deleterious effect on cell number.

A large variation in effect of liver failure plasmas is not unexpected considering the plasmapheresis samples were taken from different patients possibly with different severities of liver failure at different stages of the disease process. Due to the complex nature of plasma there may also be substances which offer a survival advantage e.g. growth factors as well as toxic molecules, and these may offer protection against some of the plasma's toxic effect as well as increasing cell proliferation. This complex nature of plasma makes it difficult to identify toxic molecules as a deleterious effect may not be due to the presence of a toxic substance but may be due to the absence of beneficial factors. However since it is the final outcome of the cells' fate that is of interest this assay does give an insight into the balance of toxins/beneficial factors in different plasma samples.

Since liver failure plasma sample 3 shows the most striking toxicity it was decided to use liver failure plasma 3 to characterise possible toxic molecules. Using the above system as a biological assay we hoped by putting the plasma through a number of different processes each time reconstituting it back to its original volume and testing its toxic effect on HepG2cells, we could further characterise "toxins" in liver failure plasma.

1.13 CHARACTERISATION OF THE MOLECULAR WEIGHT OF PUTATIVE TOXIC MOLECULES IN ACUTE LIVER FAILURE PLASMA.

In an attempt to narrow the field of search for the toxic molecules in liver failure plasma so as to make its/their purification and identification easier we fractionated whole plasma into different fractions using centrifugation across membranes with different molecular weight exclusions.

Method

HepG2 cells were trypsinised, plated at 15000/well in 100uL in a 96 well plate and incubated at 37C for 24 hours as described previously.

Centricon ultrafiltration tubes with different molecular weight cut offs were sterilised with ethanol.

10ml of liver failure plasma 3 was loaded into the upper chamber of a centricon tube with molecular weight cut off of 100 000kDa, normal plasma control was loaded in an identical way to a separate identical centrifuge tube. Tubes were then centrifuged in a swinging bucket centrifuge at 3000g for 90 minutes. 1.5ml of centrifugate was retained, the remaining 3.5ml was loaded into a second sterile centricon tube with a molecular weight cut of off 30 000 kDa and centrifuged at 3000g for 90 mins. 1.5ml of centrifugate was retained and the remaining 2ml loaded into a third sterile centricon tube with a molecular weight cut off of 10 000 kDa, this was again centrifuged at 3000g for 90 minutes. Each centrifugate was then made up to its starting volume with serum free complete alpha-MEM. 50uL of each sample was added to the HepG2 cells; after

incubating for 16 hours at 37ºC in 5% humidified CO_2 the centrifugate was removed, cells washed in HBSS x3 and then the MTT test was performed as described previously.

To confirm the molecular weights of the centrifugate, equal amounts of each sample were separated by SDS-PAGE. Protein concentration of each sample was measured using the Bradford method (see general methods). A volume equivalent to 4ug of protein of centrifugate was mixed with an equal volume of SDS-PAGE loading buffer and heated to 100ºC for 5 minutes to denature the protein; samples with protein concentrations less than 0.4mg/ml were loaded in a maximum volume (20uL) onto a 12% Tris-glycine gradient gel in pairs of normal plasma and liver failure plasma 3. A rainbow marker (RPN 800 Amersham) with a molecular weight range of 10k-250kDa was also loaded. The gel was run at 200V constant voltage for 50 minutes in MOPS running buffer. Gels were then thoroughly washed in PBS and stained using silver staining (see general methods).

Results

The MTT test showed that once liver failure plasma sample 3 had passed through the membrane with molecular weight cut off of 30kDa its toxicity was lost.

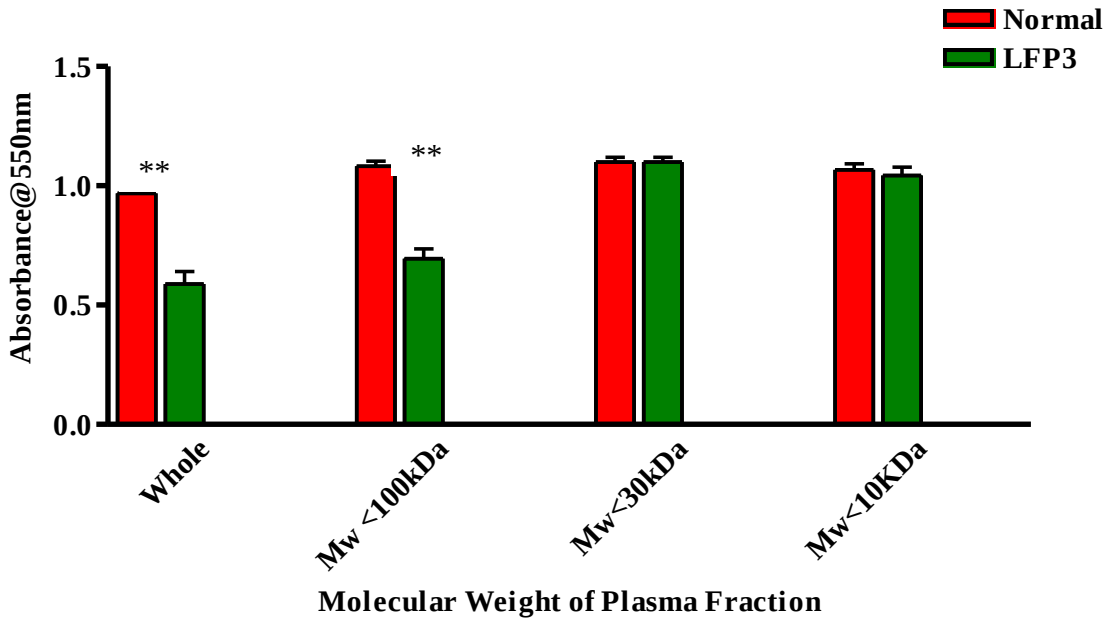


Figure 4. MTT activity as an indirect measure of cell number for HepG2 cells incubated for 16 hours in different fractions of liver failure plasma sample 3, produced by serial centrifugation across membranes with different molecular weight exclusions. Mean values ± SD. n=3 **= P value ≤ 0.005 cf Normal control.

The Bradford assay demonstrated that once the plasma had been passed through the 10 kDa molecular weight filter very little protein remained and this was confirmed by the absence of any silver staining on these samples. A densely stained band of molecular weight between 50-75kDa was clearly seen on uncentrifuged, 100kDa centrifugate, and to a far lesser extent the 30 kDa centrifugate. This band is likely to represent albumin (molecular weight 67 kDa). The reason for the appearance of a band with a molecular weight above that of the exclusion point of the membrane in the centrifuge device is that

molecules with molecular weights up to three times the molecular weight "cut off" can pass through the membrane. The manufacturer's recommendation is to select a molecular weight cut off at least three times smaller than the molecule to be retained. The stated molecular weight cut off is based on the ability of the device to retain >90% of globular protein of the defined molecular weight.

Conclusion

The toxicity of liver failure plasma sample 3 is lost after the plasma has passed through the 30 kDa centrifugation device, but the toxic molecule/s pass through the 100kDa device. This suggests that the "toxin" is a molecule with a molecular weight greater than 30kDa and is therefore unlikely to be a small water soluble molecule e.g. Urea. This suggests that the toxic molecule/s are either substances with larger molecular weight or that they are small molecular weight substances that are carried on larger molecular weight molecules e.g. Albumin. This seems a likely hypothesis since many small molecules that could be toxic to hepatocytes are carried in plasma bound by albumin. Centrifuging through a 100kDa selective centrifuge device could also be an initial step in purifying and thus identifying toxic molecules in liver failure plasma.

1.14 LIPID SOLUBILITY

Since one of the aims of this project was to identify toxic molecules in liver failure plasma an initial step in purifying it was to see if the toxic molecule/s in liver failure plasma were hydrophilic or hydrophobic using a technique originally described by Bligh and Dyer (Bligh et al, 1959).

Method

Liver failure plasma sample 3 and normal control plasma were each treated in the following way. A solution of 4 vols plasma: 5 vols chloroform: 10 vols methanol in a glass test tube was vortexed for 2 minutes in a fume hood. Additional chloroform (5vols) was added followed by further vortexing for 30 seconds. The mixture was chilled a 4ºC for 1 hour and then spun at 2000rpm for 30mins at 4C. The bottom yellow chloroform layer was drawn off and placed in a glass test-tube. The top clear layer was drawn off and stored. The protein precipitate was washed with a further 5 vols chloroform spun at 2000rpm at 4C the chloroform layer drawn off and pooled with the original chloroform layer. Samples were then frozen at -20ºC and lyophilised overnight. Samples of untreated plasma were also frozen and lyophilised to ensure the freezing and lyophilising process itself did not remove plasma toxicity. All lyophilised samples and controls were diluted in 75uL of ethanol to allow solubilisation of the lipid component, the water soluble layers and the whole plasma were treated in an identical way to ensure ethanol was present in an equal amount in all samples and controls. Samples were then made back up to there starting volume 1.5ml in Hepes buffered serum free medium. Having been filter sterilised 100uL of sample or control was added to 40% confluent HepG2 monolayers which had been plated 24 hours prior in triplicate. Cells were incubated for 16 hours. The sample was then removed and the cell monolayers washed twice with sterile medium, an MTT test was then performed as described previously.

Results

The results show that the toxicity seen in the unseparated sample of liver failure plasma persists in the aqueous fraction, but is not present in the chloroform layer.

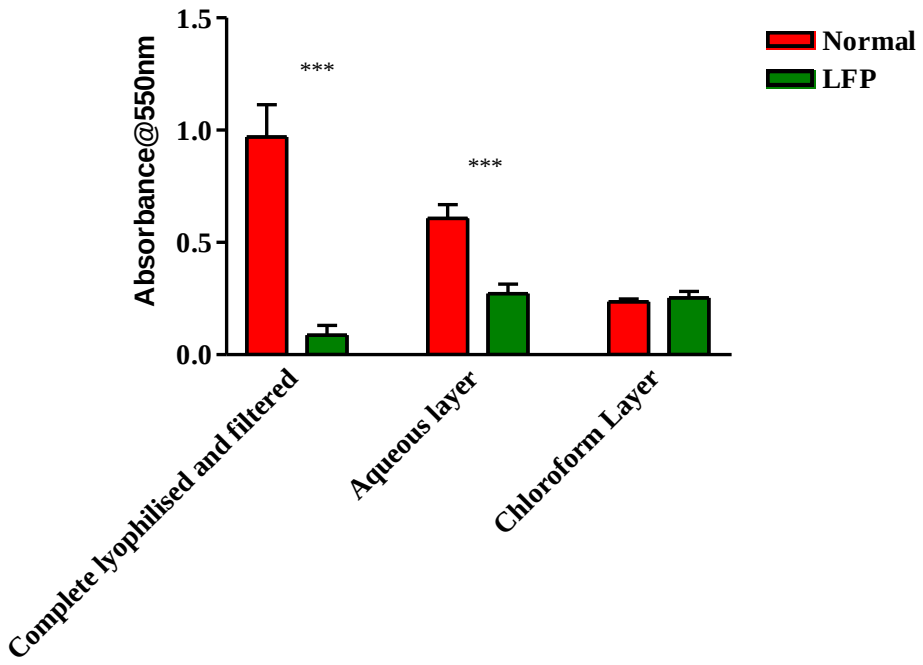


Figure 5. MTT activity as an indirect measure of cell number for HepG2 cells incubated for 16 hours in liver failure plasma sample 3, and normal plasma control separated into its lipid soluble and aqueous fractions using the technique described by Bligh and Dyer. Mean values ± SD. n=3 ***= P value ≤ 0.001 cf Normal control.

Conclusion

The toxicity in liver failure plasma sample 3 appears to be present in the sample from the aqueous phase produced by the treatment of plasma using the technique described by Bligh and Dyer but not in the lipid soluble phase; this suggests that it is a hydrophilic molecule.

1.15 HEAT LABILITY

As part of the physical characterisation of possible toxic molecules in liver failure plasma we aimed to determine if these toxins were heat labile.

Method

400uL of either liver failure plasma of normal control plasma was placed in a sterile eppendorf and placed in a waterbath at 20ºC, 45ºC, 60ºC and 70ºC for 30 minutes, then immediately removed and cooled on ice. 100uL/well of heat treated plasma sample was added to 40% confluent HepG2 monolayers in a 96 well format in quadruplicate. Cell monolayers were then incubated for 16 hours at 37ºC in 5%CO_2 and the effect on cell viability assessed by the MTT test as previously described.

Results

Heat inactivation resulted in loss of toxicity of liver failure plasma.

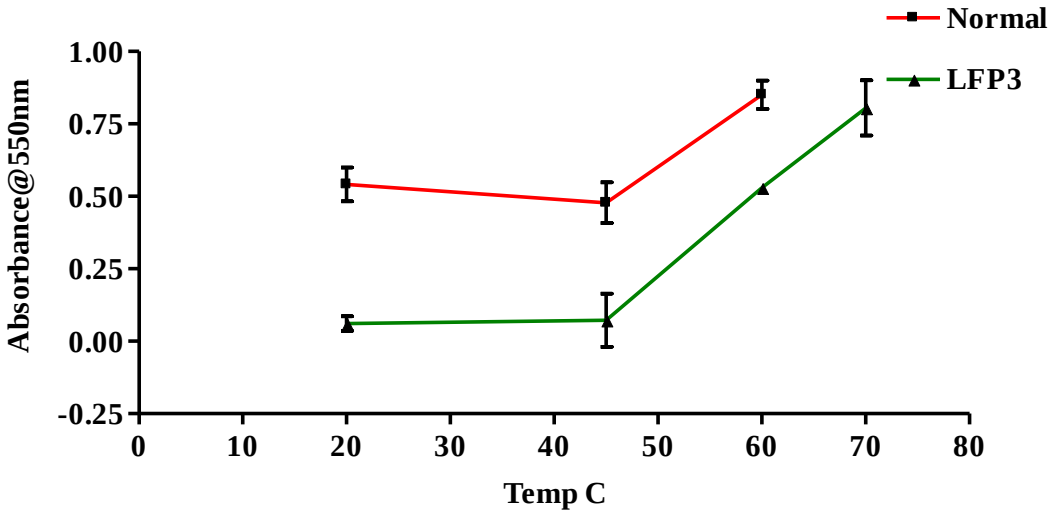


Figure 6. MTT for HepG2 cells after cells incubated for 16 hours in liver failure Plasma and Normal Plasma control. The Plasma was previously heat inactivated for 30 mins at different temperatures (x axis).

No data are available for normal plasma heat treated to 70ºC as due to the high albumin content the sample was denatured and solidified by heat treatment.

Conclusion

The toxicity of liver failure plasma sample 3 was decreased by heating to 60ºC for 30 minutes however the viability of cells treated with 60ºC heat inactivated normal plasma for 30 minutes was also increased suggesting that the improvement may not be specific to "toxic liver failure plasma" but may be due to general inactivation of substances inhibitory to cell growth in all plasma.

1.16 DIALYSIS

Introduction

Conventional dialysis allows the removal of small molecules from a sample by allowing them to diffuse down a concentration gradient across a selectively permeable membrane. To determine if the toxic molecules in acute liver failure plasma sample 3 could be removed by dialysis we dialysed human liver failure plasma against a solution of PBS and against a solution of 20% bovine albumin across a snakeskin dialysis membrane with a molecular weight cut off of 10kDa.

Method

A tubular dialysis membrane was cut into four 20cm lengths, membranes were cleaned by boiling in10mM sodium bicarbonate for 10 minutes and then boiling for 5 minutes in 10mM Na_2EDTA, repeated twice and then rinsed several times with sterile ddH2O. The bottom was tied in a double knot, and, having tested the membrane for leaks, a 1.5ml sample of liver failure plasma sample 3 was loaded into the two dialysis tubes and the tops of the tubing clamped with sterile

clamps allowing plenty of room for expansion due to solvent movement in the opposite direction. This tubing was then fully immersed in either 100ml PBS or 100ml 20% bovine serum albumin solution in PBS, which was then continuously stirred with a magnetic stirrer. The samples were left at 4ºC overnight to equilibrate with two changes of dialysis buffer every 6 hours. Two samples of plasma from a normal individual were set up in an identical way as a control. Untreated plasma was also kept at 4ºC overnight to control for any loss of toxicity due to time alone. At the end of the dialysis period the samples were removed from the dialysis fluid and their volume accurately measured. Each sample was then made up to the highest final volume of the sample, which was 1.6ml to compensate for fluid shifts and ensures their other than dialyzable solutes other molecules do not become differentially diluted/concentrated by fluid shifts. Untreated control plasma was also diluted to a final volume of 1.6ml with sterile PBS.

100ul of plasma from either LFP3 or normal plasma both undialysed, dialysed against PBS and dialysed against 20% BSA were added in triplicate to HepG2 monolayers in 96 well plates that were 40% confluent having been seeded 28 hours prior. These cells were then incubated for a further 16 hours and then plasma was removed, cell layers washed twice with medium, and MTT test performed as a measure of cell viability as described previously.

Results

Dialysis of liver failure plasma against PBS or 20% albumin solution did not remove its toxicity.

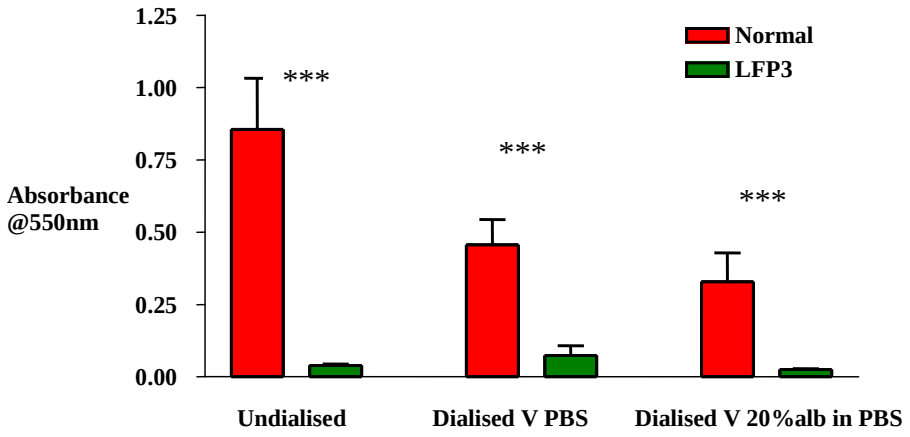


Figure 7. MTT assay for HepG2 incubated for 16 hours with toxic liver failure plasma (3). The plasma was dialyzed against PBS or 20 % Bovine Serum Albumin in PBS for 24 hours at 4ºC. Normal plasma control. Mean values ± SD. n=3 ***= P value ≤ 0.001 cf Normal control.

Conclusion

The toxicity of liver failure plasma sample 3 is retained despite dialysis against either PBS or 20% Bovine serum albumin solution. There are several plausible explanations for this. The first would be that the molecular weight of the toxic molecule itself is above the cut off of the dialysis membrane (10kDa) and is therefore not able to freely cross it and be dialysed. The second explanation is that the molecule is small but bound with a high affinity to a carrier protein and thus only a tiny fraction is in the aqueous phase and thus available to be removed by dialysis. If, for example, the toxin was bound to human albumin, this could be the case. The results support the findings of the previous work which found that toxicity was excluded from the sample by centrifugation though the centricon tube with a 10kDa cut off.

To try and differentiate between whether the molecule was bound to albumin or was itself greater than 10kDa we dialysed against a 20% albumin solution. We would have expected dialysis against albumin solution to remove some of the toxicity if the molecule was less than 10kDa but was bound to albumin. In a similar way to the use of albumin dialysis, such as is used in the MARS in the treatment of liver failure.

Dialysis against albumin did not however remove toxicity. This could be because either the toxin is not albumin bound, or that the albumin in the dialysis buffer does not have as high a binding affinity as human albumin. This may be the case as the processing of albumin often results in a large decrease in its binding capacity. Higher grades of human albumin are available and these have higher albumin specific binding capacities but are prohibitively expensive. Even the highest grade of human albumin in clinical use in MARS requires 20 minutes of recirculation across the charcoal and anion-exchange resin columns to optimise its binding capacity and remove stabilisers within the product. MARS also uses a special dialysis membrane which is pre-impregnated with albumin to facilitate transfer of albumin bound substances, we did not have this refinement. The membrane used may therefore not be suitable for dialyis of albumin bound substances.

Subsequent experiments will look at toxicity of plasma from patient's pre and post MARS treatment. These dialysis results do however confirm the results of clinical trials which suggest that standard haemodialysis of water soluble products from patients with fulminant hepatic failure does not improve the patient's outcome.

1.17 ALBUMIN BINDING

Since previous experiments to demonstrate the molecular weight of toxic molecules in liver failure plasma suggested that the toxicity co-localised to the range of molecular weight that albumin falls in, we hypothesised that the toxin/s may be albumin bound, and that the dialysis against albumin method was inadequate alone to disprove the theory that the toxin was albumin bound due to the limitations described above. To further test this hypothesis we specifically removed albumin from plasma and subsequently tested it to see if it remained toxic.

Method

Sepharose blue which has an albumin binding capacity of 7mg/ml was hydrated and washed in Tris buffer. 0.75ml of liver failure plasma sample 3 or normal plasma was added to 6ml of sepharose blue beads in a 15ml conical tube. The tubes were then turned end over end continuously for 16 hours at 4ºC. The tubes were then centrifuged at 3000g for 30 minutes. The clear supernatant was then removed. Albumin was removed from the blue sepharose beads by adding 2.5ml of sodium chloride 2M and the tubes turned end over end for 2 hours; tubes were then centrifuged at 3000g for 30mins and the supernatant removed. This sample was then desalted through a PD10 Sephadex G25 desalting column using 0.1M ammonium acetate pH 7.4 as the exchange buffer. 3.5ml of elutant was collected in 0.5ml fractions and run through a spectrophotometer at 280nm to confirm the presence of protein. Pooled fractions were then frozen and lyophilised (as was untreated control plasma and the albumin free plasma prepared earlier). Lyophilised samples were then reconstituted in 0.75ml HEPES buffered serum free medium and added to 40% confluent HepG2 cell

monolayers which had been plated 24 hours earlier in quadruplicate. Monolayers were then incubated for 16 hours at 37ºC in 5% CO_2 after which they were washed twice with sterile medium and an MTT test was performed. The removal of albumin from serum and the removal of albumin from the sepharose blue beads was confirmed by electropheresis of samples on 12% Tris-glycine gel and subsequent silver staining as described earlier.

Results

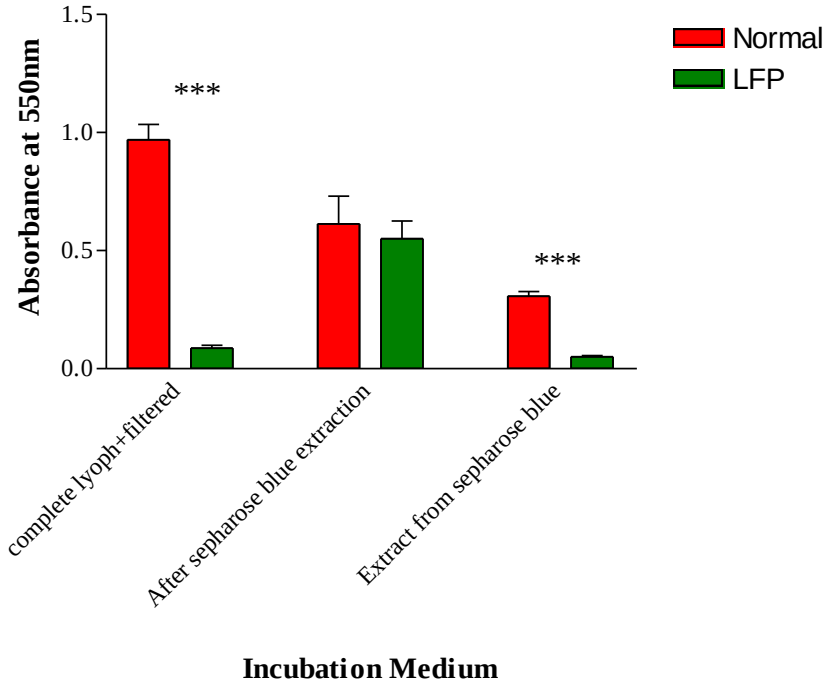


Figure 8. MTT activity as a measure of cell number for HepG2 cells incubated for 16 hours in plasma which has been treated with blue sepharose beads to remove albumin. The albumin was then removed from the sepharose blue.

Conclusion

The toxicity of liver failure plasma sample 3 is removed by removing albumin from the sample and seems to be present in the albumin recovered from the blue

sepharous beads. This confirms that the toxin is albumin bound and suggests techniques which remove albumin bound toxins e.g. MARS could theoretically render patients plasma less toxic to hepatocytes. Treatments that seek to "clean patients albumin" such as MARS or plasmapheresis which have been demonstrated to improve outcome in some trials may do so by decreasing toxicity of plasma to hepatocytes. Developing a detoxifying system using blue sepharose beads would not be efficient as blue sepharose does not have a high enough binding capacity to be used practically.

1.18 ACTIVATED CHARCOAL

Activated charcoal is known to remove a number of toxins by non-specific adsorption. Charcoal haemoperfusion has been used to treat patients with acute liver failure in the past, and is no longer used as it failed to show a survival benefit. A charcoal filter is also one of the two filters in the albumin regenerating circuit in the MARS machine. We therefore hypothesised that treatment of liver failure plasma 3 with activated charcoal could be a means of removing its toxicity.

Method

Three 15ml nunc tubes were filled with 5g of activated charcoal (sigma C9157) each; the activated charcoal was hydrated by the addition of 5ml of 0.1M buffered ammonium acetate pH 7.4. Excess buffer was removed and 0.5ml of liver failure plasma 3, normal plasma or water was added to separate tubes. The tubes were briefly vortexed and then turned continuously end over end for 30 minutes. Tubes were then centrifuged at 2000g for 30 minutes at 4ºC. The

supernatant was removed, and the charcoal washed by the addition of a further 2ml of ammonium acetate buffer vortexing and then centrifuging at 2000g for 30 minutes, the supernatant was again removed and pooled with the earlier supernatant. A further two washes were performed in this way each time pooling the supernatant with earlier fractions. Samples were filtered through a 0.2µM filter to sterilise and remove any residual charcoal. Samples were then frozen at -20ºC and lyophilised. Samples were resuspended in 0.5ml of Hepes buffered serum free medium and 100µL was added to 40% confluent HepG2 cells in monolayer in a well of a 96 well plate having been plated 24 hours earlier in quadruplicate. Cells were incubated for 16 hours at 37ºC in humidified 5%CO_2 atmosphere. Samples were then removed and the monolayers washed twice with sterile serum free medium. A MTT test was performed as described earlier.

Results

There remained a persistent toxic effect of liver failure plasma despite treatment with activated charcoal.

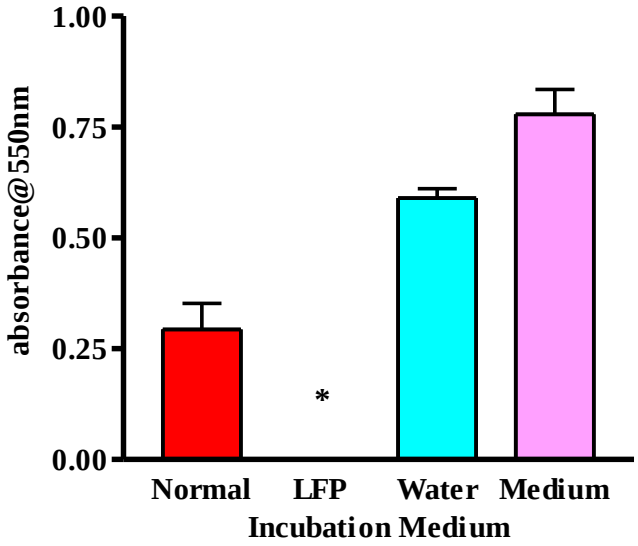


Figure 9. MTT activity for HepG2 cells that were incubated in medium or plasma (water control) that had been treated with activated charcoal. Mean values ± SD. n=3 *= P value ≤ 0.05 cf Normal control

Conclusion

These results suggest that activated charcoal does not remove the toxicity associated with liver failure plasma sample 3 and would not therefore be useful in detoxifying plasma prior to the plasma being exposed to hepatocytes in a bioartificial liver and confirms the clinical results that charcoal haemoperfusion is unlikely to improve clinical outcome in patients with fulminant liver failure.

1.19 DISCUSSION

The MTT test as an indirect measure of cell number has a number of possible flaws. The assay relies on the activity of the mitochondrial/microsomal enzyme being constant. Changes in enzyme quantity or activity which may occur as a

result of different experimental conditions could result in changes in the MTT test due to factors other than change in absolute cell number.

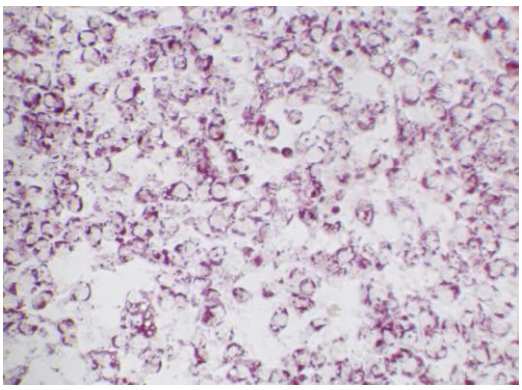
The second possible source of error is that due to the amount of time the plasma is left in contact with the cells, changes in proliferation rates by different experimental conditions could result in significantly different cell numbers at the end of the experiment. These would be misinterpreted as being due to differential effects of experimental conditions on cell death, when this may not be the case. Methods which directly measure cell death e.g. LDH release may therefore be better. It would also be preferable to develop a model which was more informative regarding the exact mechanism of cell death.

A third criticism is that the methodology means that cells not adhering to the bottom of the well at the end of the experiment are counted as dead, as they are washed away. Factors which effect cellular adhesion may therefore be wrongly interpreted as directly affecting cell death. Indeed this is a major flaw of the MTT model developed above, as the ensuing paragraphs demonstrate.

1.20 CELLULAR ADHESION

Detailed visual inspection after incubation with liver failure plasma and after addition and incubation of MTT showed large numbers of non-adherent cells in liver failure plasma sample 3. These non-adherent cells contained large amounts of purple formazan product indicating their viability (Fig 7). Tipping off the MTT prior to solubilisation results in loss of these cells and associated formazan product, and thus is an underestimate of cell number.

A.



B.

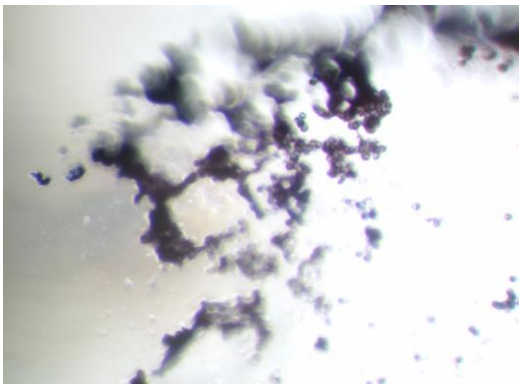


Figure 10. Photomicrographs of HepG2 cells having been incubated in MTT pre-solubilisation showing the presence of large quantities of purple formazan product in cells that have been exposed to normal plasma (A) or liver failure plasma 3 (B), note the cells although viable are not adherent.

Subsequent to these experiments Acute liver failure plasma was confirmed to be able to decrease cellular adhesion via down regulating beta-1 integrin receptor expression and activation (Newsome *et al.*, 2004). This effect is rapid occurring within a few hours, and is one of the hurdles to hepatocyte/stem cell transplantation (Potocnik *et al.*, 2000, Zvibel *et al.*, 2002). Although loss of cellular adhesion will ultimately result in cell death by apoptosis, a process termed anoikis, the MTT assay, or any assay that relies on cells remaining adherent cannot be used as a measure of cell toxicity.

In an attempt to quantify how much of liver failure plasma sample 3 toxicity was due to effects on cellular adhesion the initial experiment to assess the effect of cell toxicity was repeated. HepG2 cell adhesion was increased by pre-coating 96 well plates with collagen (see general methods) prior to plating. The method was adapted by decreasing the vigour of washing steps, replacing tipping/flicking off with gentle aspiration and inspecting washings at each step to ensure no cells were lost. Using this method the MTT assay was repeated.

Results

Having minimised the effects of changes in cellular adhesion it can be seen that there no significant variation in toxicity between liver failure plasma and normal plasma.

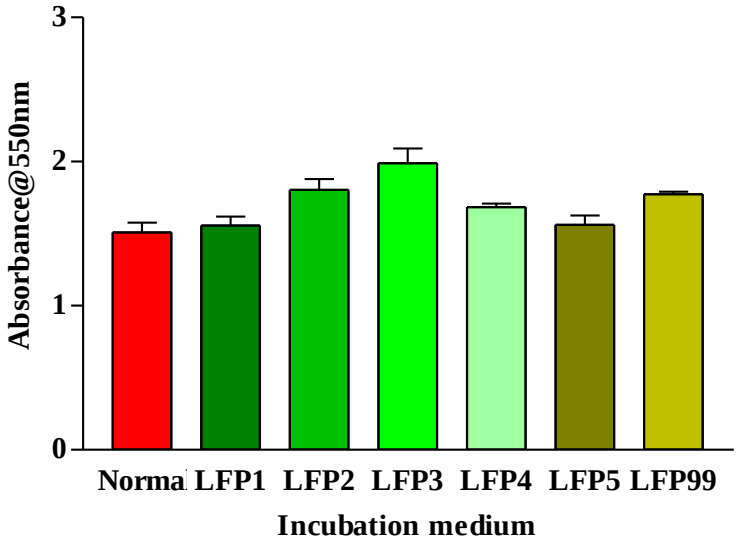


Figure 11. MTT activity as an indirect measure of cell number for HepG2 cells incubated in different plasma samples on collagen coated plastic.

1.21 TIME COURSE OF LOSS OF ADHESION

Method

To investigate the time course over which loss of adhesion occurred, experiments using non-collagen coated plastic and vigorous washing as in the original method (see appendix for general methods) were performed using acute liver failure plasma 3, but exposing HepG2cells for different time period prior to the measurement of MTT activity. Photomigrographs prior to washing under phase at each time point are also shown.

Results

Upto and including time of exposure 360 minutes there was no statistical difference between MTT activity (as a marker of cell number) between normal plasma and liver failure plasma (LFP3). From the time point 10 hours onwards

and inclusive there was a reduction in MTT activity in HepG2 cells exposed to acute liver failure plasma and an increase in MTT activity in HepG2 cells exposed to normal plasma. This resulted in a marked reduction in MTT activity in cell number in acute liver failure plasma compared to that of normal plasma for each time point ≥14 hours (P≤0.005).

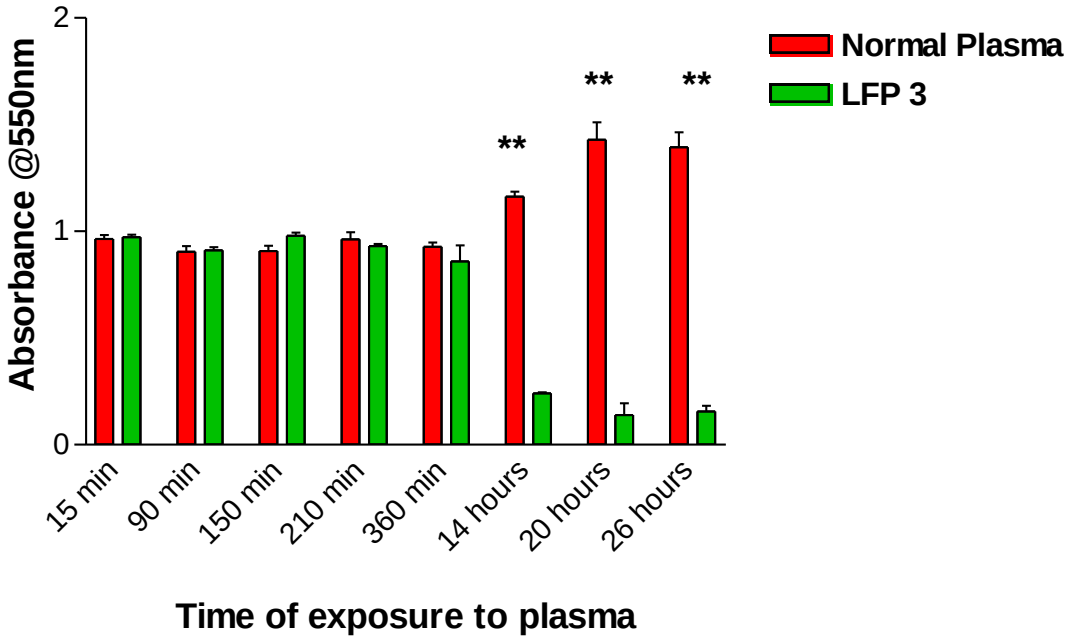


Figure 12. Time course of MTT activity (as a marker of cell number) against time of exposure for HepG2 cells exposed to LFP 3 and normal plasma (control)
** P ≤ 0.005

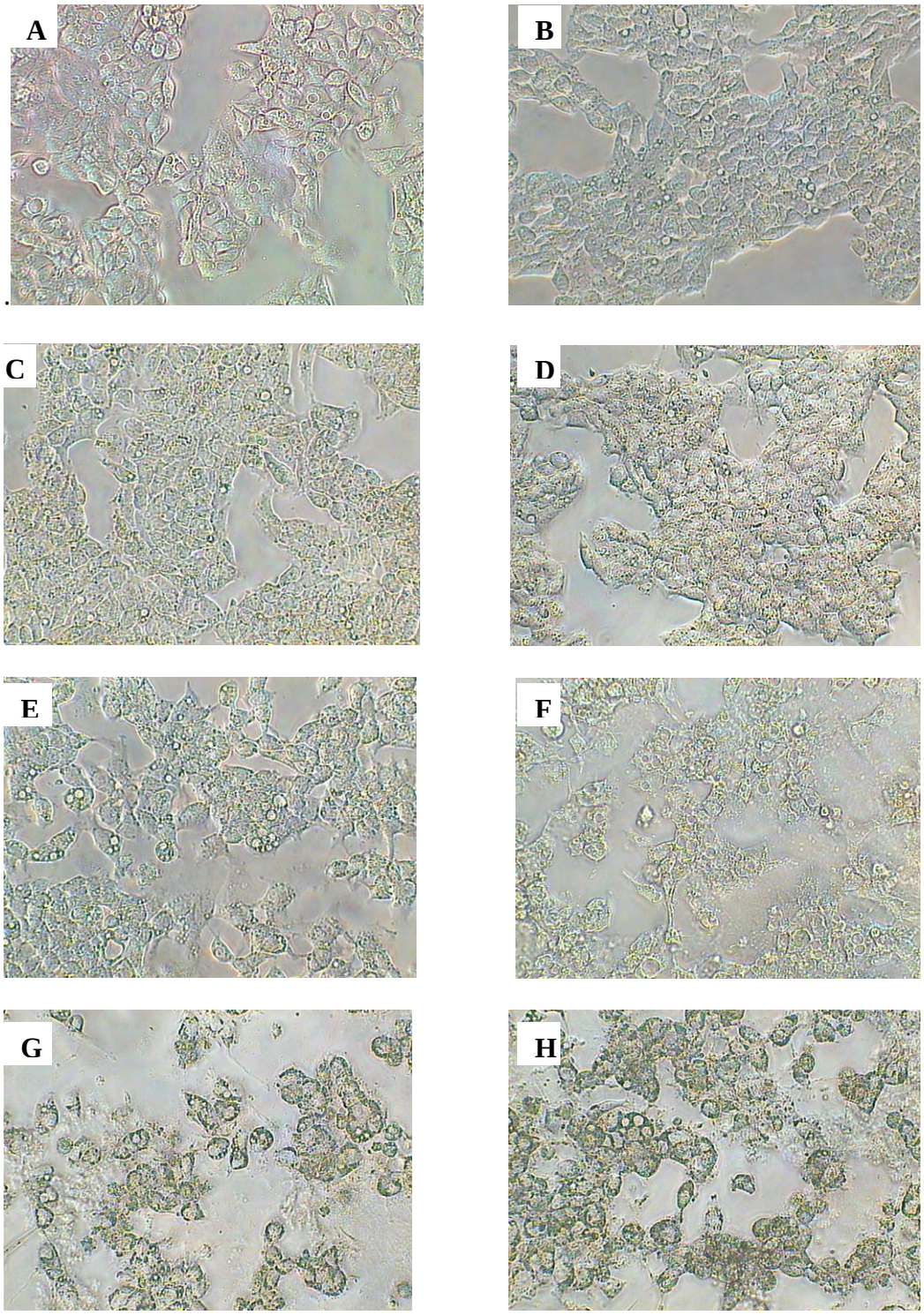


Figure 13. Photomicrograph of Hep G3 cells under phase contrast microscopy prior to washing, after exposure to Acute Liver Failure Plasma (LFP 3) for various times. A=15min. B=90min, C=150min, D=210min, E=360min, F=14hrs, G=20hrs, H=26hrs

The increase in MTT activity (cell number) in HepG2cells exposed to normal plasma over time most likely results from on-going proliferation of HepG2 cells. The reduction in MTT activity in Acute Liver failure Plasma represents loss of cell number by loss of adhesion or viability. Figure 10 shows that although unattached the cells remain viable, confirming the loss is due to cell detachment not death. The time course of loss of adhesion by Acute liver Failure plasma appears to become significant after 6 and before 14 hours of exposure.

1.22 CONCLUSION

Through this preliminary work we have identified that exposure to some samples of liver failure plasma results in changes in cellular adhesion and these factors begin to cause detachment of cells after 6 hours.

Having eliminated the problem of cellular adhesion it can be seen that there is little variation of toxicity using the MTT assay and that attempts to isolate or identify toxins in liver failure plasma would be futile as there is no liver failure sample with significant toxicity. The earlier experiments to isolate/physically characterise the toxin in liver failure plasma sample 3 were most likely isolating/physically characterising substances in liver failure plasma which affect cellular adhesion.

On examining the original literature from which the concept of toxic molecules in Acute Liver Failure was developed, it was apparent that the methodology used in these experiments and duration of exposure to the liver failure plasma means that they too where vulnerable to the effects on cellular adhesion induced by exposure to liver failure plasma. Hughes *at al* exposed primary rabbit hepatocytes to 20% liver failure plasma overnight, Anderson et al exposed

HepG2 cells to 10% liver failure plasma for 24 hours, McCloskey et al exposed HHY41cells to 100% liver failure plasma for 20 hours and Shi et al exposed HepG2cells to 10% plasma for 4 days. There experiments describe reductions in viability by 25-40% compared to normal plasma but all the methods used assume cells are lost by death only and loss by detachment, although Shi *et al* did comment that changes in morphology and adhesion were seen.

It seems intuitively correct that such large reductions in cell number would not be due to cell death, as it seems improbable that a substance so toxic, that at 20% concentration it can induce cell death in 40% of hepatocytes after overnight exposure, would be compatible with survival of the donor.

The question therefore remains if plasma from patients with Acute Liver Failure Plasma is toxic to hepatocytes.

CHAPTER 2: DEVELOPING AN IN-VITRO MODEL TO INVESTIGATE THE EFFECTS OF LIVER FAILURE PLASMA ON HEPATOCYTES.

2.0 INTRODUCTION

As indicated in the preliminary work in cheaper 1 the MTT test and other measures of cell number are insensitive methods of assessing the effects of substances such as liver failure plasma on immortalised hepatocyte cell lines. If differences between individual liver failure plasma samples, differences over time within individuals or differences before and after therapeutic manoeuvres wish to be undertaken, a more sensitive method would be required.

However in-vivo and in-vitro studies have suggested that substances in liver failure plasma are deleterious to primary non-human hepatocytes and hepatocyte cell lines (Anderson, Thabrew, & Hughes, 1999, Anilkumar, Ryan, Aslam, Poulsom, & Alison, 1997, Hughes, Cochrane, Thomson, Murray-Lyon, & Williams, 1976, Shi *et al.*, 1998). A robust reproducible in-vitro system is required to further study this phenomenon.

The ideal requirements of such a system would be for it to:

- Use Primary Human Hepatocytes rather than cell lines which by definition have deregulated life cycle pathways.
- Not be affected by anti- or pro- proliferative effects of substances i.e. non-dividing cells
- Sensitive
- Specific
- Reproducible
- Would investigate a single biological process.

As discussed in chapter 1 there is an increasing body of evidence that suggests apoptosis may be a major factor in the pathogenesis of a number of liver diseases including acute liver failure. 10% acute liver failure serum has been shown to increase apoptosis to 3.8% and 8.4% after 24 and 48 hours respectively, compared to negligible amounts in normal plasma. There are certainly a number of substances in plasma which are increased in liver failure which can induce apoptosis in hepatocytes, for example bile salts and cytokines. The aim was therefore to develop a robust in-vitro system for the study of the effects of liver failure plasma and substances within and their effects on apoptosis.

2.1 CONFIRMING ACUTE LIVER FAILURE CAUSES HEPATOCYTE APOPTOSIS AND CALCULATING TIME COURSE OF EXPOSURE

Initial experiments to assess whether liver failure plasma did induce apoptosis and to determine the time course of apoptosis were performed. Initially using HepG2 cells due to their abundance and fluorescence staining of the activated effector Caspase, Caspase 3 using Caspatag^{tm}. This test utilises a carboxyfluorescein labelled caspase inhibitor. The inhibitor binds irreversibly to active caspases and is specific to activated Caspase 3 and can be used in live cells.

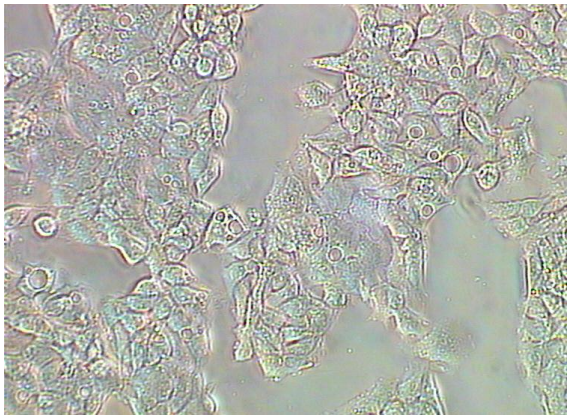
Method

HepG2 cells were trypsinised and seeded at a density of 33000/well on a collagen coated glass 8 chamber slide in 0.5ml of complete Williams E medium

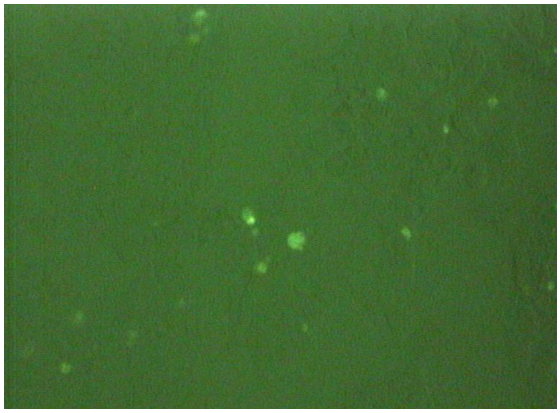
and left to adhere and proliferate for 24 hours. Medium was aspirated and replaced by 0.5ml of 100% liver failure plasma sample 3 and incubated for 0 mins, 90 mins, 150 mins, 210 mins, 360 mins, 14 hours, 20 hours and 26 hours. Plasma was then removed and replaced substituted with 300uL serum free medium. Caspase 3 staining was performed on live unfixed cells according to the protocol described in general methods. Identical fields were then viewed under phase and fluorescence microscopy.

Results

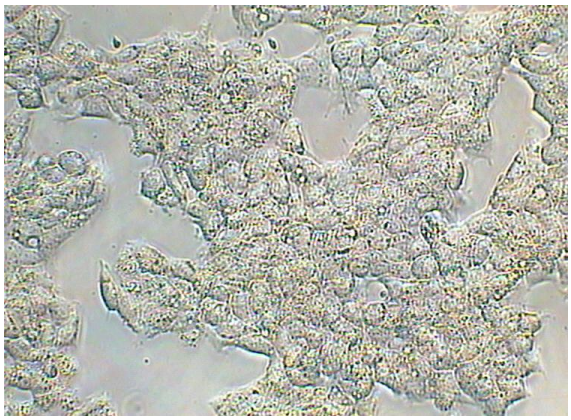
Although only semi-quantitative, liver failure plasma did induce apoptosis in HepG2 cells as measured by activation of Caspase 3. The Caspase 3 activity seemed to peak after 210 mins exposure and appeared to decline thereafter due to loss of cells presumably due to changes in cellular adhesion described earlier.



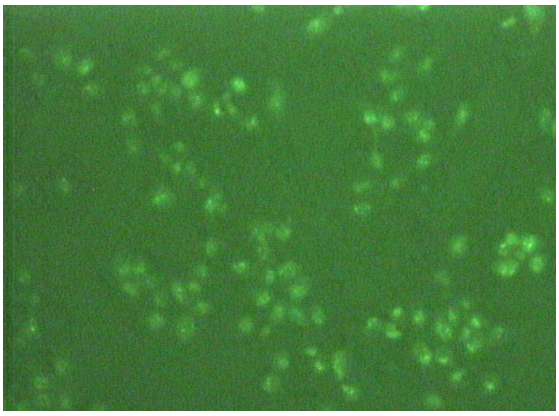
T=0 minutes phase



T=0 minutes Caspase 3 (green)



T=210 minutes phase x20



T=210 minutes Caspase 3 (green)

Figure 14. Photomicrographs of the same fields of HepG2 cells under phase and fluorescent microscopy (magnification x20) showing increased caspase 3 activation (green) after 210 minutes exposure exposure compared to control, 0 minute.

Conclusion

Caspase 3 activation appeared to occur on exposure to liver failure plasma and this appeared to peak after approximately 210 minute exposure. After this time particularly after 14 hours total cell numbers were decreased either due to loss of adhesion or due to cell death and subsequent detachment. To avoid difficulties

with cell loss it was decided to use 210 minutes as the period of exposure of hepatocytes to possible pro-apoptotic stimuli.

2.2 POSITIVE CONTROL

To develop a robust reliable system for measuring the effects of liver failure plasma on apoptosis it is necessary to develop a robust positive control. Commonly available substances that have been cited in the literature as predictable inducers of apoptosis in hepatocytes were tested. Staurosporine (a protein kinase c inhibitor (Sigma S5921)), TNF-alpha, TGF-beta and Ethanol were all tested as previous literature suggests that all can induce hepatocyte apoptosis.

Method

For an initial rapid screen of toxicity, dose response curves were made for each of the test substances by incubating 40% confluent HepG2 cells plated 24 hours earlier on a collagen coated 96 well plate in 100uL of the test dose for 16 hours. The test substance was then gently aspirated and washed gently twice with serum free alpha-MEM. An MTT test was then performed as described previously. Staurosporine 100ug was dissolved in 100ul of DMSO and stored as a stock solution at 4ºC; it was then dissolved in medium at an appropriate concentration fresh for each experiment.

Substances that induced significant cell death were further tested on HepG2 cell monolayers to ensure that the mechanism of toxicity was via inducing apoptosis and that the time course of that reaction was similar to the proposed time of exposure of plasma samples, by the following method. Glass 8-chamber slides were coated with collagen (see general methods) and then seeded at a density of

66000/well in 0.5ml complete alpha-mem and incubated for 24 hours. Test substances were then added and incubated for 4 hours. The test substance was gently aspirated and the cell monolayer and fresh serum free medium added. Live cells were then stained for Caspase 3 technique (see general methods).

Results

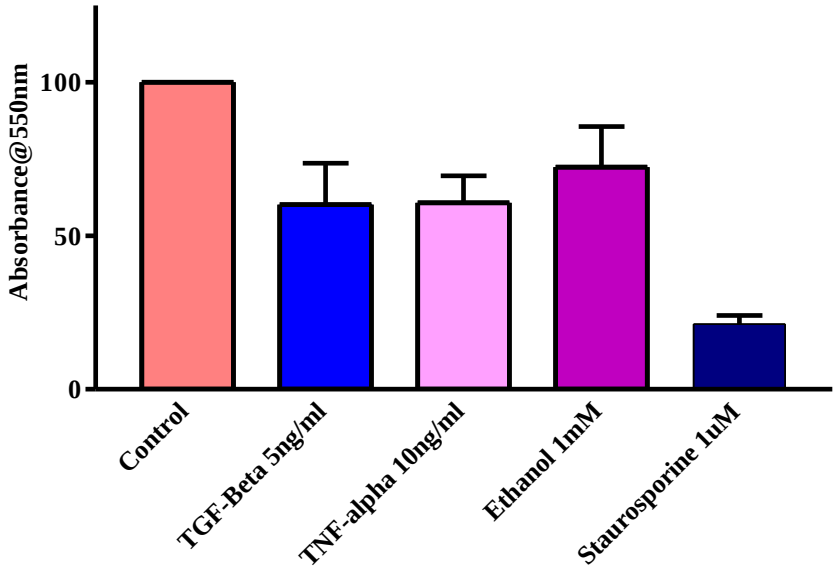
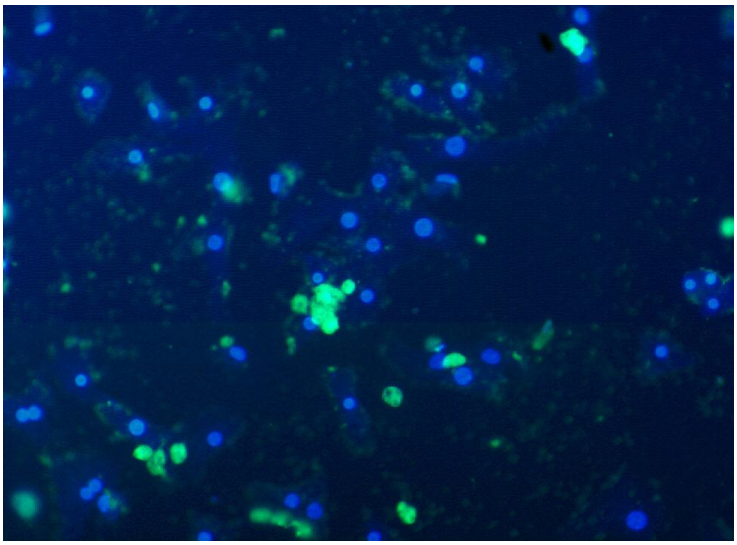


Figure 15. Graph showing MTT activity as an indirect measure of cell number, for HepG2 cells exposed to different apoptosis inducing factors for 16 hours.

These results suggested that Staurosporine 1uM was toxic and could therefore be a good positive control, as it resulted in significant loss of viable cells after 16 hours. Further study to investigate whether that loss of cell number was due to apoptosis, and to ensure the loss of cell number was due to loss of viability and not changes in adhesion, and the time course of the apoptotic process were

undertaken and confirmed Staurospaurine 1uM induced significant apoptosis in HepG2 cells over a time period of 4 hours (Fig 13).

A.



B.

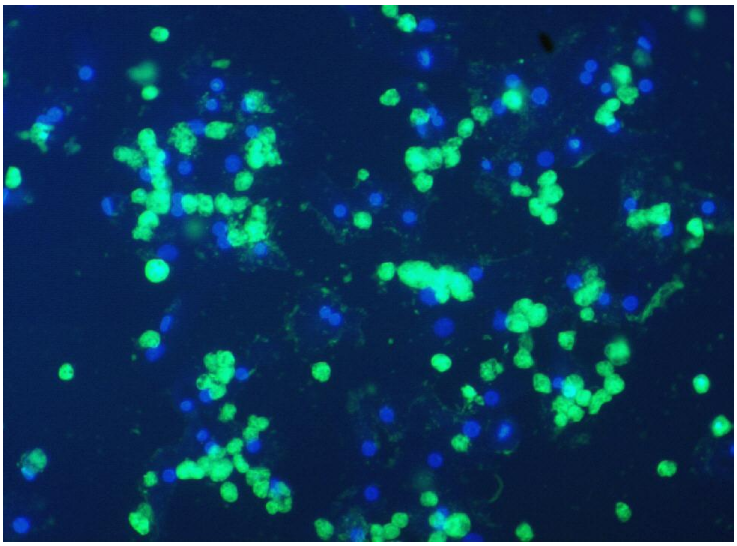


Figure 16. Photomicrographs showing increase in Caspase 3 (green) activation in Hep G2 cells after exposure to (B) Staurosporine 1uM for 4 hours compared to control (A). Nuclei counterstained with Hoescht (Blue) (magnification x40).

Conclusion

Staurosporine cause significant apoptosis over an appropriate time course and was therefore used as a positive control in this in-vitro model.

2.3 PRIMARY HUMAN HEPATOCYTES

Since the aim was to develop an in-vitro system that accurately reflects the in-vivo situation the ideal choice of cells was primary human hepatocytes.

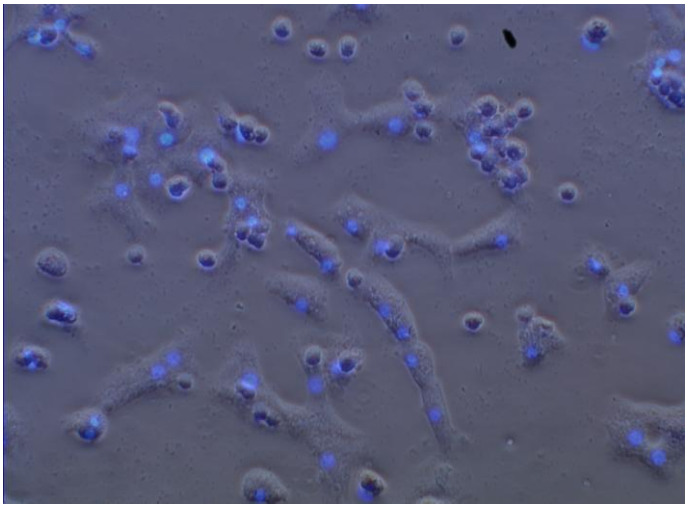
Methods

Primary human hepatocytes were isolated according to the method described above (see general methods – isolation of primary human hepatocytes). Having been counted and viability assessed using trypan-blue cell preparations with viability >95% were suspended in complete Williams E medium and seeded at a density of 66000 /well in a volume of 0.5ml on collagen coated glass 8 chamber slides (see general methods for collagen coating). Cells were then treated with Staurosporine 1uM to ensure apoptosis could be induced.

Results

The results show in figure 14 demonstrate the large healthy adherent cells with bright blue nuclei under Hoescht staining (A) compared with the shrunken spherical apoptotic cells with dark blue nuclei with Hoescht staining These apoptotic human cells have significant activation of Caspase 3 shown in green (B).

A.



B.

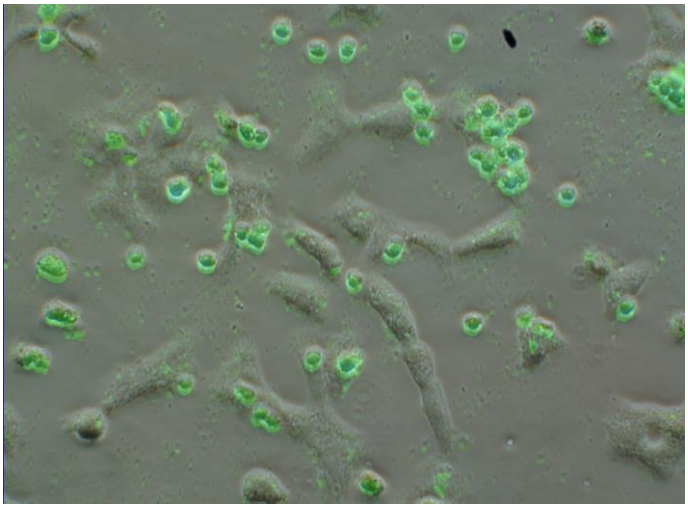


Figure 17. The same fields of Primary human hepatocytes under phase superimposed with fluorescence stained with Hoescht stain (A) and active Caspase 3 (B) having been exposed to Staurosporine 1uM for 4 hours.

The approach was therefore to use this model to investigate apoptosis induced after a period of 4 hours exposure in primary human hepatocytes by substances known to effect apoptosis in animal hepatocytes.

2.4 VALIDATION OF THE IN-VITRO MODEL IN THE ASSESSMENT OF WHETHER EPIDERMAL GROWTH FACTOR (EGF) AND HEPATOCYTE GROWTH FACTOR (HGF) CAN PROTECT PRIMARY HUMAN HEPATOCYTES AGAINST FAS-LIGAND BINDING INDUCED APOPTOSIS

As discussed earlier apoptosis is implicated in the pathogenesis of a number of human liver diseases, including acute liver failure (ALF) (Kasahara, Saitoh, & Nakamura, 2000), alcoholic Hepatitis (Ziol, Tepper, Lohez, Arcangeli, Ganne, Christidis, Trinchet, Beaugrand, Guillet, & Guettier, 2001), viral hepatitis (Rivero *et al.*, 2002),(Nasir *et al.*, 2000), Autoimmune Hepatitis (Masuichi *et al.*, 1999), Wilsons Disease (Strand *et al.*, 1998), post liver transplantation (Memon et al, 2001) and toxin related liver injury (Sakamoto *et al.*, 1998). Although the mechanisms by which hepatocyte apoptosis is controlled are not yet fully defined in these pathological states (Schuchmann & Galle, 2001), Fas, a death receptor of the TNF supereceptor family, is highly expressed in normal liver (Pinkoski *et al.*, 2000). Binding of Fas by Fas-Ligand causes activation of a cascade of Caspase enzymes resulting in apoptosis (Nagata, 1996). Fas knockout mice have hyperplastic livers suggesting Fas may be a key part of the hepatocyte apoptosis mechanism (Adachi *et al.*, 1995). In acute liver failure in humans the levels of death receptors and their ligands rise – there is increased Fas on hepatocytes (Hayashi & Mita, 1999, Rivero *et al*, 2002, Tagami *et al.*, 2003, Taieb *et al.*, 1998), increased Fas-Ligand (Fas-L) on lymphocytes and hepatocytes, elevated serum Fas-L and hepatic tumour necrosis factor (TNF-α),

TRIAL, tumour necrosis factor receptor 1, and TNF-related apoptosis-inducing ligand receptor DR5 expression (Mundt *et al*, 2003) and increased soluble Fas.

Since a significant role of the Fas receptor pathway is implicated in the pathophysiology of acute liver failure, it was decided it would be an appropriate molecule to induce apoptosis to validate our *in-vitro* model.

Understanding which factors regulate apoptosis in hepatocytes may assist in the identification of agents with therapeutic potential. Epidermal growth factor (EGF) has been shown to be protective against Fas-Ligand binding induced apoptosis in primary murine hepatocytes (Musallam *et al.*, 2001), in TGF-ß induced apoptosis in fetal primary hepatocytes (Fabregat *et al.*, 1996), and in rat hepatocytes (Roberts *et al.*, 2000).

The role of hepatocyte growth factor (HGF) is less clear, since whilst it is essential for normal liver growth and hepatocyte survival (Schmidt *et al.*, 1995), in some circumstances HGF can be pro-apoptotic (Gohda *et al.*, 1998) (Arakaki *et al.*, 1998) (Conner *et al.*, 1999). HGF has been shown in-vivo in animal models to prevent endotoxin induced hepatic failure (Kosai *et al.*, 1999), Fas-induced fulminant hepatic failure (Kosai *et al*, 1999) and carbon tetrachloride induced liver injury (Xue *et al.*, 2002), at least in part by reducing hepatocyte apoptosis. One of the mechanisms that growth factors, such as HGF, may inhibit apoptosis is via binding of the activated growth factor to death receptors sequestering them to prevent their aggregation and thus activation (Wang *et al.*, 2002); other mechanisms include interaction with anti-apoptotic proteins (Bardelli *et al.*, 1996). The different effects may depend on dose, cell type and species.

To validate this model our aim was to use the in-vitro system developed for studying apoptosis in primary human hepatocytes to evaluate the effect of EGF and HGF, both alone and in combination, in the protection of primary human hepatocytes against Fas-Ligand binding induced apoptosis. Two methods of measuring apoptosis were used, Caspase 3 activity and TUNEL staining.

Methods

Primary human hepatocytes were isolated by collagen perfusion of segments of resected normal human liver (see general methods). After washing, cells were plated on collagen coated glass slides in complete Williams E medium and incubated for 12 hours at 37ºC in 5%CO_2 to allow cell attachment. Cell layers were washed vigorously to remove unattached cells and cellular debris, and incubated for 2 hours with human EGF at a concentration of 25ng/ml, HGF at 50ng/ml, and a combination of EGF at 25ng/ml and HGF at 50ng/ml, or in complete medium alone. After 2 hours Fas-Ligand (20ng/ml) was added and incubated for a further 4 hours for Caspase activation and 6 hours for TUNEL staining. For measurement of Caspase 3 activation cells were lysed in situ and lysate activity measured using a substrate yielding a luminescent product (Caspase-glo 3/7, Promega, Madison,WI, USA) see general methods.

For TUNEL staining cells were fixed and stained using Rhodamine labelled TUNEL (Apotag R&D systems Abingdon, UK) and Hoechst nuclear stain (see general methods). Slides were visualised under fluorescent microscopy with appropriate filters and then counted. Results were recorded as TUNEL positive cells as a percentage of total cells.

Statistical analysis was performed with results tested for normality using Gaussian distribution; parametric data were analysed using paired Student's *t*-test and non-parametric data using Wilcoxon matched pairs test.

Results

Preliminary experiments showed exposure to Fas-Ligand at 20ng/ml induced similar levels of apoptosis to Staurosporine 1micmol/L. Treatment with Fas-ligand induced more apoptosis than control ($P<0.005$) (Figure 15). EGF ($P<0.05$), HGF ($P<0.05$, and EGF and HGF combined ($P<0.05$) significantly reduced apoptosis compared to Fas-L alone as measured by both Caspase 3 activation (Figure 16.) and TUNEL staining (Figure 17) graphs show means with standard error bars.

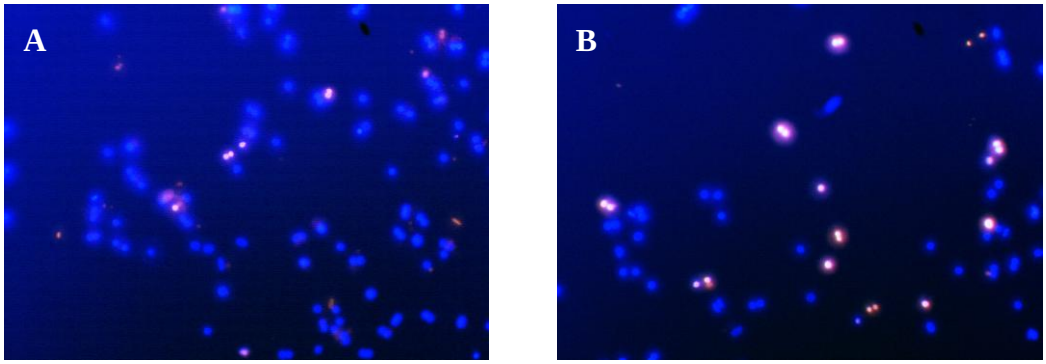


Figure 18. Photomicrograph showing increase in apoptosis in primary human hepatocytes measured by TUNEL staining (pink) with Hoescht nuclear counterstain (blue) induced by Fas-Ligand 20ng/ml (B) compared with control (A).

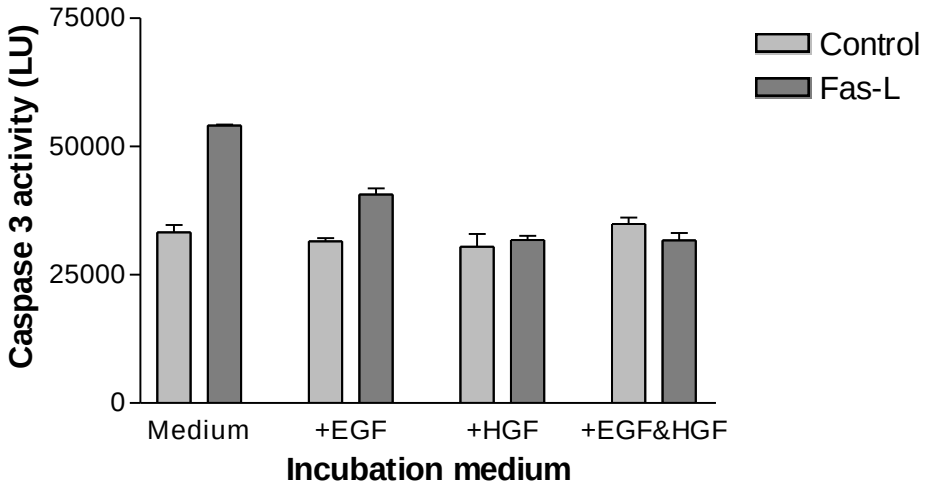


Figure 19. Protective effect of HGF, EGF and EGF and HGF in combination against Fas-L induced apoptosis as measured by Caspase 3 activity (Control= no pro-apoptotic stimulus, Fas-L= Fas-Ligand, LU luminescence units).

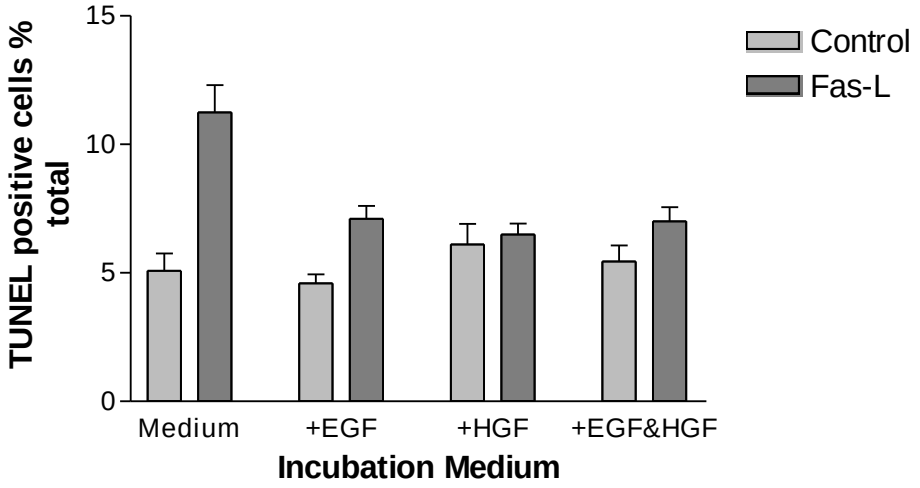


Figure 20. Protective effect of HGF, EGF and EGF and HGF in combination against Fas-L induced apoptosis as measured by TUNEL cell positivity (Control= no pro-apoptotic stimulus, Fas-L= Fas-Ligand).

Conclusion These results validated our model of apoptosis in primary human hepatocytes, which we have used in subsequent chapters. In the next chapter we explored the effect of liver failure plasma on apoptosis using this model.

CHAPTER 3. LIVER FAILURE PLASMA AND ITS EFFECTS ON APOPTOSIS AND CELL DEATH IN PRIMARY HUMAN HEPATOCYTES.

3.0 INTRODUCTION

As discussed in chapter 1 Acute liver failure is associated with the accumulation of a wide range of potentially toxic substances within the circulation as a result both of failure of normal hepatic metabolism and generation from the inflamed liver itself. As well as affecting other organs in the body giving rise to some of the clinical manifestations of this syndrome these toxins may themselves further damage the liver and inhibit liver regeneration, The processes involved in the pathophysiology of ongoing liver damage and regeneration are now being elucidated and this increased knowledge is leading to the generation of new therapies, and a better understanding of the mechanism of action of old ones. The aim of these therapies is to decrease hepatocyte loss and promote liver regeneration tipping the balance in favour of recovery (Fausto, 2000).

Apoptosis appears to be a key process in the pathogenesis of some liver diseases and may in itself result in substantial hepatocyte loss. In addition it may also be a key step in the generation of necrosis and fibrosis. A number of animal models of liver failure have shown apoptotic cells appear very early in the course of liver damage, prior to the appearance of necrosis or significant symptoms, since most of the human tissue examined for apoptosis has occurred after the onset of liver failure it may be that the degree of apoptosis earlier in the disease process is underestimated (Ledda-Columbano *et al.*, 1991). Furthermore and somewhat surprisingly, the inhibition of apoptosis in some animal models of acute liver failure by specific apoptosis inhibitors results in the absence of both apoptosis and necrosis on liver histology as well as the attenuation of symptoms of liver failure and mortality (Cursio *et al.*, 2000, Hoglen, Hirakawa, Fisher, Weeks, Srinivasan, Wong, Valentino, Tomaselli, Bai, Karanewsky, & Contreras,

2001, Jaeschke *et al.*, 1998, Kim *et al.*, 2000, Kunstle *et al.*, 1997, Rodriguez *et al.*, 1996). This challenges traditional beliefs that apoptosis is a benign process leading to cell death without inflammation. Indeed phagocytosis of apoptotic bodies by kupffer cells and infiltrating neutrophils and macrophages results in release of cytokines including TNF-alpha, TGF-β, IL-6 and IL-1β which may result in further apoptosis, increased inflammatory infiltrate and liver fibrosis (Gressner *et al.*, 2002). Thus either by generating too much phagocytosis of apoptotic bodies or by overwhelming the processes that remove apoptotic bodies resulting in the accumulation of extracellular debris, secondary necrosis will occur. Apoptosis may therefore be an essential event in the initiation and perpetuation of liver failure and a target for therapeutic manipulation.

Previous in-vitro studies using hepatocyte derived cell lines (Anderson, Thabrew, & Hughes, 1999, McCloskey, Tootle, Selden, Larsen, Roberts, & Hodgson, 2002, Shi et al, 1998), primary non-human hepatocytes (Hughes, Cochrane, Thomson, Murray-Lyon, & Williams, 1976)) and foetal hepatocytes have shown that plasma/serum from patients with Acute Liver Failure is toxic, resulting in decreased synthetic function, excretory capacity, cell proliferation, cellular adhesion and viability compared to normal plasma/serum. A recently published study adding 20% vol/vol serum to primary human hepatocytes suggested that whilst fulminant hepatic failure serum can induce apoptosis it does not do so until after at least 24 hours exposure and these effects may be secondary to changes in adhesion mediated by the β_1-integrin pathway.

Since plasma represents the internal milieu in which the hepatocytes are situated we hypothesised that substances within liver failure plasma may induce apoptosis in hepatocytes and may represent a mechanism of perpetuation of liver

damage in acute liver failure. Apoptosis induced by exposure to acute liver failure plasma also represents a significant hurdle to hepatocyte transplantation and bioartificial liver function.

Previously demonstrated effects of Acute Liver Failure Plasma have relied on the use of either tumour cell lines or non-human primary human hepatocytes. Whilst these studies have been informative, the use of cell lines derived from malignant cells, which by definition have dysregulation of apoptosis, is inappropriate for the study of apoptosis in "benign" disease, and the use of non-human primary hepatocytes again raises difficulties regarding cross-species effects. Having established a robust reliable in-vitro system for demonstrating and quantifying apoptotic effects we examined the effects of exposure of primary human hepatocytes to 100% vol/vol plasma from patients with acute liver failure (ALFP) on apoptosis. We aimed to establish if Acute Liver Failure Plasma (ALFP) induces apoptosis to a greater extent than plasma from normal controls as an early event independent of cellular adhesion.

The effect of plasma from patients with acutely-decompensated chronic liver disease on hepatocytes has not previously been established. Similarly to Acute Liver Failure there are increased quantities of a number of substances known to be capable of inducing hepatocyte apoptosis in the plasma of patients with chronic liver disease including cytokines, lipopolysacharide, and bile salts. The nature of these compounds may differ from that of acute liver failure in that the picture of liver dysfunction may be much more cholestatic, therefore some of the accumulated compounds have undergone metabolism by hepatocytes and as a consequence be less toxic or conversely substances such as bile salts may accumulate to much higher levels. Our second aim was therefore to investigate

if plasma from patients with acutely-decompensated chronic liver disease induces apoptosis in primary human hepatocytes.

Our third aim was to investigate if there was any correlation between other markers of liver injury/function and quantity of apoptosis induced for a given sample of plasma. For example if the apoptosis inducing agent accumulated as a failure of excretion then other markers of cholestasis, Bilirubin, Alkaline Phosphatase, or gamma GT may correlate with apoptosis inducing ability. If apoptosis is the major initiator of liver injury then apoptosis inducing ability should correlate with markers of hepatocyte injury such as ALT, AST or if apoptosis was induced by an albumin bound toxin then serum albumin may negatively correlate with apoptosis inducing ability as it is only unbound substances that are biologically active. Identifying such correlations may be important in developing new therapeutic strategies for the treatment of liver failure e.g. MARS removes albumin bound toxins that accumulate in liver failure, IV albumin will increase binding of unbound substances, a bioartificial liver would increase metabolism of possible toxic substances and produce substances which inhibit apoptosis, and Caspase inhibitors may decrease liver injury.

Our final aim was to investigate via which pathways Liver Failure Plasma induces apoptosis in hepatocytes. As discussed in Chapter 1 apoptosis can be initiated and executed through a number of different pathways (e.g. extrinsic (death receptor) pathway and intrinsic (mitochondrial) pathway, involving distinct though overlapping intracellular enzymes and proteases notably caspases (Fig.18). Inhibition of the enzyme activity of individual Caspases by small selective peptides (caspase inhibitors) provide tools to investigate the

intracellular pathways that lead to apoptosis, giving information on the possible substances in the plasma involved in the initiation of apoptosis as well as offering therapeutic potential.

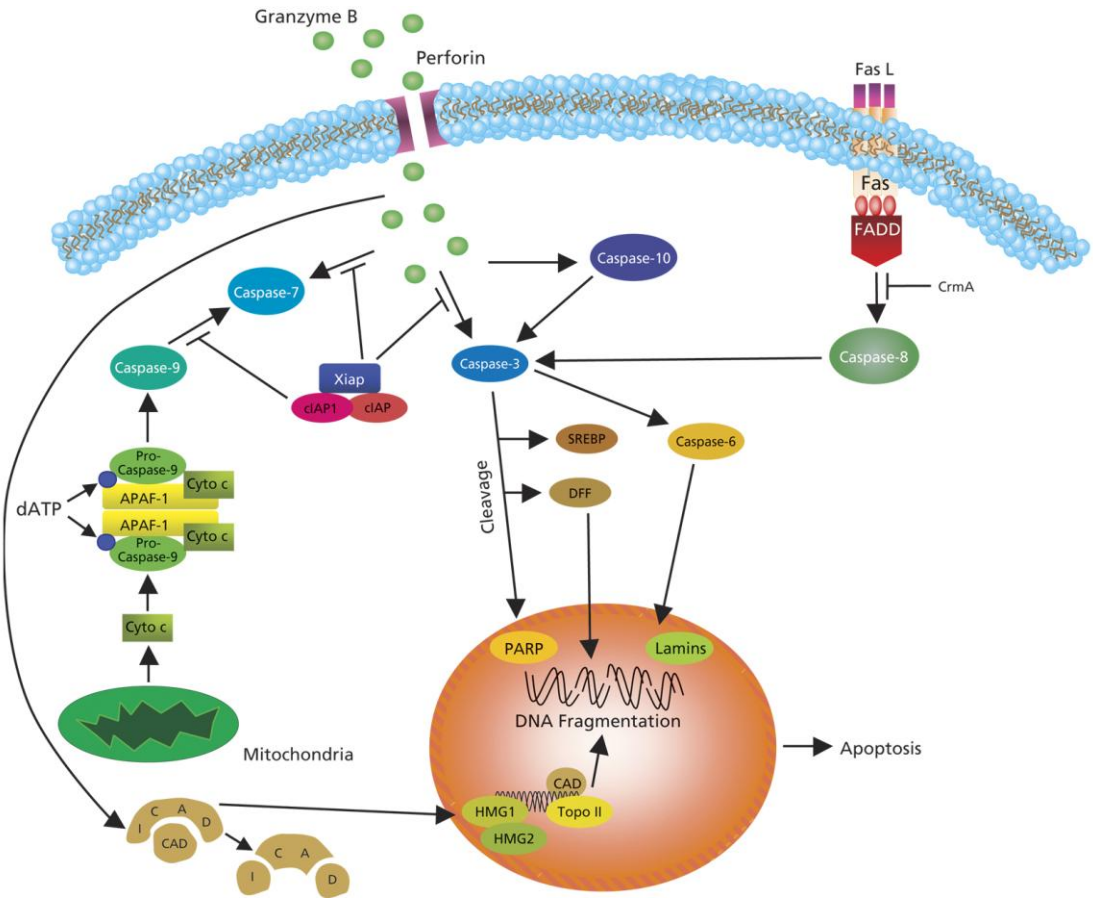


Figure 21.. Diagram outlining Caspase pathways in Apoptosis.

Picture courtesy of R&D Systems

Using our in-vitro system and inhibitors of Caspase3 (common terminal effector in both pathways), Caspase8 (initiator in death receptor pathway) and Caspase9 (activated in mitochondrial pathway) the pathway by which apoptosis is initiated by liver failure plasma was determined.

3.1 MATERIALS & METHODS

Primary human hepatocytes from normal liver resections were isolated by Collagenase perfusion (See appendix * for detailed method & suppliers) and plated in complete Williams E medium (See appendix for recipe & suppliers) at a density of 50 000/well in 100uL of medium on collagen coated sterile 96-well tissue culture plates; white sided plates were used for luminescence based Caspase 3 assay and standard clear sided for measurement of cell viability by MTT activity (See appendix for recipe & suppliers). Cells were plated on sterile Collagen-coated glass eight-chamber slides at a density of 250000/well in 0.5ml of complete Williams E medium. Cells were incubated at 37ºC in 5%CO_2/95%Air overnight to allow attachment and were then thoroughly washed three times with medium to remove unattached cells and cell debris. Cells were exposed either to 100% Liver Failure Plasma (LFP) or 100% normal plasma; complete Williams E medium was used as a negative control; and both Fas-Ligand 20ng/ml in complete Williams E medium and Staurosporine 1uM in complete medium were used as a positive controls.

Measurement of Caspase 3 activation. For measurement of Caspase 3 activation 50uL of test substance was added/well of a white sided 96-well plate in triplicate and incubated for 4 hours in 5%CO_2/95%Air at 37ºC. A 4 hour exposure period was chosen as previous experiments with HepG2 cells (see chapter 3) had shown that this represented adequate time for initiation of Caspase activity and preceded any effects on cellular adhesion. After exposure test plasma was removed and cell monolayers gently washed before the addition of 25uL of sterile PBS. Caspase 3 activity was measured enzymatically using a

substrate that produces a luminescent product (Fig) (Caspase-glo 3/7, Promega) (Figure 18).

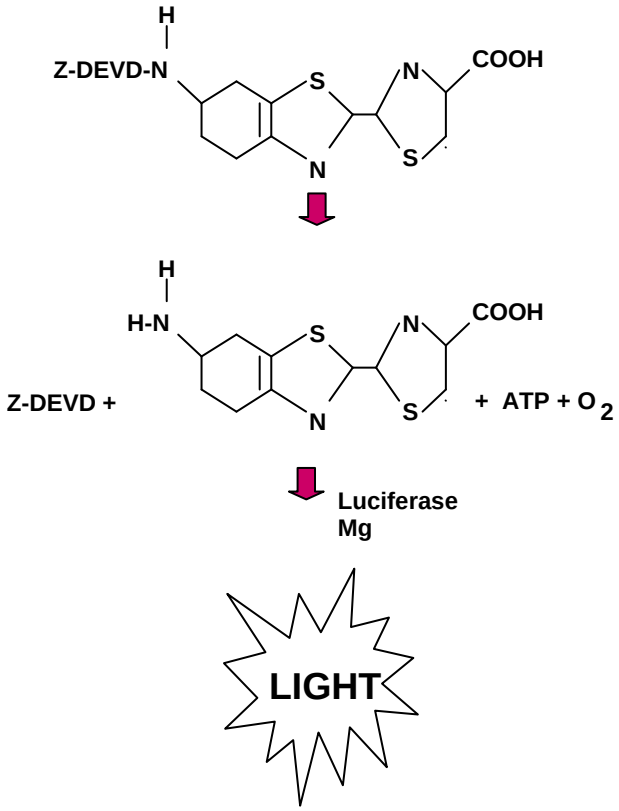


Figure 22. Schematic of luminescent reaction in Caspase glo3/7.

Addition of an equal volume of reagent (25uL/well) results in in-situ cell lysis and also contains a substrate for Caspase 3, the cleavage of DEVD (Aspartic acid -Glutamic acid – Valine – Aspartic acid) from this substrate produces a product that is a substrate for Luciferase (also in the reagent) resulting in the generation of luminescence, thus luminescent activity is directly proportional to Caspase 3 activity. Appropriate blanks of PBS (no cells) were subtracted from readings to eliminate any effects due to background luminescence. After addition of the reagent the 96-well plates were incubated and gently agitated for 1 hour at room temperature. Light emission per well was recorded over 1

second. Luminescence readings in light units (LU) are directly proportional to Caspase 3 activity per well. Since the method is performed in-situ and hepatocytes were plated at identical densities luminescence readings within each experiment are directly comparable.

TUNEL staining. Cells on collagen-coated 8-chamber glass slides were exposed to test plasma/medium/control for 4 hours. Plasma was gently removed and cell monolayers were gently washed with PBS three times. Cells were then fixed and stained using Rhodamine labelled TUNEL and Hoechst nuclear counterstain (Apotag R&D Systems) (see appendix). Cells were visualised under high power (magnification x40) fluorescent microscopy with appropriate filters and counted. A minimum of 1000 cells/well were counted. Results were recorded as TUNEL positive cells as a % of total cells.

MTT activity. MTT activity was used as a measure of cell viability. Cells in 96-well plates were exposed to test plasma for 16 hours following which cells were washed three times, 50μL of the tetrazolium salt, MTT (0.75mg/ml in PBS), was added to each well and the plates incubated at 37^{o}C for 3 hours in the dark. Excess MTT solution was then flicked off leaving the purple granules of formazan product within the cells attached to the bottom of the well. To solubilise the formazan product 100μL/well of 0.004M Hydrochloric acid in iso-propranol was added. The 96-well plate, wrapped in clingfilm to prevent evaporation, was agitated on a platform shaker for 30 minutes to ensure complete dissolution of the formazan product. The optical density at 550nm was read by an automated plate reader. Optical density is therefore proportional to live cell number.

Each experiment was repeated on at least three separate occasions each time using hepatocytes prepared from a different liver resection in an attempt to eliminate individual variation in hepatocyte susceptibility. Results shown are representative of all experiments.

Plasma Samples

Heparinised plasma from 15 individuals with acute liver failure due to various aetiologies and severity (Table 1), 31 patients with acutely-decompensated chronic liver disease of various aetiology and severity (Table 2), and 4 normal controls, was acquired and stored at -20^{o}C. In patients with acute liver failure due to paracetamol overdose samples were taken after a minimum of 48 hours after ingestion by which time Paracetamol was undetectable in the plasma. Subjects (or if unable their advocates) gave informed consent for the acquisition and experimental use of tissue samples in accordance with local ethics committee guidelines. Liver function tests were measured using standard laboratory based methods by the clinical biochemistry department.

Having confirmed data had a Gaussian distribution, data was subsequently analysed for statistical significance using a Student's t-test. To investigate the relationship between clinical parameters and apoptosis/cell viability, linear regression and correlation analyses were performed.

Table 3- Acute Liver Failure plasma.

ID	Age	Sex	Aetiology	Liver & Renal Function								
				Ur	Cr	BIL	ALP	AST	ALT	GGT	TPR	ALB
1	32	M	POD	5.3	525	77	69	99	411	44	44	28
2	51	M	POD	16.1	150	188	94	357	668	281	34	24
3	47	F	Hepatitis Non A-E	12.2	213	29	60	2427	3058	32	38	34
4	45	F	POD	3.8	252	248	61	329	66	11	37	27
5	34	M	POD	2.8	117	206	34	5.0	6.0	48	48	26
12	27	M	Alcoholic Hepatitis	27.6	278	487	252	51	91	390	43	26
14	34	M	Hepatitis Non A-E	5.2	87	162	172	248	274	426	72	37
22	51	M	POD	26.4	633	544	123	42	62	107	54	31
25	72	M	Hepatitis Non A-E	11.3	171	256	374	308	194	126	65	25
38	25	M	POD	6.2	97	52	98	9441	3930	186	45	20
39	48	F	POD	11.2	206	40	89	687	2362	154	30	12
87	38	F	Hepatitis Non A-E	4.2	157	439	263	405	229	141	64	23
92	28	F	Hepatitis Non A-E	3	167	484	314	358	188	133	45	20
99	22	M	POD	8.3	167	141	41	142	163	193	49	21
52	42	F	Wilsons Disease	23.7	237	825	26	109	52	31	57	29

Table 4-Chronic liver disease patients.

ID	Age	Sex	Aetiology	Liver & Renal Function								
				Ur	Cr	BIL	ALP	AST	ALT	GGT	TPR	ALB
7	59	M	Acutely decompensated alcoholic Cirrhosis	14.1	173	441	196	173	129	61	52	28
9	42	M	Alcoholic Hepatitis	12.1	173	569	147	81	32	104	41	24
10	45	F	Decompensated PBC	16.8	428	207	763	96	69	248	58	27
11	44	F	Decompensated Autoimmune Hepatitis	4.0	141	517	87	249	181	294	50	33
12	27	F	Alcoholic Hepatitis	27.6	278	487	252	51	91	390	43	26
15	58	M	Acutely decompensated alcoholic Cirrhosis	7.1	132	351	158	51	46	16	60	30
16	50	M	Acutely decompensated alcoholic Cirrhosis	8.5	155	358	91	117	42	106	51	25
18	61	M	Acutely decompensated alcoholic Cirrhosis	7.8	136	118	267	69	34	628	59	29
19	58	M	Acutely decompensated alcoholic Cirrhosis	4.8	115	354	431	176	107	164	58	22
21	52	F	Acutely decompensated alcoholic Cirrhosis	23	245	135	901	39	55	758	48	25
23	44	M	Acutely decompensated alcoholic Cirrhosis	3.2	132	400	212	158	83	288	45	22
24	48	F	Decompensated PBC	7.5	87	102	141	140	113	114	55	28
28	28	F	CAH	5.4	139	447	481	202	445	879	51	31
31	45	M	Acutely decompensated alcoholic Cirrhosis	20.6	431	384	147	52	36	83	76	39
32	54	M	Acutely decompensated alcoholic Cirrhosis	2.8	79	49	80	37	22	110	61	25
33	52	M	Acutely decompensated alcoholic Cirrhosis	20.3	192	317	419	223	215	311	42	30
34	42	M	Acutely decompensated alcoholic Cirrhosis	4.4	128	228	230	144	101	25	55	23
36	29	M	Acutely decompensated alcoholic Cirrhosis	4.5	109	124	131	122	52	582	55	30
37	86	M	Obstructed CBD, alcohol Cirrhosis	5.0	129	252	1052	137	121	888	55	30
73	59	F	Acutely decompensated alcoholic Cirrhosis	32.1	136	569	352	169	93	311	44	34
86	33	F	Acutely decompensated alcoholic Cirrhosis	2.9	135	434	188	111	56	584	59	34

124/DW	31	M	Acutely decompensated alcoholic Cirrhosis	16.8	136	646	117	54	101	88	43	28
120/DS	64	M	Acutely decompensated alcoholic Cirrhosis	35.4	649	297	251	105	47	39	69	23
117/MR	46	F	Acutely decompensated alcoholic Cirrhosis	2.3	90	273	162	115	85	73	72	37
113/KB	46	F	Acutely decompensated alcoholic Cirrhosis	20.1	235	451	333	93	56	410	46	30
93/NS	33	F	Alcoholic Hepatitis	3.3	132	561	149	92	40	-	56	32
78/TN	33	F	Acutely decompensated alcoholic Cirrhosis	2.6	125	150	97	58	97	-	75	32
67/AG	58	M	Acutely decompensated alcoholic Cirrhosis	13.4	264	691	254	90	54	60	52	31
SR	54	F	Alcoholic Hepatitis	12.2	132	296	238	139	105	296	54	24
JW	34	M	BRIC	8.2	187	677	334	147	294	73	60	37
40/SD	39	M	Acutely decompensated alcoholic Cirrhosis	8.6	143	245	143	80	62	56	51	28

3.2 RESULTS

Acute Liver Failure Plasma

Primary human hepatocytes showed increased numbers of apoptotic cells (as demonstrated by TUNEL staining (Fig 3) after exposure to Acute Liver Failure Plasma (LFP) compared to normal plasma.

This was confirmed by the demonstration of significantly increased Caspase 3 activity in primary human hepatocytes exposed to liver failure plasma compared to normal plasma (Fig. 20).

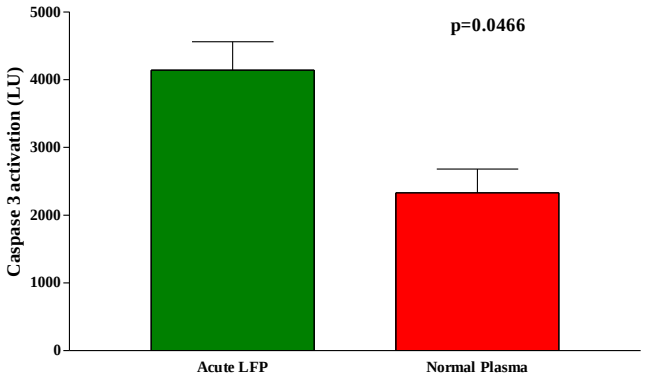


Figure 23. Caspase 3 activation in primary human hepatocytes exposed to samples of acute liver failure plasma (green n=15) and normal plasma (red n=4).

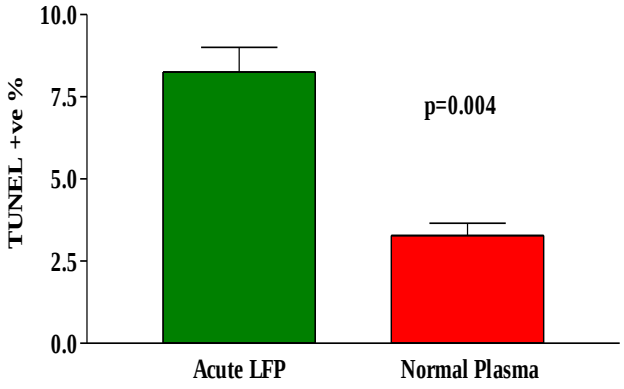


Figure 24. Mean (%) total primary human hepatocytes TUNEL +ve after exposure to samples of acute liver failure plasma (green n=15) and normal plasma (red n=4).

There was significant variation in quantity of apoptosis between individual samples of acute liver failure plasma with nine of the fifteen samples exhibiting significantly more apoptosis, two of the samples significantly less and four of the samples no difference in the amount of apoptosis as measured by Caspase 3 activation compared to the mean for normal controls.

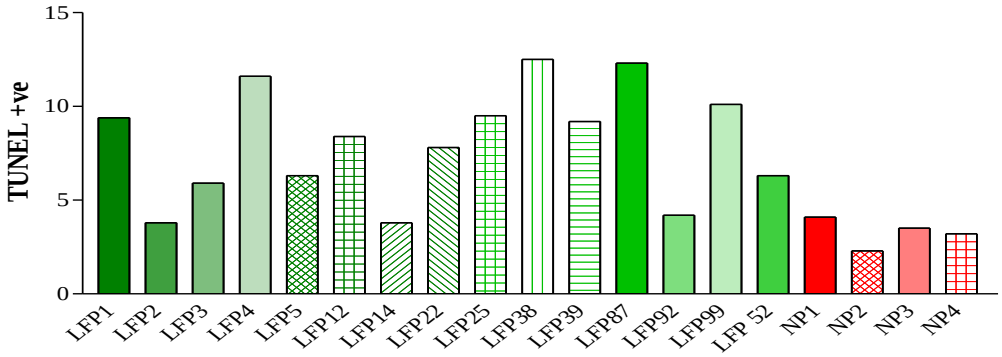


Figure 25. Percentage cells TUNEL +ve after exposure to samples of acute liver failure plasma (green) and normal plasma (red)

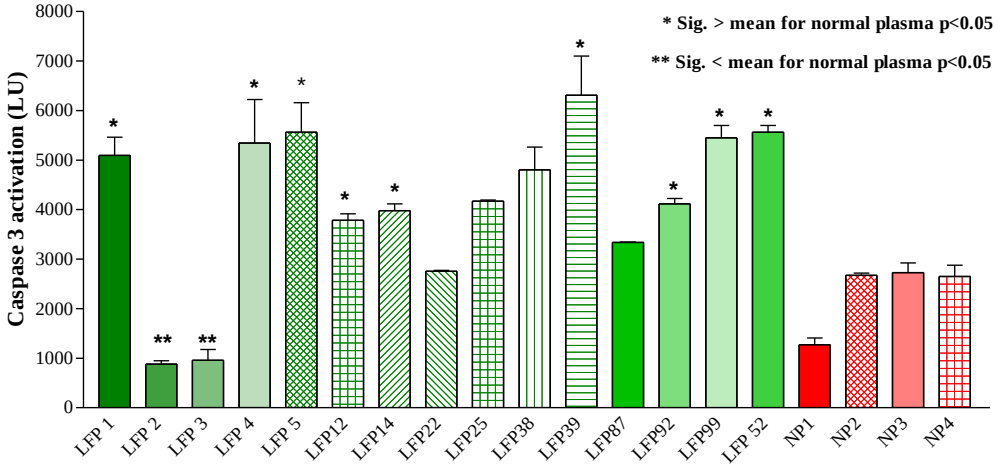


Figure 26. Caspase 3 activation in primary human hepatocytes exposed to samples of acute liver failure plasma (green) and normal plasma (red).

Quantification of apoptosis by TUNEL staining and by measurement of Caspase 3 activity showed a good correlation Pearsons r = 0.6150, p=0.0051. This confirms that both methods are reliable in the detection of apoptosis.

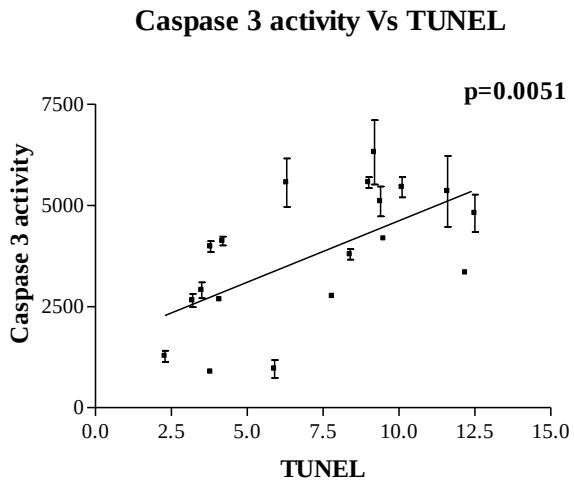
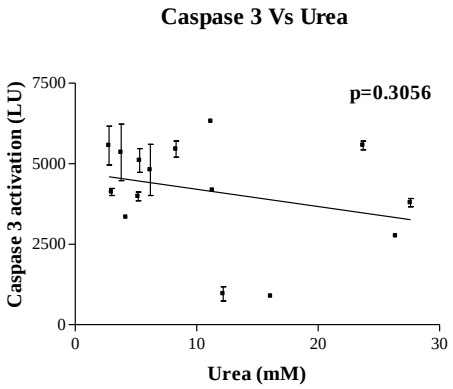
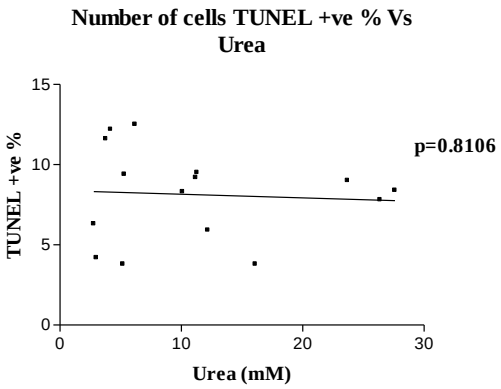


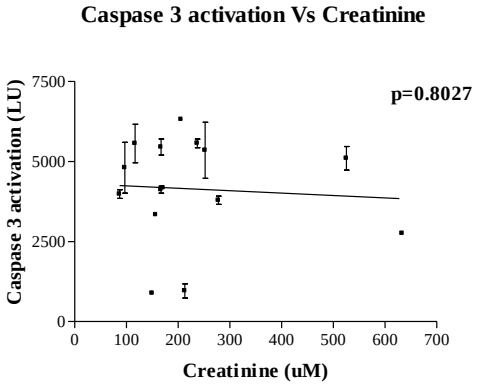
Figure 27. Correlation between TUNEL cell positivity and Caspase 3 activity in primary human hepatocytes exposed to liver failure plasma.

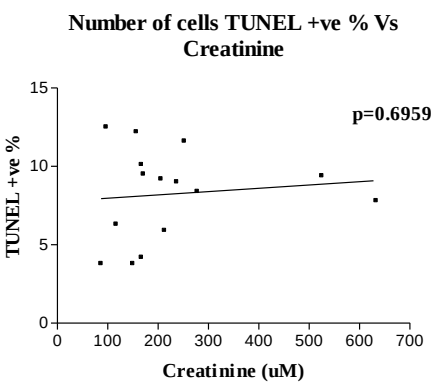
Clinico-Pathological correlates - Acute Liver Failure Plasma

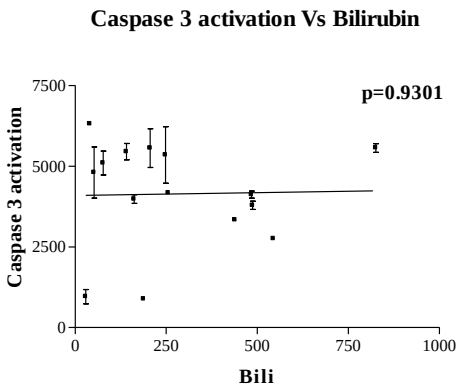
There was no correlation between amount of apoptosis induced in hepatocytes by exposure to acute liver failure plasma measured either by Caspase 3 activation or TUNEL staining and markers of cholestasis (Bilirubin, ALP, GGT), hepatocellular injury (AST, ALT) or serum albumin.

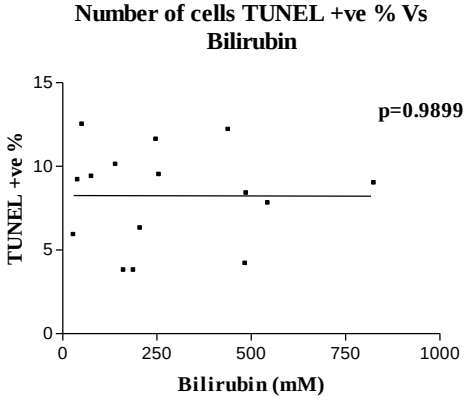

Caspase 3 Vs Urea
p=0.3056
Caspase 3 activation (LU)
7500
5000
2500
0
0
10
20
30
Urea (mM)


Number of cells TUNEL +ve % Vs Urea
p=0.8106
TUNEL +ve %
15
10
5
0
0
10
20
30
Urea (mM)

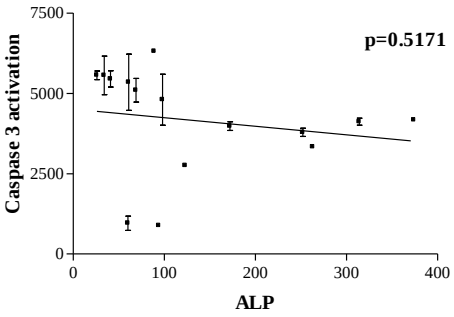

Caspase 3 activation Vs Creatinine
p=0.8027
Caspase 3 activation (LU)
7500
5000
2500
0
0
100
200
300
400
500
600
700
Creatinine (uM)


Number of cells TUNEL +ve % Vs Creatinine
p=0.6959
TUNEL +ve %
15
10
5
0
0
100
200
300
400
500
600
700
Creatinine (uM)

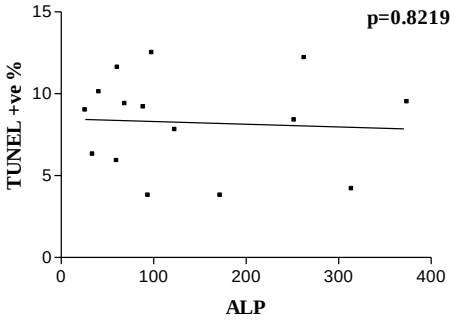

Caspase 3 activation Vs Bilirubin
p=0.9301
Caspase 3 activation
7500
5000
2500
0
0
250
500
750
1000
Bili


Number of cells TUNEL +ve % Vs Bilirubin
p=0.9899
TUNEL +ve %
15
10
5
0
0
250
500
750
1000
Bilirubin (mM)

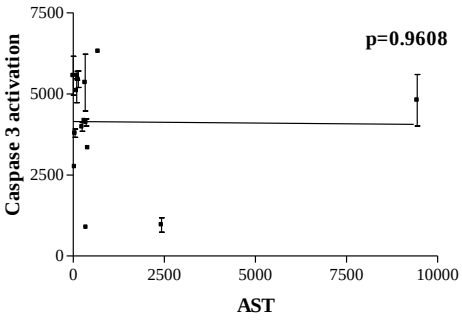
Caspase 3 activation Vs ALP



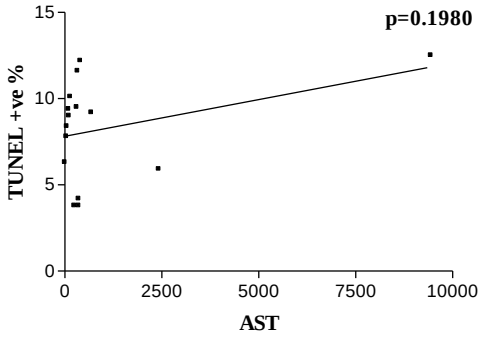
Number of cells TUNEL +ve % Vs ALP



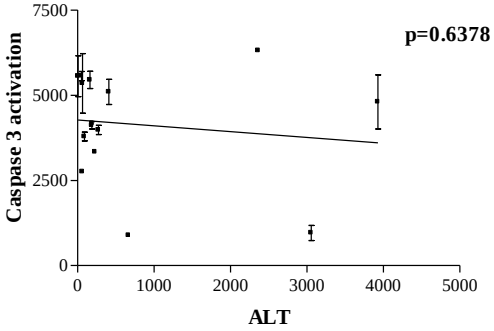
Caspase 3 activation Vs AST



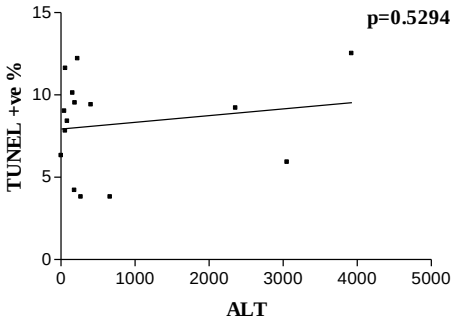
Number of cells TUNEL +ve % Vs AST



Caspase 3 activation Vs ALT



Number of cells TUNEL +ve % Vs ALT



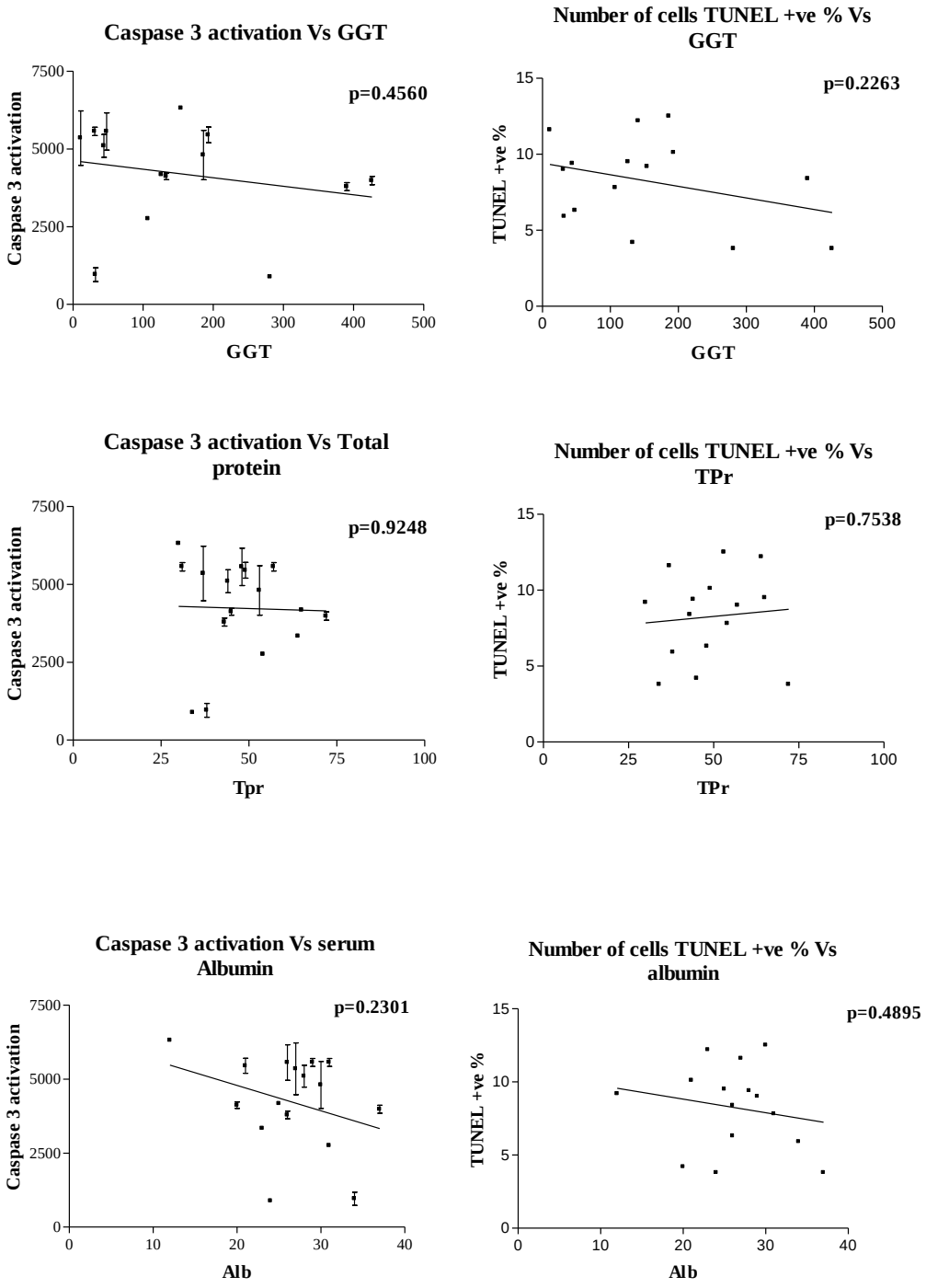


Figure 28. Clinico-Pathological correlates - Acute Liver Failure Plasma

Chronic Liver Disease Plasma

Unlike Acute liver failure plasma, plasma from patients with chronic liver disease did not induce significantly more apoptosis than normal plasma as measured by TUNEL cell positivity or Caspase 3 activity. However both liver failure plasma and normal plasma showed significantly reduced apoptosis compared to medium. Positive controls Fas-Ligand and Staurosporine showed significant increases in apoptosis demonstrating that the failure to demonstrate any difference between normal plasma and chronic liver disease plasma was not due to technical failure.

Again there was large variability in apoptotic responses between individual chronic liver failure plasma samples and some samples did induce increased apoptosis whilst others decreased apoptosis (Figure 28.).

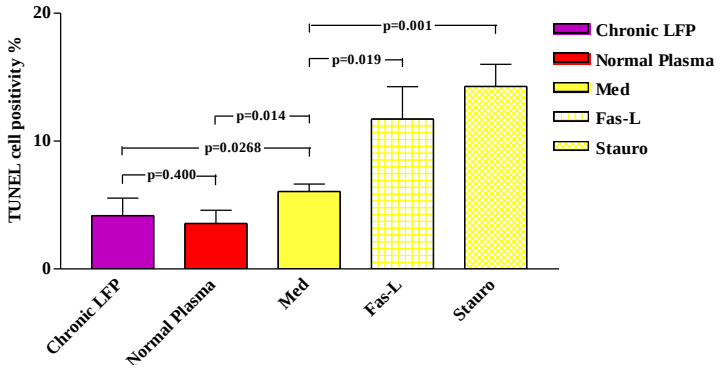


Figure 29. Mean TUNEL cell positivity in primary human hepatocytes exposed to chronic liver failure plasma n=31 (purple),normal plasma n=4 (red) and controls (yellow).

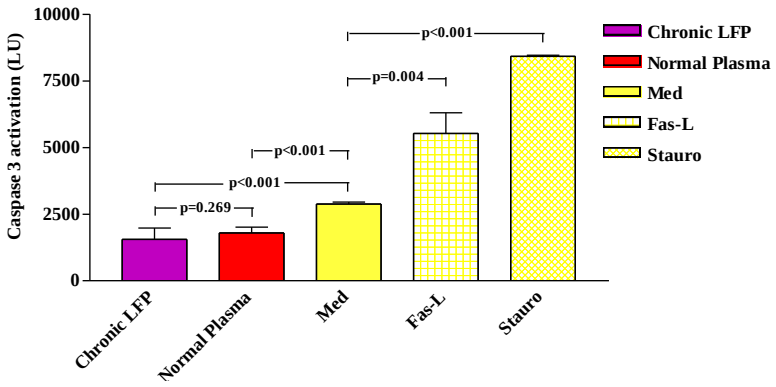


Figure 30. Mean Caspase 3 activation in primary human hepatocytes exposed to chronic liver failure plasma n=31 (purple),normal plasma n=4 (red) and controls (yellow).

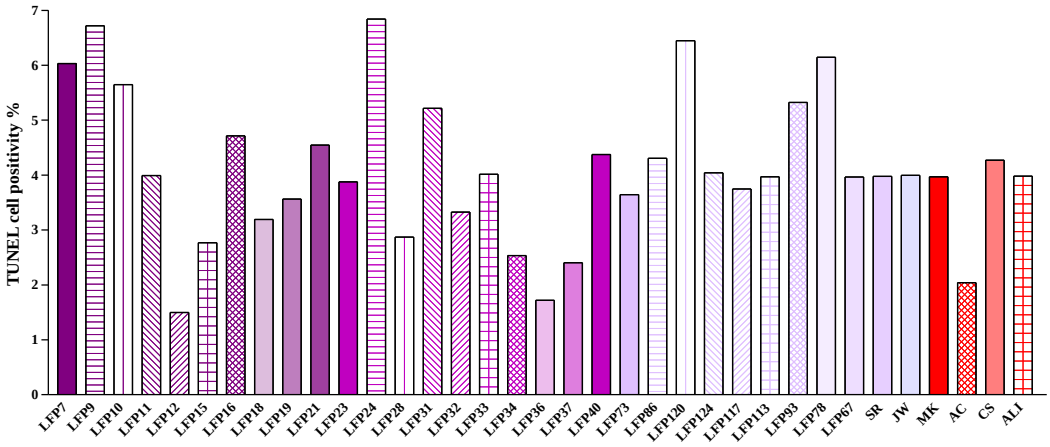


Figure 31. TUNEL cell positivity for primary human hepatocytes exposed to samples of acute liver failure plasma (green) and normal plasma (red)

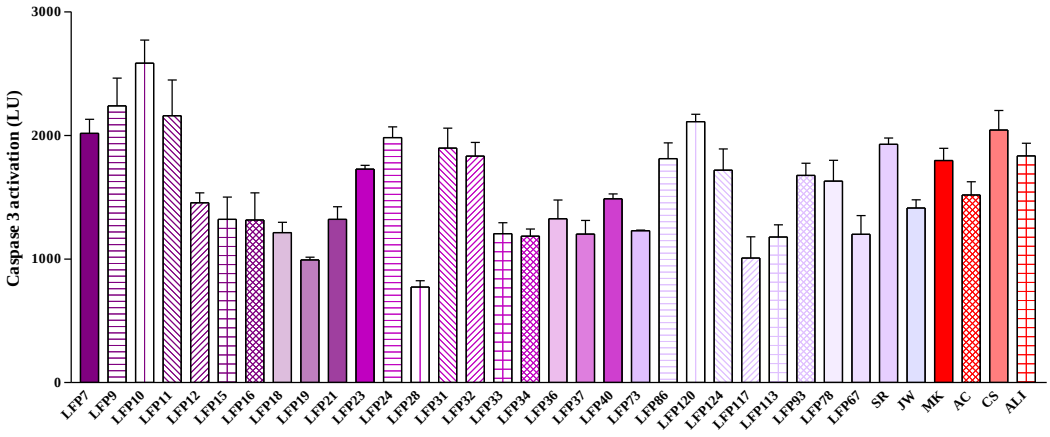


Figure 32. Caspase 3 activation in primary human hepatocytes exposed to samples of acute liver failure plasma (green) and normal plasma (red)

As with previous experiments there was good correlation between apoptosis as measured with TUNEL and Caspase 3 activity.

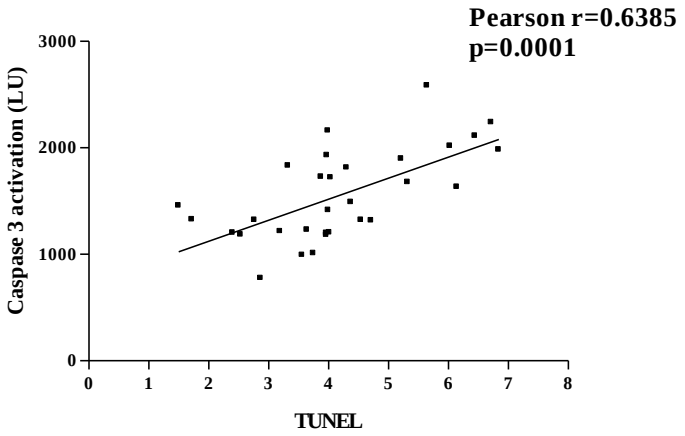
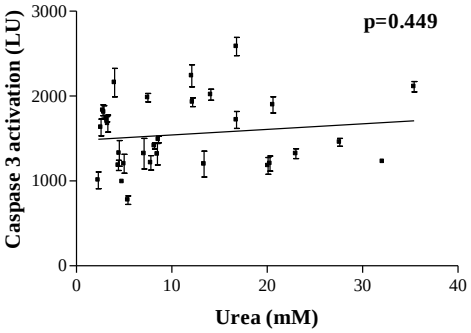


Figure 33. Correlation between Caspase 3 activation and percentage cells TUNEL positive for chronic liver failure plasma.

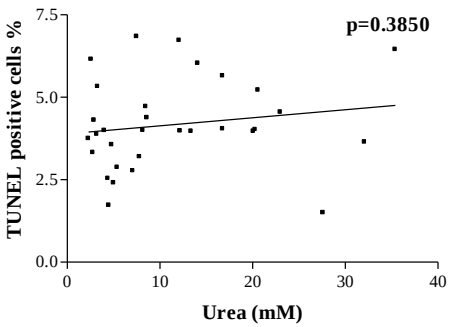
Clinico-pathological correlates for chronic liver failure plasma

There was no correlation with markers of liver injury, cholestasis, renal function or synthetic function and apoptosis induced by plasma from patients with chronic liver disease.

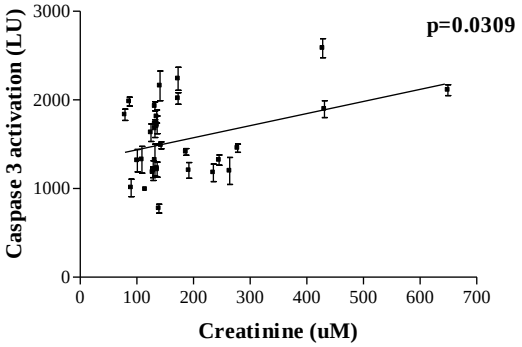
Caspase 3 Vs Urea for acutely decompensated chronic liver disease



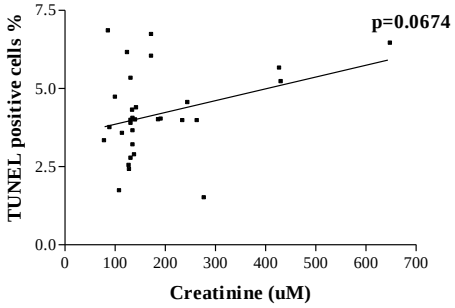
TUNEL cell positivity Vs Urea for acutely decompensated chronic liver disease



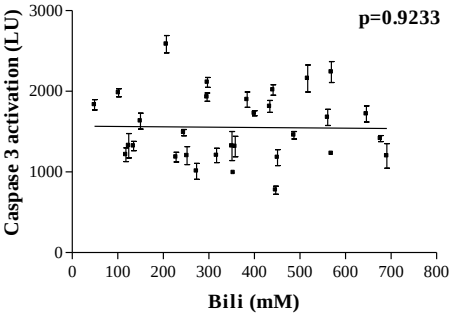
Caspase 3 activation Vs Creatinine for acutely decompensated chronic liver disease



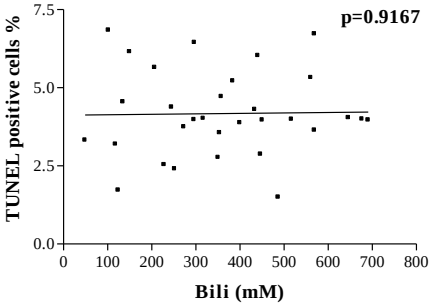
TUNEL positive cells % Vs Creatinine for acutely decompensated chronic liver disease

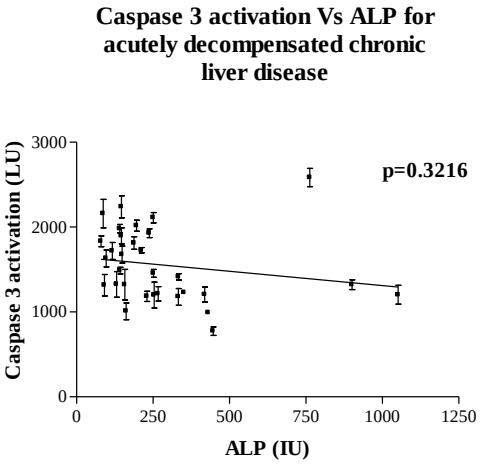


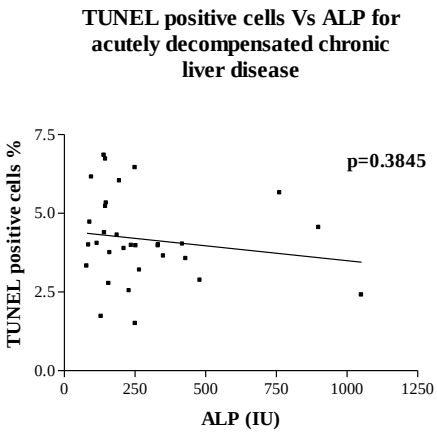
Caspase 3 activation Vs Bilirubin for acutely decompensated chronic liver disease

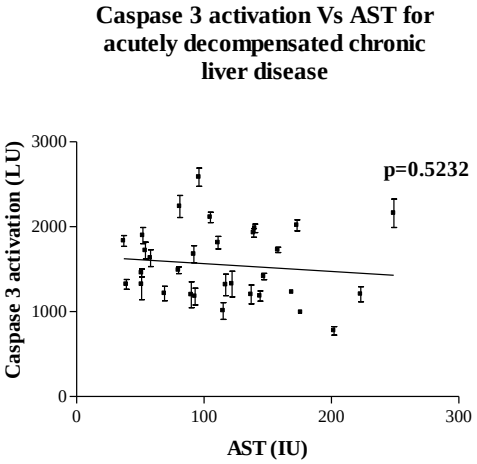


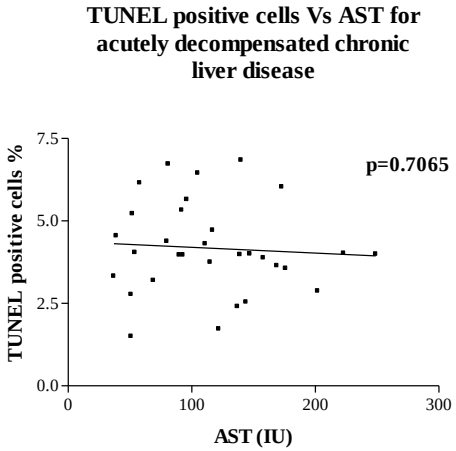
TUNEL positive cells Vs Bilirubin for acutely decompensated chronic liver disease



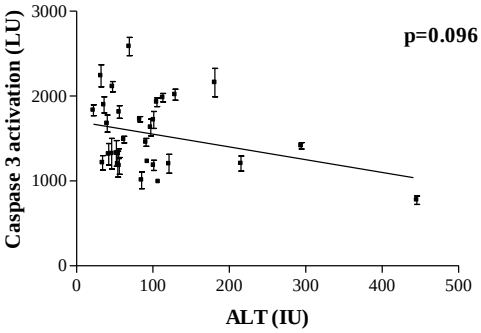

Caspase 3 activation Vs ALP for acutely decompensated chronic liver disease
p=0.3216
Caspase 3 activation (LU)
ALP (IU)
0
1000
2000
3000
0
250
500
750
1000
1250


TUNEL positive cells Vs ALP for acutely decompensated chronic liver disease
p=0.3845
TUNEL positive cells %
ALP (IU)
0.0
2.5
5.0
7.5
0
250
500
750
1000
1250

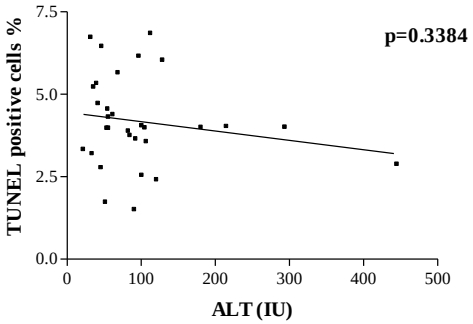

Caspase 3 activation Vs AST for acutely decompensated chronic liver disease
p=0.5232
Caspase 3 activation (LU)
AST (IU)
0
1000
2000
3000
0
100
200
300


TUNEL positive cells Vs AST for acutely decompensated chronic liver disease
p=0.7065
TUNEL positive cells %
AST (IU)
0.0
2.5
5.0
7.5
0
100
200
300

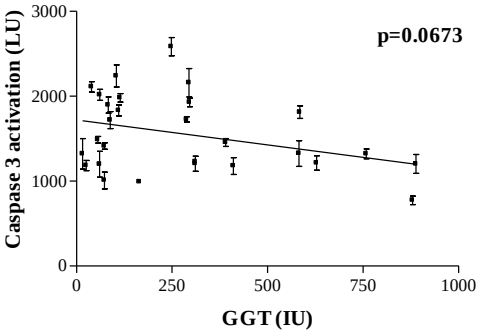
Caspase 3 activation Vs ALT for acutely decompensated chronic liver disease



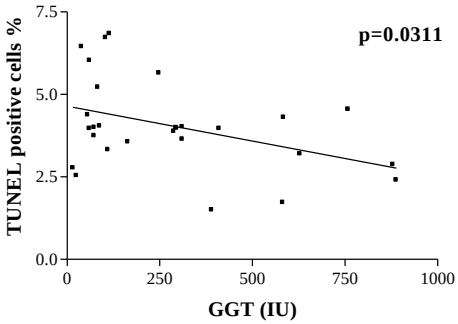
TUNEL positive cells Vs ALT for acutely decompensated chronic liver disease



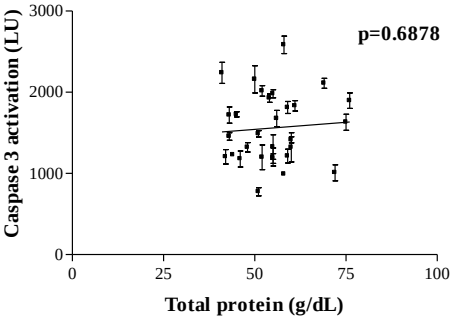
Caspase 3 activation Vs GGT for acutely decompensated chronic liver disease



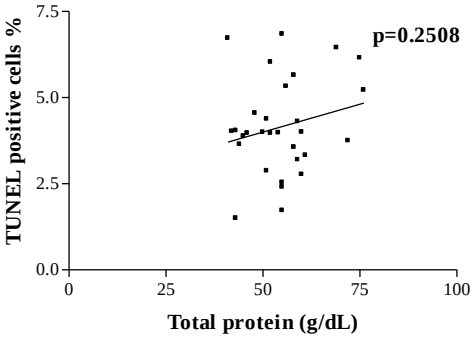
TUNEL positive cells Vs GGT for acutely decompensated chronic liver disease



Caspase 3 activation Vs Total protein for acutely decompensated chronic liver disease



TUNEL positive cells Vs Total protein for acutely decompensated chronic liver disease



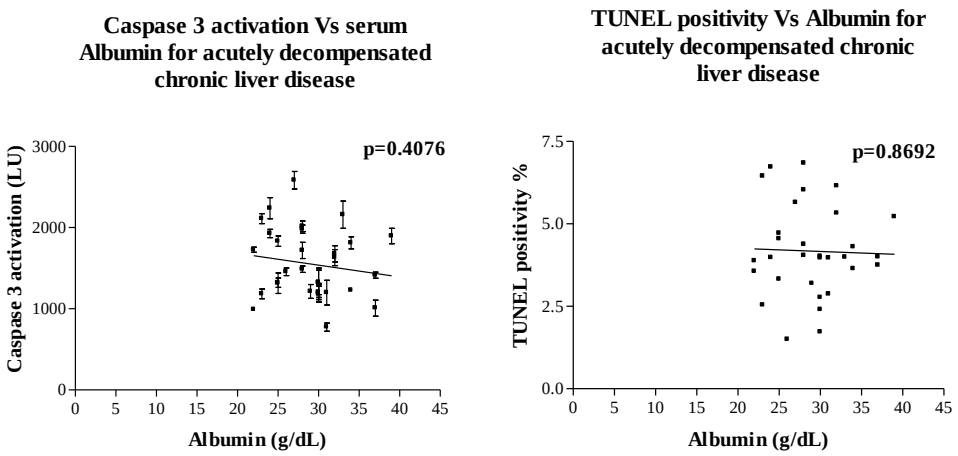


Figure 34. Clinico-pathological correlates for chronic liver failure plasma.

As with acute liver failure plasma chronic liver disease plasma showed no association of cell viability with markers of liver injury, cholestasis, renal function or synthetic function.

3.3 CASPASE INHIBITORS

Acute Liver Failure plasma samples that had been shown to increase hepatocyte apoptosis were used to assess the effect of Caspase inhibitors.

Materials & Methods

Caspase inhibitors were purchased from R&D Systems and stored at -20C until use. Having been solubilised in DMSO according to manufacturers' instructions a solution of 100-fold the final required strength in Williams E medium (no additives) was prepared. DMSO at the same concentration in Williams E was

used as control (no inhibitor) to eliminate the effects of DMSO on apoptosis. Caspase inhibitors or control were diluted 1:100 with plasma, mixed thoroughly and applied to cell layers for 4 hours before TUNEL analysis as before. A Caspase 3 inhibitor Z-VAD-FMK (z-Valine – Alanine – Aspartic Acid-fluoromethylketone) (C3I) (20μMol), a Caspase 8 inhibitor Z-IETD-FMK (z-Isoleucine - Glutamic Acid - Threonine – Aspartic Acid – flouromethylketone) (C8I) (20μMol), and a Caspase 9 inhibitor Z-LEHD-FMK (z-Leucine – Glutamic Acid – Histidine – Aspartic Acid) (C9I) (20 μMol) were all used at final concentrations specified. For experiments with Caspase inhibitors Caspase 3 activity analysis of cell layers was performed after removal of plasma and washing thrice with sterile PBS to remove any Caspase inhibitor which may interfere with the assay. Data were tested for statistical significance using a paired student t-test.

Results

The increase in apoptosis induced by Acute liver failure plasma (n=9) measured by both % cells TUNEL positive and increased Caspase 3 activity was significantly inhibited by the presence Caspase 3, and Caspase 8 inhibitors but not by a Caspase 9 inhibitor.

Table 5. Apoptisis induced by Acute Liver Failure Plasma in the presence of Caspase 3, Caspase 8 and Caspase 9 inhibitors.

	Normal Plasma	**LFP**	**LFP+Casp 3 inhibitor**	**LFP+Casp 8 inhibitor**	**LFP+Casp 9 inhibitor**
Caspase3 activity(LU)	3426 $^{+}$/.520 p<0.05	6145 $^{+}$/.494	4140 $^{+}$/.228 p<0.01	5187 $^{+}$/.516 p<0.01	5332 $^{+}$/.223 p=0.10
TUNEL+ve Cells %	3.9 $^{+}$/.0.44 p<0.05	9.4 $^{+}$/.0.49	4.9 $^{+}$/.0.25 p<0.01	5.8 $^{+}$/.0.43 p<0.01	7.0 $^{+}$/.0.88 p=0.07

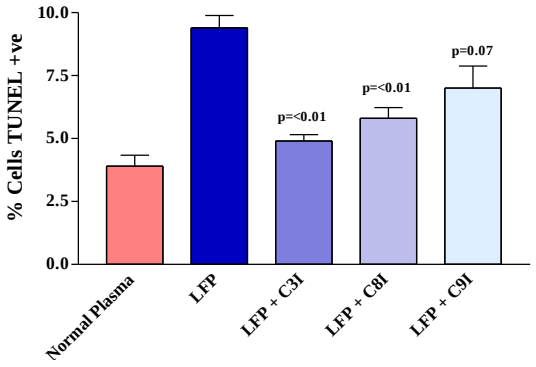


Figure 35. Percentage cells TUNEL positive for primary human hepatocytes incubated in Acute Liver Failure Plasma +/-Caspase inhibitors

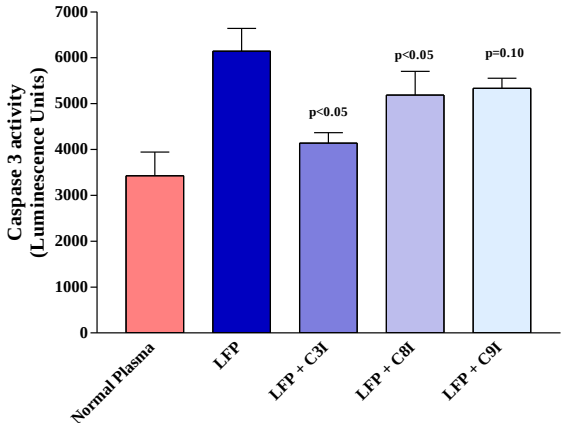


Figure 36. Caspase 3 activity (LU) for primary human hepatocytes incubated in Acute Liver Failure Plasma +/-Caspase inhibitors

Discussion

The presence of Caspase 3 inhibitors significantly inhibited apoptosis. Caspase 3 is an effector caspase utilised by both intrinsic and extrinsic pathways. Caspase 8 is the major caspase in the propagation of signals from cell surface death receptors to effector caspases; the protective effect of Caspase 8 inhibitors

suggests that the toxic substances in liver failure plasma that induce apoptosis in hepatocytes do so by activation of cell surface death receptors. Caspase 3 and 8 inhibitors may therefore have therapeutic potential in acute liver failure. Caspase 9 inhibition did not significantly inhibit apoptosis although there was a trend towards a reduction. This is may be due to the inhibition of Caspase 9 activation downstream of Caspase 3 activation and cytochrome c release. Caspase 9 is activated by cytochrome c release and forms part of the amplifying signal after initiation of apoptosis.

In summary there are substances in acute liver failure plasma (but not in chronic liver failure plasma which induce apoptosis in primary human hepatocytes via a Caspase 8 dependent pathway. Further experiments were undertaken to define the physico-chemical properties of the molecule.

3.4 DOSE RESPONSE CURVES

Dose response curves of normal and acute liver failure plasma mix were constructed having diluted plasma vol/vol with complete Williams E medium. Heat inactivated fetal calf serum was used as a control. Paradoxically both liver failure plasma and normal plasma significantly decrease the Caspase 3 activity they induce as they become more concentrated i.e. the exact opposite of the expected curve for a toxic substance. This interesting finding is consistent with the previous work in HepG2 cells (chapter 2) which showed that human plasma from either normal controls or liver failure patients resulted in improved cell viability compared with medium. Also at 100% vol/vol plasma the liver failure plasma induces more caspase activity than normal plasma as in previous experiments.

Why human plasma is more effective at promoting hepatocyte survival is unknown, but the presence of either heat labile (inactivated in FCS) or species specific protective factors are a possibility.

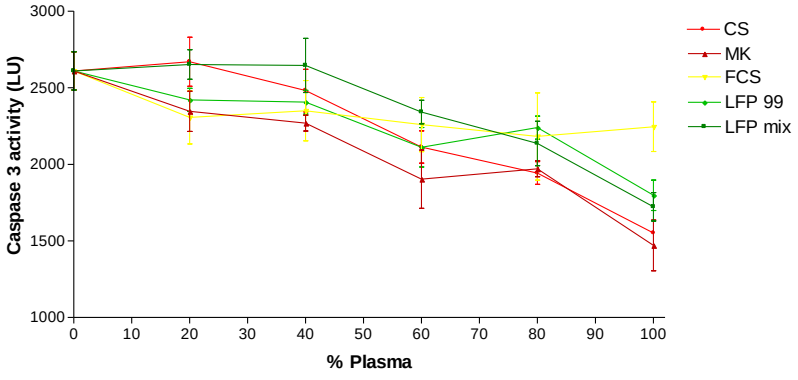


Figure 37. Caspase 3 activity in primary human hepatocytes after exposure to plasmas diluted with complete medium (red=normal plasma, green=liver failure plasma).

3.5 HEAT INACTIVATION OF ACUTE LIVER FAILURE PLASMA

Earlier experiments demonstrated that Acute Liver Failure plasma increased apoptosis in hepatocytes compared to normal plasma. This is consistent with experimental work from other groups using rat hepatocytes and cell lines, and has been the main evidence for suggesting that there are toxic substances within acute liver failure plasma. However both normal plasma and liver failure plasma were better for cells than Complete Williams E medium or 100% FCS (heat inactivated). An alternative suggestion could therefore be that there are substances within human plasma that are protective for hepatocyte cell death and that Acute Liver Failure contains less of these substances than normal plasma. All previous published work used normal plasma as control rather than medium/FCS. If there was a heat labile toxic substance within liver failure plasma we would expect that heat activating would result in decreased Caspase 3 activation, whereas if plasma contained heat labile protective factors then we would expect that heat inactivation would result in increased Caspase 3 activation. This section explores these two alternatives.

Materials & Methods

Human plasma was heat inactivated by warming to 56ºC in a water bath for 30 mins. Heat inactivated plasma and normal plasma was then applied to hepatocytes in 96 well plates and after 4 hours Caspase 3 activity measured as previously described.

Results

Heat inactivation of plasma resulted in a significant increase in Caspase 3 activity in hepatocytes compared with untreated control plasma in 3 of the 4 samples of acute liver failure plasma and in all the samples of normal plasma. It

can be seen that the increase in apoptosis induced by heat inactivation was far greater for normal plasma than liver failure plasma. Interestingly once heat inactivated apoptosis induced by 100% vol/vol human plasma was not significantly different from apoptosis induced by 100% vol/vol heat inactivated FCS. Suggesting that once heat inactivated there is no cross species effects.

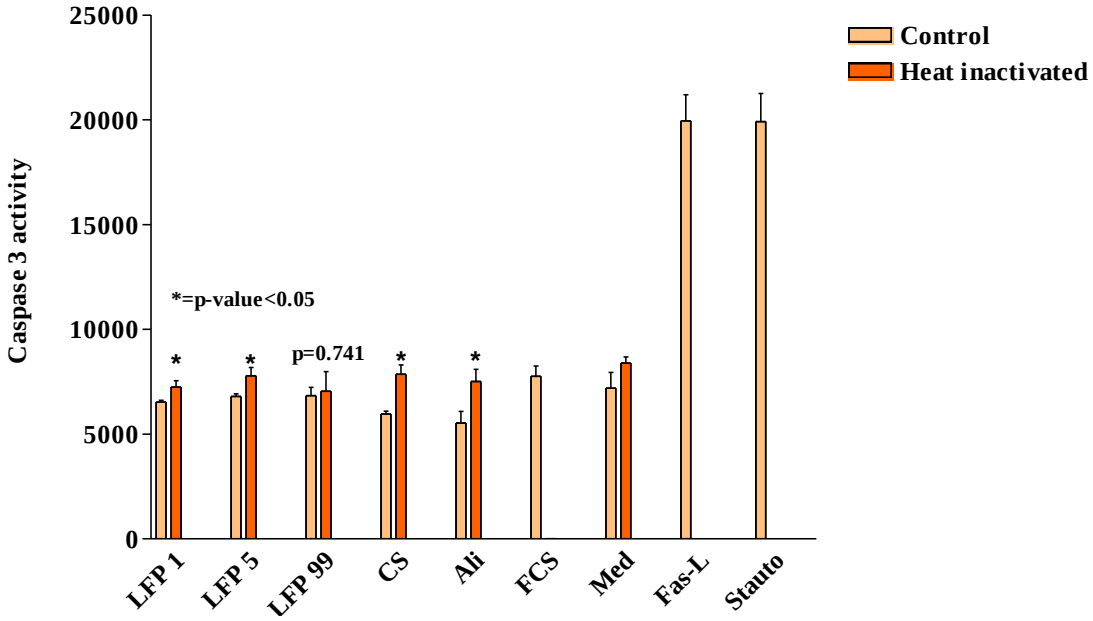


Figure 38. Heat inactivation of Acute Liver Failure Plasma (LFP1, LFP 5, LFP99) and Normal Plasma (CS, Ali)

Discussion

These results suggest that there may be a heat labile protective factor in human plasma and that this protective factor is diminished in acute liver failure. So we investigated this possibility in the following section.

3.6 PROTECTIVE EFFECTS OF HUMAN PLASMA

To demonstrate if there are protective factors in human plasma we investigated whether human plasma attenuated apoptosis induced by Fas-Ligand or Staurosporine; and in addition if normal plasma attenuated the apoptosis to a greater extent than acute liver failure plasma.

Material & Methods

Fas-Ligand stocks of 400ng/ml, 200ng/ml, 100ng/ml, 20ng/ml, and 2ng/ml in Complete Williams E were made and 20uL added to 380uL of plasma resulting in a 1:20 dilution; 20uL of Complete Williams E was added to plasma for control "No-Fas-Ligand". Staurosporine stocks of 20uM, 10uM, 5uM, 1uM and 0.1uM were made in Complete Williams E and 20uL was added to 380uL of plasma resulting in a 1:20 dilution, 20uL of Complete Williams E was added to plasma for control (no staurosporine). After thorough mixing Fas-Ligand or Staurosporine in plasma was applied to hepatocytes for 4 hours and Caspase 3 activity measured as described previously

Results

Neither acute liver failure plasma, normal plasma nor FCS attenuated apoptosis induced by Fas-ligand as evidenced by the similar slope of the curves; the different position of the curves is due to the different starting point of the curves due to the differences in plasmas as demonstrated in previous experiments. Both liver failure plasma and normal plasma seem to attenuate apoptosis induced by Staurosporine with normal plasma exerting a greater protective effect than acute liver failure plasma.

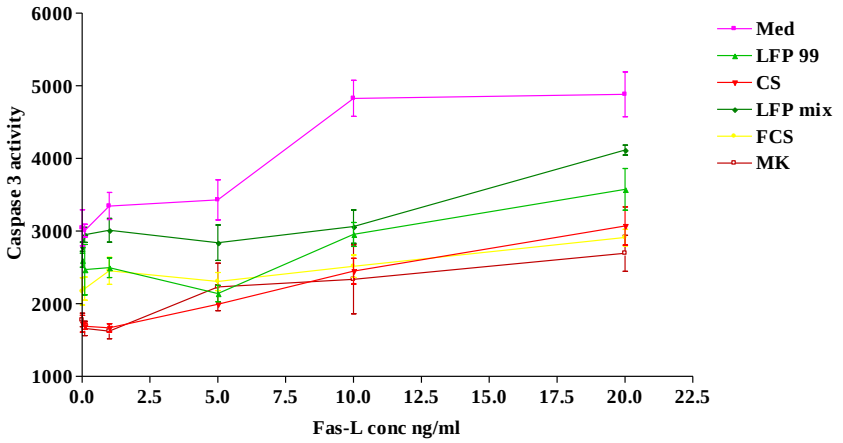


Figure 39. Caspase 3 activity in primary human hepatocytes exposed to increasing concentrations of Fas-L diluted in either medium (pink), FCS (yellow), normal plasma (red), or liver failure plasma (green).

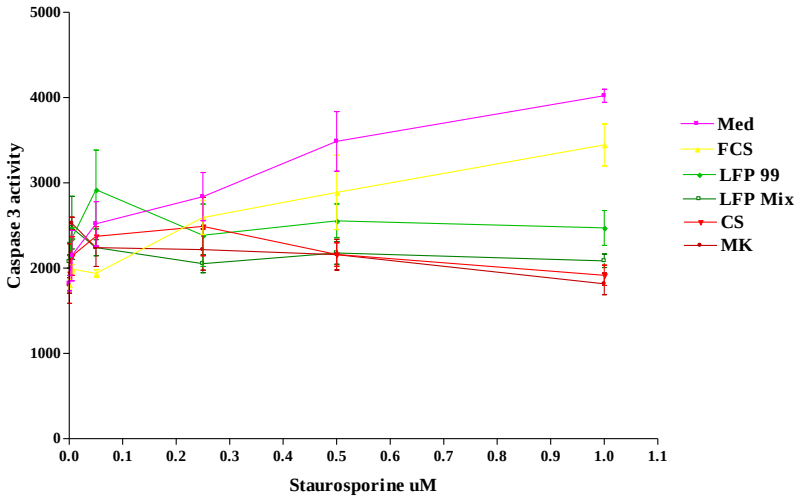


Figure 40. Dose response curves for Staurosporine diluted in acute liver failure plasma, normal plasma, FCS and Complete Williams E medium

3.7 DISCUSSION

Many substances which are potentially toxic are increased in the circulation of patients with acute liver failure plasma and other possibly protective factors are reduced.

These substances are of importance as in theory they may perpetuate cell loss in liver failure, affect transplanted liver cells (both in organ transplantation, and stem cell transplantation and they may affect the function of a bioartificial device. As a result there has been much research aiming to identify toxic substances in-vivo and to identify methods of reducing these toxic effects.

Theoretically fractionation and purification of plasma could identify the molecules involved in liver failure plasma toxicity, but due to the complex nature of plasma, the possible interplay of perhaps more than one toxic substance, the decrease in possible protective factors and the use of a biological assay as an end-point requiring proteins not to be denatured and not to be contaminated with toxic substances arising from contamination during processing, this is a formidable task. An alternative is to test the substances known to be increased in acute liver failure independently dissolving them in plasma and observing their effects (Hughes, Cochrane, Thomson, Murray-Lyon, & Williams, 1976); again this is difficult as plasma contains a vast array of substances many of which are unidentified and these substances may have additive or diminutive effects when used in combination. Thus to test all known substances in all combinations again becomes an impossible task.

A more pragmatic approach is not to try to identify these toxins but to investigate their effects and to attempt to identify the mechanism of action of

toxicity and thus therapeutic strategies. The design of such studies is however fraught with difficulties and compromises.

Previously used models have shown that plasma/serum from patients with acute liver plasma is toxic to cell lines and primary non-human hepatocytes. These vitro studies are limited by several factors. The first is that the demonstration of in-vitro toxicity of a purified substance applied in tissue culture medium does not necessarily translate to in-vivo toxicity as the presence of other factors in plasma (many of which are unidentified) may negate such toxicity.

The second is the choice of cells used in any in-vitro system. Tumour cell lines whilst readily available have by definition dysregulated apoptosis and are therefore not suitable as a model of apoptosis in non-neoplastic disease. An alternative is to use primary non-human hepatocytes, however, even human plasma from normal individuals has been shown to have deleterious effects on non-human cell viability, possibly due to cross species effects. In addition there are large variations in activity of specific hepatic metabolic pathways, particularly via the cytochrome p450 system between species again making applicability of results to human disease difficult.

A further difficulty is whether to use serum or plasma. Previous studies have mainly used serum, this contains many substances released from components of blood by activation of the coagulation cascade and therefore does not represent the internal milieu of the patients' hepatocytes. Plasma would therefore appear to be a better choice particularly since hepatocytes in a bioartificial liver would be exposed to this. The plasma used was not heat inactivated as this would destroy many of the substances of interest but as a consequence it contains proteins from the complement cascade that can directly lyse cells in the absence

of any cellular immune component. For this reason the ideal choice of cell is human hepatocytes from a blood group O donor. This reduces effects due to major incompatibility of blood group however minor effects due to antibodies in plasma activating complement cascade due to individual cross-reactions between plasma and hepatocytes cannot be entirely eliminated. A final difficulty noted by ourselves (unpublished) and others (Shi, Gaylor, Cousins, Plevris, Hayes, & Grant, 1998) is that acute liver failure plasma may have effects on cellular adhesion resulting in cell detachment after prolonged exposure. Studies should ideally either express results standardised for cell number or expose cells for shorter durations, otherwise over-estimates of toxic effects due to loss of cells by detachment may occur.

We have developed a robust in-vitro model for the study of apoptosis in primary adult human hepatocytes using two different methods for quantifying apoptosis, TUNEL staining and Caspase 3. The two methods showed a good correlation suggesting that both methods are accurate and reproducible measures of apoptosis. We have used this model to investigate the effects of liver failure plasma on hepatocyte apoptosis.

Acute Liver Failure Plasma

We have demonstrated that exposure to acute liver failure plasma induced increased apoptosis in adult primary human hepatocytes compared to exposure to normal plasma. This suggests that there are toxic substances within liver failure plasma which cause hepatocyte loss by apoptosis. Since apoptosis has been shown in animal studies to be an important step in the pathophysiology of acute liver failure these results confirm that apoptosis induced by substances

within acute liver failure plasma may be important in the perpetuation of acute liver failure in humans.

It was noted that there was a wide variation in degree of apoptosis induced by acute liver failure plasma and not all samples were toxic; indeed two individuals plasma showed significantly less apoptosis than control normal plasma. This is not surprising considering the diversity of the patients' clinical conditions both between individuals and within individuals over time.

Dose response curves of Caspase 3 activity for hepatocytes diluting plasma with complete Williams E were generated to demonstrate this toxic effect, paradoxically the cells incubated with more concentrated plasma demonstrated less apoptosis than at lower concentrations. This is consistent with the observation that apoptosis is always greater and cell viability less in hepatocytes incubated in complete Williams E medium compared to all plasma samples. It suggests that complete Williams E is not the perfect milieu for primary human hepatocytes and as a consequence there is a background rate of cell loss by apoptosis which is greater than that seen in plasma. Thus to construct a dose response curve is impossible unless we can identify a medium which is more favourable than plasma and generates minimal apoptosis to dilute the plasma with.

This demonstration of both normal and liver failure plasma inducing less apoptosis than medium suggests a possible alternative explanation for the differences in toxicity seen with normal and acute liver failure plasma. Instead of an increase in apoptosis due to an increase in toxic substances within liver failure plasma compared to normal plasma, there maybe a general background level of apoptosis *in-vitro* and normal plasma protects against this to greater

extent than acute liver failure plasma. In summary the increased toxicity observed in liver failure plasma compared to normal plasma maybe due to decreased factors that protect against apoptosis in acute liver failure plasma compared to normal plasma rather than an increase in toxic factors in acute liver failure plasma. This is consistent with previous papers in which the differences between normal and acute liver failure plasma are demonstrated but amounts of apoptosis in medium alone are not shown.

Clinico-Pathological correlates – Acute Liver Failure Plasma

There was no correlation between apoptosis induced by the patients' plasma, or cell viability and the patients' renal or liver function tests measured. We had hypothesised that if apoptosis was a cause of hepatocyte injury leading to secondary necrosis that other markers of hepatocellular damage, e.g. AST, ALT, may positively correlate with apoptosis inducing ability of plasma. No such correlation could be demonstrated. Although this may be taken as evidence to reject this hypothesis results have to be interpreted with caution as no correlation may be due to differences in the time course of release of these markers.

We also hypothesised that if apoptosis was induced by toxins that had accumulated in the plasma as a result of failure of liver excretion that markers of excretion/cholestasis bilirubin, ALP, GGT would positively correlate with apoptosis inducing capacity of plasma; again no such correlation could be demonstrated, suggesting that the toxic nature of plasma was not related to failure of hepatic excretion of hepatotoxins.

Finally, we hypothesised that if albumin binds to lipid soluble hepatotoxins and that it is unbound toxins that induce hepatocyte apoptosis that serum albumin

would negatively correlate with apoptosis inducing ability of plasma. Whilst this correlation failed to reach statistical significance there was a trend in this direction. This may have been due to the direct effect of unbound toxins, due to albumin itself having protective effects against apoptosis or be an indirect association as patients with lowest serum albumin represent the "sickest" patients. Greater patient numbers may be required to confirm this correlation. Some of these hypotheses could be tested by measuring apoptosis inducing ability of plasma from non-liver critically ill patients as these also have low serum albumin and increased inflammatory cytokines and patients with chronic liver disease who also have low albumin.

Chronic Liver Disease Plasma

Plasma from patients with acutely-decompensated chronic liver disease did not significantly increase apoptosis, measured by both TUNEL staining and Caspase 3 activation, compared with plasma from normal individuals. However both normal and liver failure plasma were associated with less apoptotic hepatocytes than complete Williams E medium. As with acute liver failure plasma there was a wide variation in quantity of apoptosis associated with each sample, with some samples increasing apoptosis and some decreasing it compared with normal plasma. Again cell viability measured by MTT activity did not correlate with apoptosis and the possible explanations for this are the same as for acute liver failure plasma.

Apoptosis, measured by Caspase 3 activation, positively correlated with serum creatinine $P<0.05$); apoptosis, measured by TUNEL cell positivity, also showed a trend toward a positive correlation, although this did not reach statistical significance ($p=0.07$). It is known that renal failure is a significant cause of

mortality in chronic liver disease with the onset of impaired renal function often heralding the terminal stages of this disease. Serum creatinine is therefore considered a good prognostic indicator and is used to calculate the MELD score and the creatinine corrected Childs-Turcott-Pugh Score, both of which have been shown to be of greater prognostic value compared to Childs-Turcott-Pugh Score alone which does not consider renal function. The correlation of apoptosis with creatinine therefore suggests a possible mechanism for this is association. The onset of renal impairment may result in the accumulation of substances normally excreted renally that are capable of inducing apoptosis in hepatocytes, thus decreasing the likelihood of recovery. From this it could be theorised that removal of these substances by haemofiltration/dialysis could decrease plasma toxicity and improve outcome in acutely decompensated chronic liver disease. Since haemofiltration is an integral part of MARS therapy this hypothesis will be investigated in the following chapter.

Apoptosis, measured by TUNEL cell positivity, is negatively associated with GGT activity ($p<0.05$), there is also a trend towards this association when apoptosis is measured by Caspase 3 activation although this does not reach statistical significance ($p=0.07$). The explanation for this is unclear but both methods of measuring apoptosis indicate a similar result, making a type II error unlikely. Gamma-GT is induced by a number of liver diseases, the function and mechanism of this induction is unclear. Apoptosis was not associated with other markers of cholestasis and the correlation is negative suggesting accumulation of toxins is not an explanation. It may be that the induction of GGT represents some form of protective mechanism in liver disease and this induction may be associated with activation of other protective mechanisms, for example those

that protect against hepatocyte apoptosis such as NFκβ activation and increased expression of proteins of the Bcl-2 family. However until more is known about the role of GGT in liver disease no further comment can be made.

Caspase Inhibitors

Having shown that acute liver failure plasma is associated with an increase in apoptosis in hepatocytes when compared to normal plasma, we investigated by which pathway apoptosis was mediated by using specific caspase inhibitors.

Inhibition of Caspase 3 resulted in a significant (and the greatest) decrease in apoptosis, Caspase 3 is the major effector caspase and is utilized by both intrinsic and extrinsic pathways, it would therefore be expected that inhibition of Caspase 3 would result in maximal inhibition of apoptosis.

Caspase 8 inhibition also resulted in a significant inhibition of apoptosis, Caspase 8 is the major caspase activated by binding of cell surface "death receptors" to their ligands, including Fas and TNF-α receptors.

This result suggests that the increase in apoptosis seen in hepatocytes incubated in acute liver failure plasma is induced by substances which act on cell surface death receptors. It also suggests that Caspase 8 inhibitors may have therapeutic potential in Acute Liver Failure. Caspase 9 inhibition did reduce apoptosis marginally but this effect did not reach statistical significance. This is not entirely unexpected as the caspase cascade in the intrinsic and extrinsic pathways are not exclusive and there is cross activation of pathways. In the case of Caspase 9 release of cytochrome c from the mitochondria (the defining point of apoptosis for cells, common to all pathways), results in oligomerisation of the protein apoptotic protease activating factor-1 and this combines with

cytochrome c and Caspase 9 resulting in the formation of the apoptosome which processes Caspase 9 to its active form. This activated Caspase 9 further activates Caspase 3 thus acting as a positive feedback loop amplifying the apoptotic signal. Thus inhibition of Caspase 9 may result in some diminution of apoptotic response whichever pathway it is triggered by as a result of knocking out its downstream amplificatory function.

Inhibition of apoptosis by Caspase inhibitors 3 & 8 indicate possible pathways by which apoptosis is induced by liver failure plasma and could have therapeutic potential in the treatment of acute liver failure. The role of caspase inhibition as a therapeutic modality for Acute Liver Failure will be investigated further in chapter 7.

Heat inactivation of Acute Liver Failure Plasma

Heat inactivation resulted in an increase in Caspase 3 activity induced by exposure to both normal plasma and to two of the three samples of acute liver failure plasma compared to each's non-heat inactivated control. This suggests that heat inactivation of plasma results in inactivation a substances(s) within it which are protective against apoptosis. Having been heat inactivated there was no significant difference between the amounts of apoptosis induced by normal, acute liver failure plasma, 100%FCS and Complete Williams E medium suggesting there are no cross-species differences after heat inactivation.

Protective effects of Acute Liver Failure Plasma

Previous results suggested that the differences in plasmas` apoptosis inducing effect was due to differences in their content of factors which protect against apoptosis, rather than differences in their content of "toxic molecules". To test

this hypothesis we diluted substances which are known to induce apoptosis in hepatocytes (Fas-L and Staurosporine) in plasma from patients with Acute Liver Failure and normal controls to see if there were differences in response to these apoptosis-inducing substances in the different substrates.

Neither acute liver failure plasma, normal plasma, nor FCS attenuated apoptosis induced by Fas-ligand; whereas both liver failure plasma and normal plasma attenuated apoptosis induced by Staurosporine with normal plasma exerting a greater protective effect than acute liver failure plasma.

The reason for the differences in protective effects against the two apoptosis inducing agents is unclear but may be due the different pathways by which apoptosis is induced (Staurosporine is a protein kinase c inhibitor whereas Fas-L activates cell surface death receptors), or may be due to differences in the physical properties of the substances once dissolved in different agents. For example if Staurosporine becomes albumin bound its toxic effect may become attenuated due to decreased unbound compound. In this case substances with greater albumin concentration or greater albumin binding capacity may exert a greater protective effect against apoptosis induced by Staurosporine.

Summary

We have demonstrated that acute liver failure plasma is toxic to hepatocytes by inducing apoptosis, but that plasma from patients with acutely decompensated chronic liver disease does not. These patients may have equally deranged failure of excretion of hepatotoxins (using bilirubin as a marker of toxin load) as in acute liver failure. These data challenges the simple theory that liver failure plasma is toxic due to accumulation of toxins not excreted by the liver. What, therefore could explain the differences between acute and chronic liver failure?

One possibility is the release of toxic substances, such as cytokines, from the inflamed liver may be responsible for acute liver failure plasma toxicity. This has radical implications in the development of therapeutic modalities for liver failure and may be at least in part be responsible for the fact that detoxicatory therapies have never shown any survival benefit in acute liver failure (Novelli *et al.*, 2002a). It suggests that therapies aimed at decreasing inflammatory cytokines or preventing their effect may be more rewarding in acute liver failure, or that any detoxifying treatments must have metabolic capability to conjugate toxins prior to excretion. Although MARS and other detoxicatory therapy can potentially remove cytokines, which are albumin bound substances, their capacity to do so is relatively small, studies to show reduction of inflammatory cytokines before and after treatment have not been consistent and indeed initially cytokine production may be increased due to exposure of plasma to artificial membranes with the device.

These results do not suggest that detoxifying treatments are of no benefit to all liver disease patients, indeed they may be a necessary part of any bioartificial device that would have no excretory system, but we suggest that the theory of detoxifying plasma to directly promote hepatocyte viability is flawed. MARS therapy has shown improvement in liver function in-vivo, and we suggest that removal of vasoactive mediators e.g. nitric oxide and the resultant increase in blood pressure and regional perfusion is responsible for this improvement rather than removal of direct hepato-cellular toxins. Removal of toxins may still be of benefit to the patient's clinical condition by improving encephalopathy where evidence for a direct effect for toxic molecules is greater. We suggest that liver support devices should be thought of in two distinct categories: Devices such as

MARS therapy, plasmapheresis and adsorption columns which promote excretion of liver toxins, and detoxifying treatments which have metabolic capability i.e. bioartificial livers or extracorporeal liver perfusion. The former may be of greatest value to patients in whom cholestasis is predominant. Bile salts are known to be cholestatogenic and removal of these compounds may thus break the vicious cycle that appears in these patients. The latter would be able to metabolise unconjugated substances and would be perhaps of greatest benefit to non-cholestatic patients with acute liver failure.

CHAPTER 4: ANIMAL MODELS OF ACUTE LIVER FAILURE AND INHIBITORS OF APOPTOSIS IN THIOACETAMIDE INDUCED LIVER INJURY

4.1 APOPTOSIS AND ACUTE LIVER FAILURE

Apoptosis is thought to be an important process in the pathophysiology of a number of liver diseases with an enhanced rate of apoptosis above the background rate of 1-5 cells per 10,000 hepatocytes having been reported in many forms of human liver disease. Particularly striking is the increase in rate of hepatocyte apoptosis in acute liver failure (Kasahara, Saitoh, & Nakamura, 2000). The aetiologies of acute liver failure in which increased (Ryo, Kamogawa, Ikeda, Yamauchi, Yonehara, Nagata, & Hayashi, 2000) hepatocyte apoptosis has been demonstrated have previously been described in the first chapter. The association between liver failure and apoptotic hepatocytes has been firmly established in human tissue through conventional histological analysis and detailed nuclear morphology demonstrating chromatin fracturing using the TUNEL technique (Kasahara, Saitoh, & Nakamura, 2000, Ryo, Kamogawa, Ikeda, Yamauchi, Yonehara, Nagata, & Hayashi, 2000);

Apoptosis leads directly to significant hepatocyte loss and although only a small percentage of total hepatocytes may be seen to be apoptotic on histological specimens at any given time, the speed of the apoptotic process and lack of accumulation of apoptotic debris means that even low rates of apoptosis can be directly responsible for loss of large amounts of liver cell mass over short periods of time.

In addition apoptosis is known to be capable of inducing the development of secondary necrosis with inflammatory cell infiltrates, activation of kupffer cells, lymphocytes and macrophages, release of cytokines, and activation of hepatic stellate cells stimulating fibrogenesis all of which further contribute to liver damage. Inhibition of apoptosis may therefore be beneficial in the treatment of acute liver failure both by preventing loss of liver cell mass and reducing the liver's inflammatory response.

There are several key targets which could be harnessed to therapeutically inhibit apoptosis including manipulation of death receptors and their ligands, Caspase inhibitors, inhibitors of apoptosis proteins (IAPs), Bcl-2 family proteins and mitochondrial membrane transition potentials.

Interventions that reduce quantity or activity of ligands known to stimulate apoptosis have already been used with success in several non-malignant human diseases (see table 7).

Table 6. Summary of clinical trials using anti-death receptor antagonism.

Target	**Reagent**	**Principle**	**Experimental Effects/Disease**	**Trial status**	**Manufacturer & References**
TRAIL receptors			Hepatitis C		
CD95/ Fas	CD95-Fc	Humanised CD95 Fc-decoy construct	Inhibition of CD95 signalling in spinal cord injury.	Preclinical	Apogenix (Demjen *et al.*, 2004)
TNF & Fas-Ligand	Anti-TNF & Anti-FasLigand	Soluble anti-Fas ligand antibody antibody	Mouse stoke model, decreased infarct volume & Mortality	Animal Models	(Martin-Villalba *et al.*, 2001)
TNF	Infliximab	Mouse/human anti-TNF antibody	Inhibition TNF-α & Apoptosis of surface TNF expressing cells e.g. Macrophages IBD, RA, beneficial Alcoholic Hepatitis (increased mortality)	Routine clinical use	Centercor/Schering-plough (Targan *et al.*, 1997) (Naveau *et al.*, 2004)
CD95/ Fas-Ligand	Anti-Fas ligand antibodies	Intra-thecal Soluble anti-Fas ligand antibody antibody	Decreased inflammation in experimentally induced mouse encephalomyeilitis. An animal model of Multiple Sclerosis	Animal model	(Okuda *et al.*, 2000)
CD95/ Fas-Ligand & TNF	Anti-Fas ligand & anti-TNFantibodies	Systemic administration of soluble neutralising antibody	Amelioration of mouse model of graft versus host disease	Animal model	(Hattori *et al.*, 1998),
CD95/ Fas-Ligand	Pooled human immunoglobulin	Interference with Fas-Ligand binding	Toxic epidermal necrosis	Clinical trial	(Viard *et al.*, 1998)
TNF	Adalimumab	Recombinant human anti-	Inhibition of TNF-α	Routine clinical	CAT/Abbott (Hanauer *et*

		TNF antibody	IBD, RA, Psoriasis, Ankylosing Spondylitis	use	*al.*, 2006, Sandborn *et al.*, 2004)
TNF	Etanercept	Recombinant TNFR2/IgG fusion protein	Anti-inflammatory RA, IBD. Small case series in Alcoholic Hepatitis	Routine clinical use	Amgen/Wyeth (Menon *et al.*, 2004)
TNF	ISIS 104838	2'-O-Methoxyethyl antisense construct against TNF-α	Anti-inflammatory RA, IBD, psoriasis.	Phase II Trials for RA, Crohns, Psoriasis	
TNF	Pentoxyfylline	Reduces TNF secretion, + Downstream effects on CPP32-like protease.	Anti-inflammatory and renal protective effects, in Alcoholic Hepatitis.	Phase III Trials	(Okamoto, 1999) (Akriviadis *et al.*, 2000)

IBD = Inflammatory bowel disease RA = Rheumatoid arthritis

Since death receptor activation is known to induce liver failure, inhibition/antagonism of these pathways should improve outcome in liver disease. TNF-neutralising antibodies, anti-Fas ligand antibodies, TNF decoy receptors (Kondo, Suda, Fukuyama, Adachi, & Nagata, 1997), pentoxyfylline (Okamoto, 1999), and physical removal of toxins such as bile salts have all been shown to reduce apoptosis and have/are undergoing clinical trials in liver disease. Inhibition of the CD95 receptor/ligand system using CD95 neutralising antibodies have shown benefit in-animal models decreasing disability after spinal cord injury (Demjen et al, 2004).

The targets of these therapies, often cytokines, do however serve multiple functions some of which may be beneficial. For example TNF-alpha although capable of inducing hepatocyte apoptosis is also implicated in hepatocyte proliferation and survival and thus its removal/antagonism may worsen liver regeneration and clinical outcome. In addition the antagonism of TNF is highly immunosupressing increasing the risk of septic complications, particularly tuberculosis. Complications already described after the use of Infliximab

include lymphoma, infections, congestive cardiac failure, demyelinating disease and lupus-like syndrome (Bratcher & Korelitz, 2006).

More specific inhibition of apoptosis would avoid these effects and could be enabled by inhibiting the downstream intracellular machinery leading to apoptosis. A further advantage of manipulating apoptosis downstream is the avoidance of the administration of these large recombinant proteins which are expensive, unstable, require systemic administration and may provoke immunogenicity resulting in hypersenstitivity reactions and loss of efficacy.

4.2 CASPASE INHIBITION

The molecular mechanisms/pathways involved in the execution of apoptosis are described in detail in the introductory chapter, however in brief activation of apoptosis via surface cell death receptors or mitochondrial permeablisation results in activation of a series of serine proteases called Caspases. These enzymes not only result in transmission of the apoptotic trigger to effector substances e.g. other caspases and endonucleases which result in cellular dismantling but also amplify the apoptotic signal resulting in a rapid all or nothing response. Caspases are therefore ideal targets for antagonism in specific prevention of apoptosis and have been studied in various animal models and human diseases. Caspases may be classified according to their function. Caspases 1, 4 & 5 are predominantly involved in the maturation of pro-inflammatory cytokines e.g. Interleukin-1(Thornberry *et al.*, 1992). Caspases 8, 9 & 10 are initiator caspases, whereas others (e.g. 3,6 & 7) are executioner caspases. Studies of transgenic and caspase knockout mice have shown that diseases such as stroke, myocardial infarction, neurodegenerative disease and liver injury are all attenuated by caspase inhibition and synthetic caspase inhibitors have already been successfully used in several animal models including spinal cord injury, arthritis, sepsis, stroke, renal and mesenteric ischaemia and liver disease (see table below).

Table 7. Summary of clinical trials using Caspase inhibition.

Target	Reagent	Model	Experimental Effects/Disease	Trial status	References
Caspase inhibitor		Mice Acute Spinal cord injury	Decreased cell death, Improved hind leg activity		(Li *et al.*, 2000, Springer *et al.*, 1999)
Broad spectrum Caspase	z-VAD-fmk	Mouse Acute liver failure induced by Jo-2 (activating anti-Fas antibody antibody	Attenuated liver injury		(Rodriguez et al, 1996)
Broad spectrum Caspase	z-VAD-fmk	Mouse Acute liver failure induced by Jo-2 (activating anti-Fas antibody antibody) to TNF-alpha	Attenuated liver injury		(Kumgstle et al, 1997)
Caspase 1 inhibition		Mouse Arthritis models	Decreased disease severity		(Miller *et al.*, 1995)
Broad spectrum Caspase	z-VAD-fmk	Mouse sepsis	Decreased severity &Mortality		(Hotchkiss *et al.*, 1999)
Caspase 1 inhibition			Rat model of cardiac ischaemia reperfusion injury.		(Yaoita *et al.*, 1998)
Pan-caspase inhibitor,	boc-aspartyl(OMe)-fluoromethylketone	Rat stroke model	Reduced neuronal death and infarct size		(Cheng *et al.*, 1998)
	IGF-1 and ZVAD-fmk	Rat renal ischaemia reperfusion injury	Decrease apoptosis and necrosis		(Daemen *et al.*, 1999)
Pan-case inhibitor	ZVAD (N-benzyloxy carbonyl Val-Ala-Asp-Ome-fluoromethylketone)	Mouse small intestine ischaemia reperfusion injury	Decreased apoptosis and inflammation.		(Farber *et al.*, 1999)

Irreversible pan-caspase inhibitor	IDN-6556		Anti-apoptotic, Anti-inflammatory, Ant-i-fibrotic effects in liver damage Decreased serum transaminases in healthy volunteers Preservation of livers during transplantation	Phase 2 trials HepC, open label trials Hep B and ischaemia reperfusion post liver transplantation	IDUN (Canbay *et al*,2004, Hoglen *et al*, 2001) (Valentino *et al.*, 2003)
Caspase inhibitor	IDN-6734		Decreases heart muscle damage in animal models	Phase 1 studies acute MI	IDUN
VX-799	Small molecule Caspase inhibitor		Effective in sepsis and neuronal death models	Phase II trials for septic multiorgan failure started 2003	Vertex/Serono
MX-1013	Dipeptide pancaspase inhibitor		Prevents apoptosis in animal models MI, Stroke, Acute Liver Failure	Preclinical	Maxim (Jaeschke *et al.*, 2000, Yang *et al.*, 2003)
M-920	Broad spectrum Caspase Inhibitor		Mouse model septic shock	Preclinical	Merck Frost (Hotchkiss *et al.*, 2000)
IDN-11104	ICE-inhibitor			Preclinical	IDUN
VX-740	ICE-inhibitor		Anti-inflammatory effects in Rheumatoid and OA studies	Phase II Rheumatoid arthritis	Vertex/Aventis

M-826	Reversible Caspase 3 inhibitor		Reduces neuronal loss in mouse model of Huntington's disease and stroke	Preclinical	Merck Frosst (Han *et al.*, 2002, Toulmond *et al.*, 2004)
M-791	Caspase-3 inhibitor		Effective in mouse model of septic shock	Preclinical	Merck Frosst (Hotchkiss *et al*, 2000)
Xyz 033	Broad spectrum Caspase inhibitor		Concavalin A in mice		(Kim *et al* , 2000)

Animal models of liver disease in which caspase inhibitors have been utilized include the warm ischaemia reperfusion rat model, in which a broad spectrum Caspase inhibitor decreased apoptosis, necrosis and mortality (day 7 Caspase inhibitor group 5%, Control 30%) (Cursio, Gugenheim, Ricci, Crenesse, Rostagno, Maulon, Saint-Paul, Ferrua, Mouiel, & Auberger, 2000); a rat model of haemorrhagic shock in which a broad spectrum Caspase inhibitors did not prevent hepatocellular injury (Mauriz *et al.*, 2003); the Endotoxin & D-galactosamine mouse model (a TNF-alpha mediated model) in which caspase inhibition decreased apoptosis, necrosis and mortality (at 24 hours 0% mortality in pancaspase inhibitor group, 100% in controls) (Jaeschke, Farhood, Cai, Tseng, & Bajt, 2000) and in the same model a specific Caspase 8 inhibitor also attenuated liver injury (Bajt, Vonderfecht, & Jaeschke, 2001, Kunstle et al, 1997, Rouquet, Pages, Molina, Briand, & Joulin, 1996); Mice treated with Jo-2 antibody (a Fas activating antibody) to induce apoptosis, showed apoptosis, necrosis and mortality were decreased both by a specific Caspase 8 inhibitor (Bajt *et al.*, 2000) and a broad spectrum caspase inhibitor (Rodriguez et al, 1996, Yang et al, 2003); A cold ischemia warm reperfusion rat liver transplant model showed a pancaspase inhibitor added to the preservation solution reduced SEC apoptosis in explants by 55% and Caspase 3 activity by 94% (Natori, Higuchi, Contreras, & Gores, 2003). Decreased acute and chronic injury was seen in the bile duct ligated mouse model using a pancaspase inhibitors (Canbay, Feldstein, Baskin-Bey, Bronk, & Gores, 2004); Concavalin A in a murine model of T-cell

mediated apoptosis via the Fas receptor pathway akin to autoimmine hepatitis showed reduced apoptosis, necrosis and mortality using a specific Caspase 3 inhibitor and a broad-spectrum Caspase inhibitor (Kim, Kim, Park, Park, Chang, Park, Chung, & Kang, 2000); in contrast in a mouse model of acetaminophen toxicity Capsase 3 activity was not increased and Caspase inhibitors did not attenuate injury (Lawson *et al.*, 1999).

Caspase inhibitors, although showing mixed results depending on the mechanism of liver injury, do show considerable promise as therapies both for endogenous hepatocytes in acute liver failure and also provide a method by which apoptosis can be inhibited in exogenous hepatocytes during transplantation and use in a bioartificial device.

Since the commonest causes of acute liver failure in the U.K. are toxin mediated (i.e. paracetamol toxicity and alcoholic hepatitis) and we have demonstrated in earlier chapters that there are toxic substances within acute liver failure plasma which may perpetuate liver failure which are inhibited in-vitro by Caspase inhibitors we wished to study the effect of Caspase inhibition *in-vivo* on a toxin induced animal model of acute liver failure, the thioacetamide (TAA) model of AHF.

4.3 THIOACETAMIDE MODEL

In this experimental model Thioacetamide is metabolised to toxic metabolites thioacetamide sulfine (sulphoxide) and sulfene (sulfone) (Hunter *et al.*, 1977, Ramaiah *et al.*, 2001). Hepatocyte damage involves generation of free radicals and superoxide anions causing lipid peroxidation (Diez-Fernandez *et al.*, 1996a). Cellular consequences include interruption of cellular calcium homeostasis (Diez-Fernandez *et al.*, 1996b), disruption of membranes of the endoplasmic reticulum and failure of amino acid incorporation into liver proteins with resultant hepatic necrosis (Ramaiah, Apte, & Mehendale, 2001) and apoptosis (Ledda-Columbano, Coni, Curto, Giacomini, Faa, Oliverio, Piacentini, & Columbano, 1991) associated with activation of Caspase 3 (Hayami *et al.*, 1999).

Thioacetamide causes hepatic failure in rats, the severity of which varies according to intra-species variation, age, diet, and also concomitant supportive

therapy (Caballero *et al.*, 2001, Diez-Fernandez *et al.*, 1998, Zimmermann *et al.*, 1989). Previous work of this group characterised the clinical, biochemical, metabolic and histological pattern of thioacetamide-induced hepatic failure in the Wistar rat, to create a model of substantial severity (~70% mortality), which fulfils the criteria of reproducibility, reversibility and provision of a therapeutic window, suggested by Terblanche et al.(Terblanche & Hickman, 1991) for animal models of AHF(Rahman & Hodgson, 2003). This model induces lethargy within 6 hours of the first injection of Thioacetamide, and by 12 hours 70% of the animals demonstrated Grade 1 encephalopathy lasting 4-12 hours and then developed Grade II-III encephalopathy by 48 hours. The model had a 50% mortality by 48 hours with a 70% mortality by 96 hours (Rahman & Hodgson, 2003).

Using this model the ability of a broad spectrum caspase inhibitor VE-453, and a selective Caspase 3 inhibitor Z-VAD, to attenuate liver injury was assessed.

4.4 EXPERIMENT TO DEMONSTRATE IF THERE IS INCREASED APOPTOSIS IN THIOACETAMIDE INDUCED LIVER FAILURE.

A preliminary experiment to confirm the presence of apoptosis of hepatocytes and delineate its time course was performed.

Method

Animals and treatments. Male Wistar rats weighing 250-300 grams were fed with standard rat chow diet ad libitum, allowed free access to water and housed in standard facilities with a 12-hour day/night cycle at a room temperature of 25^{o}C. Animals were handled and the method licensed according to home office criteria (see appendix for licence). Rats were rested for 2 weeks after

transportation. All the animals used in these experiments were cared for in this way.

Thioacetamide at a concentration of 62.5mg/ml was dissolved in sterile Normal saline. A dose of 500mg/kg was injected intraperitoneally, twice 6 hours apart in eight rats. Vehicle only was injected in four rats as controls. Rats (two thioacetamide and one vehicle control) were sacrificed in accordance with home office regulations at 6, 12, 24 and 36 hours. Liver tissue was taken and samples were fixed in formalin and embedded in Paraffin blocks; also samples were placed in tissue mounting medium on cork tiles and snap frozen in Iso-pentane (for frozen cryostat section histology). Liver tissue was snap frozen in liquid nitrogen and stored at -80ºC for subsequent analysis. Paraffin embedded tissue sections were cut and placed on APES coated slides and allowed to dry. Sections were then stained with TUNEL according to the protocol in general methods.

Numbers of apoptotic cells (TUNEL -Rhodamine positive nuclei) and normal nuclei (Hoescht – Blue) were counted under x40 magnification with immunofluorescence using appropriate filters.

Results

Increased numbers of TUNEL positive apoptotic cells were seen in Thioacetamide treated rats compared with vehicle only controls. The TUNEL positive cells were particularly distributed in areas of increased cellularity around central veins. The time course appeared to show increasing apoptosis up to 36 hours.

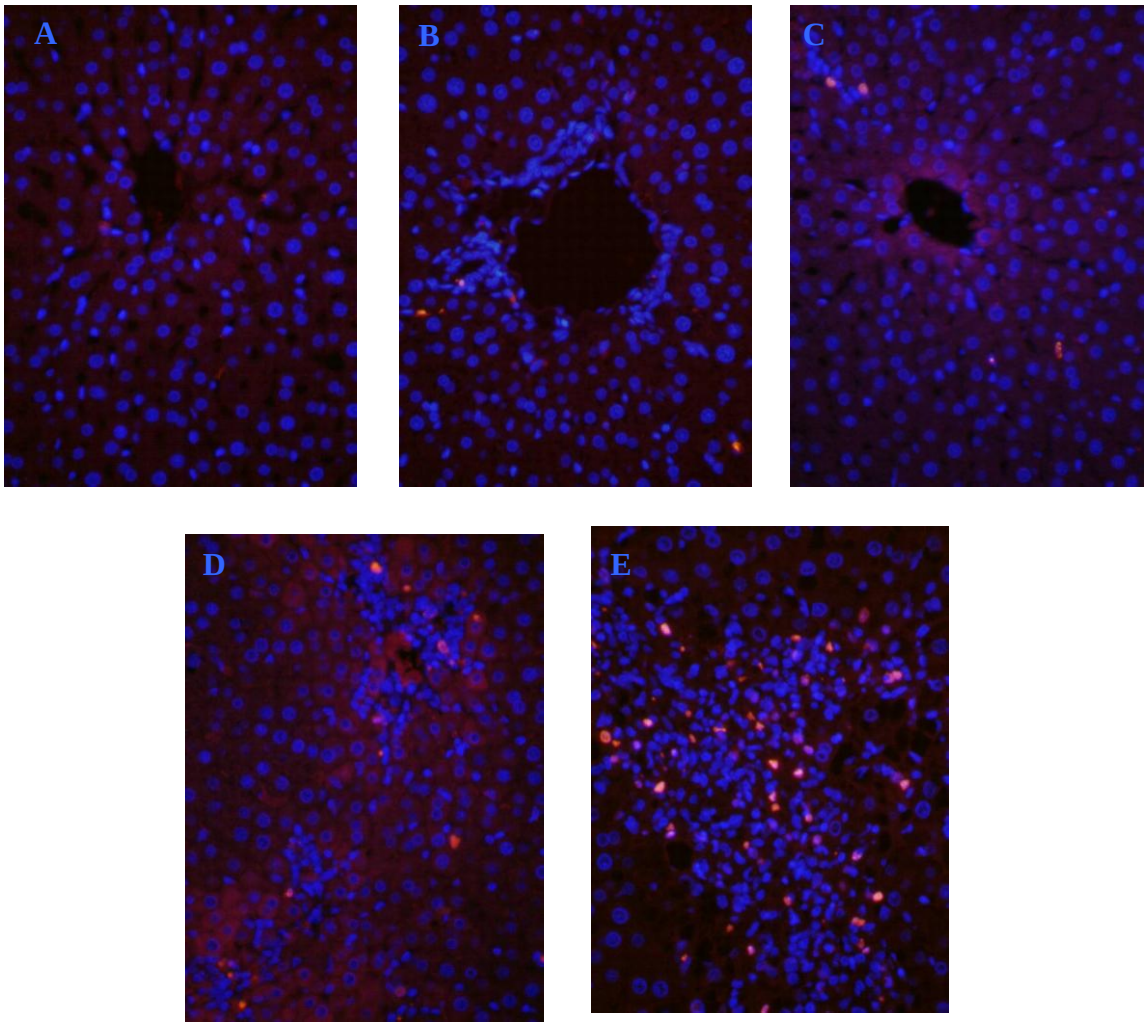


Fig. 41. Photomicrographs showing sections of rodent liver treated with Thioacetamide at (B) 6 hours, (C) 12 hours, (D) 24 hours and (E) 36 hours compared to normal control (A) magnification x40. TUNEL positive cells red, Hoescht nuclear counterstain blue.

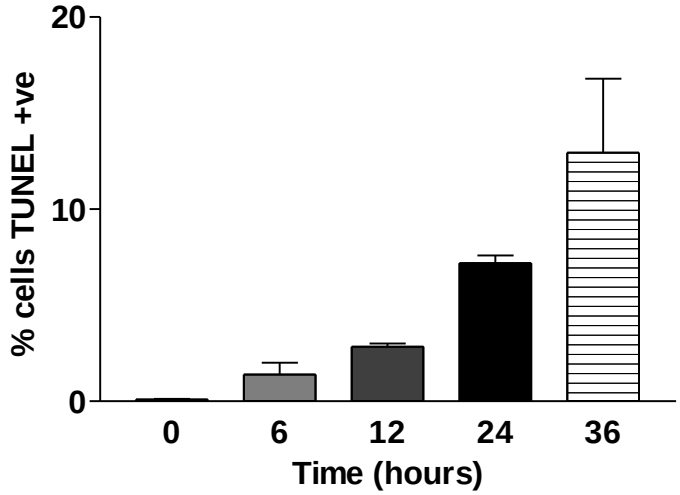


Figure 42. Percentage of total hepatocytes TUNEL positive in rat liver after Thioacetamide administration at 0, 6, 12 and 24 hours.

4.5 THE EFFECTS OF CASPASE INHIBITORS ON THE THIOACETMIDE INDUCED LIVER INJURY RAT MODEL.

The effect of a broad spectrum caspase inhibitor VE-453 (Vertex Pharmaceuticals, Didcot), and a selective Caspase 3 inhibitor Z-VAD (Vertex Pharmaceuticals, Didcot) on morbidity and mortality of the Thioacetamide rat model was assessed.

Method

Animals and treatments. Male Wistar rats 250-300grams in weight, cared for as described, were weighed and labelled immediately prior to experimentation.

Caspase inhibitors were made up in accordance with manufacturer's instructions. Z-VAD and VE-453 (stored at -20ºC) powder was allowed to come to room temperature to allow water absorption prior to weighing (powders

being very hydroscopic). 30.25mg of Z-VAD powder and 90.75mg of VE-453 powder were individually solubilised initially in 3 ml of 100% PEG300 and then diluted 1:10 vol/vol with PBS to a final volume of 33ml of vehicle (10%PEG300, 90%PBS). A dose of 10mg/kg body weight of Z-VAD (approximately 3ml), 30mg/kg body weight of VE-453 (approximately 3ml) or vehicle alone (approximately 3ml) was drawn up in labelled individual 5ml syringes, volumes drawn up were adjusted according to each animals weight.

Thioacetamide 2.888g (Sigma) was dissolved in 35mls sterile normal saline and thoroughly vortexed. A dose of 600mg/kg body weight (approximately 2ml) volume adjusted for individual animals weight, was drawn up in 2ml syringes.

Three groups of five animals were randomly selected; Group 1 Z-VAD + Thioacetamide; Group 2 VE-453 + Thioacetamide; and Group 3 Vehicle alone + Thioacetamide. At time=0 hours, and t=6 hours animals were injected intraperitoneally with TAA followed by either a Caspase inhibitor or vehicle alone with an orange needle. Animals were examined for the presence of encephalopathy 6 hourly (table). Animals with grade 3 encephalopathy were reviewed hourly and animals with Grade 4 encephalopathy were terminated in accordance with the home office animal licence. Animals were weighed 24 hourly as a marker of general well being. Animals were given subcutaneous maintenance fluids of normal saline 70ml/kg/24 hours in four divided doses (six hourly). A primary endpoint of mortality rate at 96 hours (the end of the study period) was selected. Secondary end points were degree of grade of encephalopathy and weight loss.

Table 8. Encephalopathy scoring system for rodents.

Stage of HE	Prominent neurobehavioral change	Total Reflex Score (Maximum score=30)
I	Lethargy	20-25
II	Lethargy and Ataxia	15-20
III	Gradual loss of reflexes	10-15
IV	Coma	0-10

Results

Table 9. Data from Thioacetamide toxicity rodent model

Animal Code	Substance	Wght T=0	Enc T=0	Enc T=6	Enc T=12	Enc T=18	Wght T=24	Enc T=24	Enc T=30	Enc T=36	Enc T=42
C1	Control	273	0	0	0.5	0.5	252	1	1	2	3
C2	Control	257	0	0	0.5	1	244	2	1	2	1.5
C3	Control	268	0	0	0.5	0.5	260	1	2	2	1.5
C4	Control	271	0	0	0.5	1	275	2	2	3	D
C5	Control	254	0	0	0.5	0.5	237	1	1	2	2
C6	Control	278	0	0	0.5	1	258	0.5	0	1	1
C7	Control	273	0	0	0.5	1	257	0.5	0	1	1
C8	Control	260	0	0	0.5	1	241	0.5	0	1	1
V1	VE-453	254	0	0	0.5	1	248	0	3	D	D
V2	VE-453	262	0	0	0.5	0.5	251	1	2	1.5	0.5
V3	VE-453	262	0	0	0.5	1	258	1	1	1.5	0.5
V4	VE-453	271	0	0	0.5	0.5	262	0	1	1.5	0.5
V5	VE-453	265	0	0	0.5	1	259	1	0.5	0.5	0.5
Z1	Z-VAD	260	0	0	0.5	1	257	1	1	2	0.5
Z2	Z-VAD	263	0	0	0.5	0.5	244	1	1	2	1.5
Z3	Z-VAD	253	0	0	0.5	0.5	244	1	1	2	0.5
Z4	Z-VAD	245	0	0	0.5	0.5	236	1	1	2	0.5
Z5	Z-VAD	245	0	0	0	0	240	0	1	2	0.5

Animal Code	Wght T48	Enc T48	Enc T54	Enc T60	Enc T66	Wgt T72	Enc T72	Enc T78	Enc T84	Enc T90	Wght T=96	Enc T96
C1	247	0.5	1	0.5	1	240	0.5	1	1	1	240	0.5
C2	241	0.5	1	0.5	1	228	1	1	1	1	228	0.5
C3	246	0.5	1	0.5	1	246	0.5	1	1	1	246	0.5
C4	D	D	D	D	D	D	D	D	D	D	D	D
C5	230	0.5	I	0.5	1	221	0.5	1	1	1	221	0.5
C6	246	0.5	0	0	0	242	0	0	0	0	238	0
C7	245	0.5	0	0	0	240	0	0	0	0	247	0
C8	231	1	0.5	0.5	0.5	223	0	0.5	0.5	0	230	0
V1	D	D	D	D	D	D	D	D	D	D	D	D
V2	237	0.5	1	0.5	0.5	236	0	0.5	0.5	0.5	244	0
V3	225	0.5	0.5	0.5	0.5	239	0	0.5	0.5	0.5	252	0
V4	245	0.5	1	0.5	0.5	237	0.5	0.5	0.5	0.5	240	0
V5	216	0.5	1	0.5	0.5	232	0	0.5	0.5	0.5	230	0
Z1	239	0.5	1	1	1	233	0	1	1	1	239	0.5
Z2	226	0.5	0.5	1	0.5	229	0	1	1	1	230	0.5
Z3	230	0.5	1	1	0.5	217	0	1	1	1	212	0.5
Z4	219	0.5	2	1	0.5	210	0	D	D	D	207	D
Z5	228	0.5	1	1	1	221	0	1	1	1	220	0.5

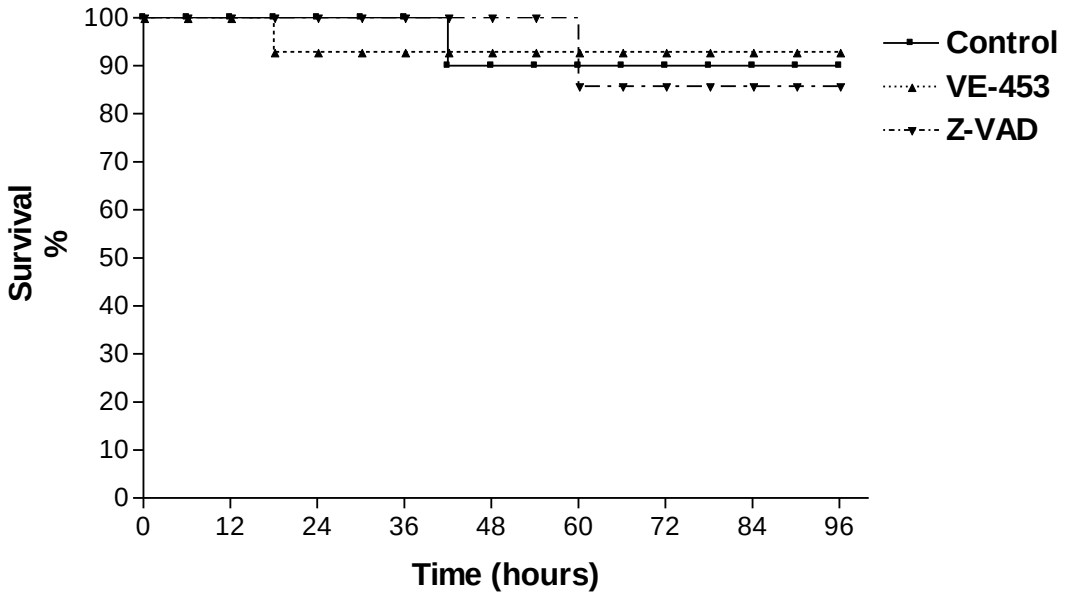


Figure 43. Kaplin-Meyer Survival Curves for rats given 500mg/kg Thioacetamide +/- Caspase inhibitors

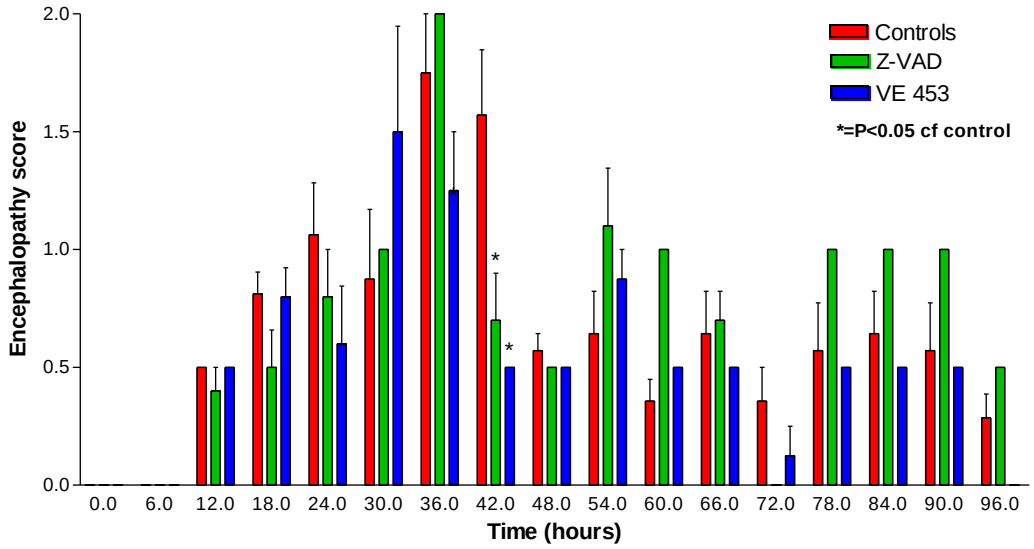


Figure 44. Encephalopathy score over time for male Wistar rats treated with Thioacetamide 500mg/kg (control), and Thioacetamide and Caspase inhibitors zVAD or VE 453

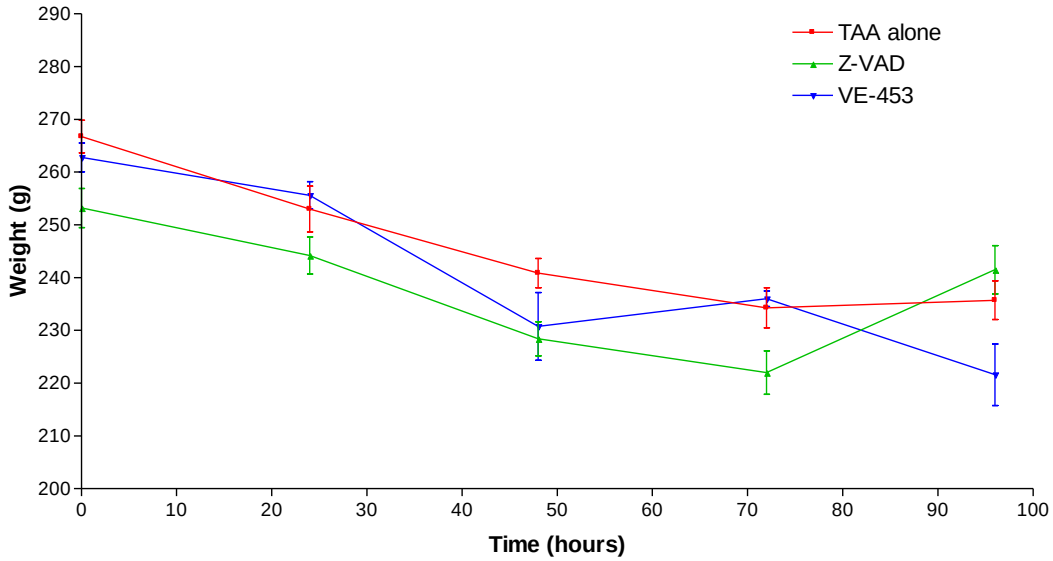


Figure 45. Weight loss of animals given Thioacetamide +/-Caspase inhibitors Z-VAD or VE-453

There was no statistically significant difference in the primary endpoint of mortality at time 96 hours post Thioacetamide injection. There was also no difference at any time point for weight loss. Encephalopathy was statistically significantly lower at t=42 hours but not at any other time point.

These results suggest that caspase inhibitors do not significantly improve clinical outcome in the Thioacetamide induced liver failure. However the mortality in the control group, Thioacetamide only was not as high as predicted from previous studies, despite using the same breed, source and feed of rat. Experiments were therefore repeated using a higher dose (and new batch) of Thioacetamide. The protocol was identical to that described above but instead a dose of 600mg/kg body weight of Thioacetamide was used.

Results

Animal Code	Substance	Wght T=0	Enc T=0	Enc T=6	Enc T=12	Enc T=18	Wght T=24	Enc T=24	Enc T=30	Enc T=36	Enc T=42
Red1	Control	308.2	0	1	2	1	290	1	1	0.5	0.5
Red2	Control	314.6	0	1	3	2	301	1.5	1	0	0
Red3	Control	312.5	0	1	1.5	1	291	1	1	0	0
Red4	Control	301.2	0	1	2	1	275	1	0.5	0	0
Red5	Control	315.3	0	1	2	1	296	0.5	0.5	0	0
Red 6	Control	265	0	0.5	0.5	1.5	241	1	1	0.5	0.5
Red 7	Control	270.3	0	0.5	0	1.5	253	1	1	0.5	0.5
Red 8	Control	254.4	0	0.5	0.5	2	243	1.5	3.5	D	D
Grn1	VE-453	307	0	0.5	1	1.5	288	0.5	0.5	0	0
Grn2	VE-453	293	0	0.5	2	1.5	277	1	0.5	0	0
Grn3	VE-453	297	0	1	1.5	1.5	288	1.5	0.5	0	0
Grn4	VE-453	337	0	0.5	1	1.5	310	1	0.5	0	0
Grn5	VE-453	314.1	0	0.5	1	1	291	0.5	0.5	0	0
Blu1	Z-VAD	334.3	0	0.5	1	1	318	1	0.5	0	0
Blu2	Z-VAD	289	0	1	1	1	260	1.5	0.5	0	0
Blu3	Z-VAD	350	0	0.5	2	1.5	331	0.5	0.5	0	0
Blu4	Z-VAD	329.6	0	0.5	1	1.5	318	1	0.5	0	0
Blu5	Z-VAD	307.4	0	1	2	2	289	1	0.5	0	0

Animal Code	Wght T=48	Enc T=48	Enc T=54	Enc T=60	Enc T=66	Wght T=72	Enc T=72	Enc T=78	Enc T=84	Enc T=90	Wght T=96	Enc T=96
Red1	278	0	0	0	0	268	0	0	0	0	268	0
Red2	284	0	0	0	0	282	0	0	0	0	281	0
Red3	276	0	0	0	0	265	0	0	0	0	262	0
Red4	263	0	0	0	0	257	0	0	0	0	259	0
Red5	285	0	0	0	0	275	0	0	0	0	275	0
Red6	231	0	0	0	0	229	0	0	0	0	230	0

Red7	240	0	0.5	0.5	0.5	241	0	0	0	0	243	0
Red8	D	D	D	D	D	D	D	D	D	D	D	D
Grn1	273	0	0	0	0	270	0	0	0	0	272	0
Grn2	262	0	0	0	0	256	0	0	0	0	251	0
Grn3	285	0	0	0	0	266	0	0	0	0	266	0
Grn4	302	0	0	0	0	257	0	0	0	0	289	0
Grn5	273	0	0	0	0	271	0	0	0	0	274	0
Blu1	300	0	0	0	0	294	0	0	0	0	301	0
Blu2	277	0	0	0	0	252	0	0	0	0	257	0
Blu3	D	-	-	-	-	-	-	-	-	-	-	-
Blu4	295	0	0	0	0	290	0	0	0	0	292	0
Blu5	268	0	0	0	0	269	0	0	0	0	275	0

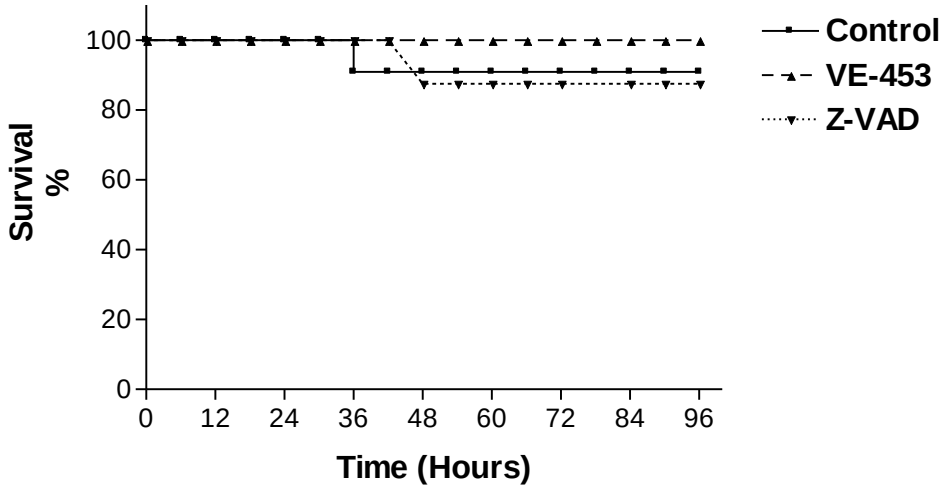


Figure 46. Encephalopathy score over time for male Wistar rats treated with Thioacetamide 600mg/kg (control), and Thioacetamide and Caspase inhibitors zVAD or VE 453

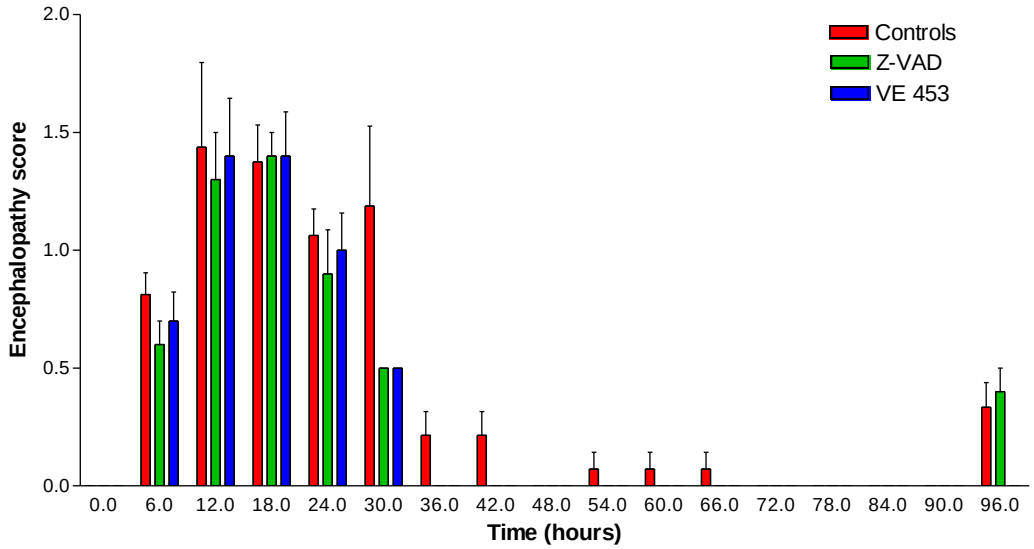


Figure 47. Graph showing encephalopathy score over time for male Wistar rats treated with Thioacetamide 600mg/kg (control), and Thioacetamide and Caspase inhibitors zVAD or VE 453

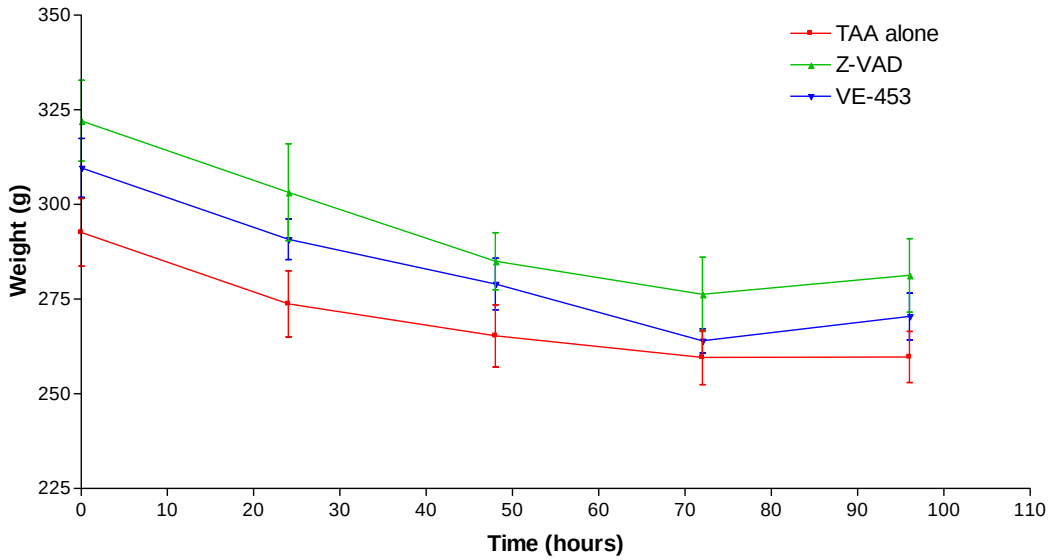


Figure 48. Graph showing weight loss of animals given 600mg/kg Thioacetamide +/-Caspase inhibitors Z-VAD or VE-453

No statistically significant difference could be detected in any of the primary or secondary clinical endpoints. Despite using an increased dose of Thioacetamide the mortality within the control group of Thioacetamide + vehicle alone remained lower than expected from previous studies.

Caspase 3 activation demonstrated by histology

Liver tissues were stained immunohistochemically using an antibody to activated Caspase 3 to investigate if TUNEL staining was accurately identifying apoptotic cells; to investigate if apoptosis induced by Thioacetamide was occurring via Caspase 3 activation; and to quantify if the caspase inhibitors used in the above animal model had inhibited Caspase 3 activation. The primary antibody chosen was an anti-human/mouse Active Caspase 3 antibody (R&DSystems AF835). Manufacturers recommendations stated that due to high degrees of homology between mouse and rodent Caspases and previous work, the antibody should bind with a high degree of specificity to activated Caspase 3 in both human and rodent tissue. We confirmed this using Human Tonsil, which is known to contain large numbers of lymphocytes undergoing apoptosis via a Caspase 3 dependent pathway, and used this as a biological positive control.

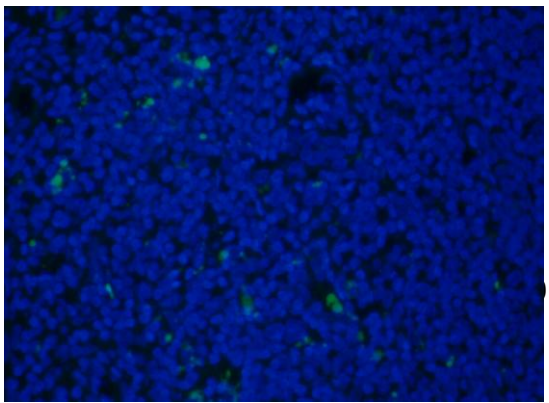
Method

Tissue on Apes coated slides was deparaffinised by thrice sequential 5 minute washes in 100% xylene, followed by rehydration with two 5 minute washes in absolute ethanol, a 3 minute wash in 95%ethanol, a 3 minute wash in 70% ethanol and a 5minute wash in Phosphate Buffered Saline (PBS).

Antigen retrieval was performed by heating for 5 minutes in citrate buffer (0.1M Citric acid 9mls, 0.1M Sodium Citrate 41mls, 450mls ddH_20, pH to 6.0) and then washed in PBS.

Cross reacting antigens to secondary antibody species were blocked by 15 minute incubation with 10% goat serum in PBS. The primary antibody Polyclonal Rabbit Anti-human mouse Active Caspase 3 antibody (R&DSystems AF835 stored -20ºC) was diluted to working strength (0.3µg/ml in PBS) was applied to slides and incubated for 1 hour at room temperature in a humidified chamber. Slides were then thrice washed with 15 minute washes in PBS before application of the secondary antibody Goat anti-Rabbit human, mouse, and rat adsorbed polyclonal IgG (BD Biosciences 554020 store 4ºC protected from light) working strength 10µg/ml, and incubation for 1 hour at room temperature in a humidified chamber. Antibody solution was removed and slides washed thrice for 15 minutes in PBS. Nuclei were counterstained by incubating with Hoechst stain (5uLin 1ml PBS) for 5 minutes followed by a PBS wash.

Autofluorescence (particularly high in liver tissue due to lipofuscin) was reduced by immersion of slides for 20 minutes in 10mM Copper sulphate in 50mM ammonium acetate Buffer pH5 for 20minutes, followed by three five minute washes in PBS. Slides were mounted in Antifade mountant.



Results

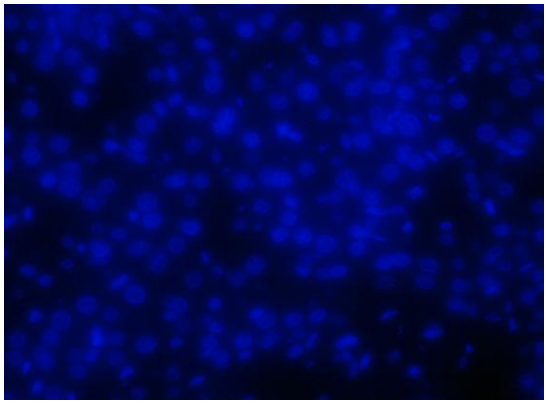


Figure 49. Human Tonsil positive control for Active Caspase 3 (Left) and Thioacetamide treated t=36 hours rat liver (Right) x40.

Caspase 3 positive cells (green) were clearly seen on the slides of human Tonsil (positive control), but no Caspase 3 positive cells could be seen in Thioacetamide treated rat liver.

The inability to identify Caspase 3 positive cells in liver tissue, at a time after the administration of Thioacetamide when apoptosis had been identified by TUNEL cell positivity could have a number of explanations.

The negative Caspase staining in rat tissue could represent a true finding. This would suggest that if the TUNEL cell staining is a true positive that Thioacetamide induces apoptosis via a non-Caspase 3 dependent pathway. Apoptosis can occur by direct activation of mitochondria and be Caspase 3 independent, it may also explain why Caspase 3 inhibitors failed to have any effect on clinical outcomes in protecting the rodents from Thioacetamide induced liver injury. However previous studies have shown increased Caspase 3 activity in rat liver and plasma after Thioacetamide induced injury (Hayami, Ikeda, Sun, Tanaka, & Kojo, 1999).

Alternatively the TUNEL staining could be a false positive marker for apoptosis and be concordant with the negative Caspase 3 activity, it has been previously suggested that TUNEL staining may also identify necrotic cells (Hahn *et al.*, 2001) and Thioacetamide is known to induce liver necrosis at some doses.

Finally lack of specificity of the primary antibody for rodent activated Caspase 3 could explain the lack of staining, if there is insufficient homology between rat and human Caspase 3 the primary antibody could fail to recognise rat Caspase 3 and coupled with background non-specific Caspase 3 antibody binding and tissue autofluorescence this could result in falsely negative staining.

Finally the timing of the Caspase 3 activation could be earlier than the timing of peak TUNEL cell positivity.

To try to differentiate between the above possibilities, a number of experiments were performed, the main aim of which was to identify a rat tissue positive control.

The D-galactosamine + LPS rat model in which increased apoptosis and Caspase 3 activity have already been demonstrated was prepared, and liver tissue samples treated according to the same methodology, but despite demonstrating increased apoptosis using TUNEL cell positivity in the treated rat (Picture A), compared to normal liver tissue (picture B), increased Active Caspase 3 could not be demonstrated using immunohistochemistry (Figure 76).

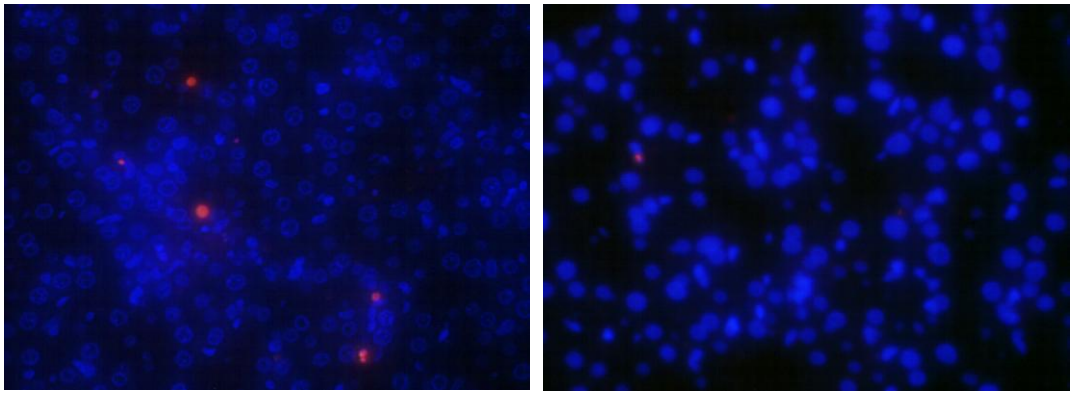


Figure 50. Photomicrograph A- TUNEL cell positive cells (red) seen in liver tissue of a rat treated with D-GAL/LPS to induce liver injury (left), compared to normal rat liver tissue (right), Magnification x40.

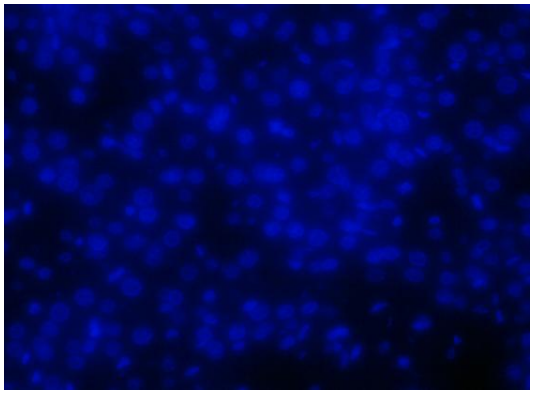


Figure 51. Photomicrograph of liver tissue from D-GAL/LPS treated rat labelled with primary anti-activated Caspase 3 antibody, and appropriate secondary FITC labelled antibody, demonstrating absence of activated Caspase 3 (green).

Subsequent experiments were performed using a new batch of identical antibody, an alternative, anti-active Caspase3 antibody from a different manufacturer, secondary labelling with a non-fluorescent horseradish peroxidase system, amplification of signal using an additional biotin step (ABC) and different techniques of antigen retrieval but no activated Caspase 3 could be identified in rodent tissue using immunohistochemistry despite consistently positive human Tonsil controls.

Western blotting of rodent liver tissue was also undertaken using cell lysates as positive controls but again no positive activated Caspase 3 could be identified in rat tissue.

4.6 DISCUSSION

Having identified in earlier chapters that humoral substances within acute liver plasma can induce apoptosis in primary hepatocytes, and that this can be attenuated by Caspase inhibition in-vitro, we aimed to identify if this inhibition would translate to an in-vivo model of acute liver failure, the Thioacetamide induced acute hepatic failure model. Unfortunately, and for reasons that are unclear the Thioacetamide model control group did not have its usual predictably high mortality, despite identifying increased apoptosis by TUNEL cell positivity in Thioacetamide treated animals and animals suffering significant encephalopathy and weight loss. Thus any possible decrease in mortality or improved clinical outcome was not identifiable in the Caspase inhibitor groups.

Also due to the inability to identify a reliable antibody to rodent activated Caspase 3, further experiments quantifying inhibition of Caspase 3 by inhibitors could not be undertaken.

4.7 FUTURE WORK WITH ANIMAL MODEL

We demonstrated apoptosis in liver tissue of animals with Thioacetamide induced liver injury but this failed to have its usual high mortality. If time and resources had allowed we would initially have investigated the Thioacetamide model, to re-establish its validity; having done this the next step would be to identify and validate an alternative method to quantify Caspase 3 activation via an enzymatic method. Initial studies in small numbers of animals could then be initially undertaken looking at alternative non-clinical markers of liver injury, particularly liver histology and plasma markers including serum transaminases. Having established that Caspase inhibitors did decrease these parameters of liver injury then a larger animal study could be undertaken looking at mortality as a primary outcome.

If inhibition of apoptosis was shown to attenuate acute liver injury then further studies on the functionality of surviving hepatocytes and the ability of hepatocytes to proliferate allowing regeneration and longterm safety would need to be investigated.

CHAPTER 5: THERAPEUTIC METHODS FOR REMOVAL OF ALBUMIN BOUND TOXINS – MARS.

5.1 INTRODUCTION

Artificial liver devices

Liver transplantation cures approximately 90% of patients with acute liver failure, however a shortage of donor organs, unsuitability of patients to receive a transplant combined with the fact that many patients will become too ill to undergo transplantation, means despite this mortality remains high (Williams & O'Grady, 1990). Therapeutic strategies that allow temporary liver support buying time for transplantation or endogenous liver recovery are therefore required.

By performing the detoxifying function of the liver it is hoped an "artificial liver" would not only prevent other end-organs from damage and preserve life, but by removing toxic substances from the plasma may make the general environment of the patient's own hepatocytes less toxic thus decreasing liver cell death and promoting regeneration and repair, breaking the vicious circle of liver failure (Riordan & Williams, 2000). The aim of this chapter was to investigate whether one such form of liver support – the molecular Adsorbents Recirculating system (MARS) would remove the pro-apoptotic effect of acute liver failure plasma (demonstrated in earlier chapters) on primary human hepatocytes. Removal of the pro-apoptotic effect would suggest that artificial liver support devices may improve patient outcome by improvements in endogenous liver function, and these systems could be valuable adjuncts to the protection of hepatocytes given either therapeutically (organ or cell transplantation) or in a bioreactor as part of a liver support device. A brief summary of artificial liver devices follows.

Extracorporeal organ support: The earliest forms of liver support used cross-circulation of animal livers initially baboons (Abouna, 1968) and later porcine organs (Shimahara *et al.*, 2001) for periods up to several days in duration; there was some improvement in the patients' condition, and human bile salts were found to be excreted by the non-human organ suggesting that they were indeed performing detoxicatory function, however ultimately due to immunological intolerance, risk of zoonosis, poor efficiency, and procedural complexity very few patients were treated. The procedure demonstrated proof of principle that replacement of endogenous liver function could improve a patients' clinical condition, but alternative methods would have to be derived.

Whole blood exchange: Also in the 1970's removal of toxins by whole blood exchange in acute liver failure was used but with limited success (Barlyn *et al.*, 1971, Demedts *et al.*, 1974) and subsequent animal experiments have demonstrated that whole blood exchange may result in removal of factors necessary for liver regeneration limiting its use (Eguchi *et al.*, 1998).

Plasmapheresis: High volume plasmapheresis, is a much more selective method for removal of toxins from plasma (Inoue, 1981), it has been shown to improve neurological status (Freeman *et al.*, 1986), decreases intracranial pressure, increase mean arterial blood pressure (Clemmesen *et al.*, 1997) and improve cerebral blood flow(Larsen *et al.*, 1994a) and oxygenation and possibly improves survival in acute liver failure (De Silvestro *et al.*, 2000, Larsen, Hansen, Jorgensen, Secher, Kirkegaard, & Tygstrup, 1994b). Large randomised trials are lacking but again the technique suggested that selective removal of substances within plasma of patients with liver failure may be beneficial (Clemmesen *et al.*, 2001).

Haemodialysis and haemofiltration: Simultaneously advances in the development of haemodialysis and haemofiltration membranes, which could efficiently remove water soluble substances, including abnormal middle-sized molecules seen on the chromatograms of sera in patients with liver disease (Matsubara *et al.*, 1990), showed hope of removing toxic substances that may accumulate in liver failure. Some trials did demonstrate improvement of level of consciousness, however they did not reverse liver failure or reduce mortality (Denis *et al.*, 1978, Hughes & Williams, 1993, Opolon, 1979, Opolon, 1981, Rakela *et al.*, 1988).

Charcoal haemoperfusion, another technique which removes water soluble substances was developed but again controlled trials in acute liver failure were disappointing, failing to show significant removal of albumin bound substances or reduction in complications of liver failure e.g. cerebral oedema and renal failure, or mortality (Gimson *et al.*, 1982, O'Grady *et al.*, 1988b, Silk & Williams, 1978)

The consistent inadequacy of these systems has been interpreted as due to the inability to remove significant amounts of albumin-bound substances (Hughes *et al.*, 1992). Activated charcoal which showed the capability of binding albumin bound substances such as bilirubin had been developed but was not biocompatible (exposure of whole blood to activated charcoal resulted in activation of cytokine and coagulation cascades) limiting its usefulness; alternative resins with improved biocompatibility and efficiency of removal of albumin bound toxins were required. Amberlite XAD-7 was used for this application and whilst *in-vitro* it removed albumin bound toxins, including cytokines, from plasma, and albumin coating improved biocompatibility (Ton *et*

al., 1979), *in-vivo* trials showed limited removal of toxins (Hughes *et al.*, 1979). Its binding capacity was inadequate, rapidly becoming saturated, the volume of resin required meant that columns would be too large for practical use and further development was abandoned.

All of these early non-cell based artificial liver support devices failed to show any significant survival benefit in either acute liver failure or in acute-on-chronic liver failure in open label studies although some did improve encephalopathy (Ash, 1991, Kjaergard *et al.*, 2003b).

Bioartificial liver support: Due to the complexities of liver function it seemed unlikely that any purely mechanical artificial device would be able to reproduce the myriad of functions performed by the liver. Bio-artificial liver support systems containing hepatocytes in a bio-reactor were therefore proposed in an attempt to replicate normal liver function. In 1987 Matsumura et al described the first case report of extracorporeal bioartificial support using rabbit hepatocytes in a plate dialyzer to treat a patient with an inoperable bile duct tumour (Matsumura *et al.*, 1987). Subsequently in 1989 Margulis et al published a large controlled trial of 126 patients (59 subjects and 67 controls) with acute liver failure using porcine hepatocytes in an AV shunt cell, which showed a reduction in mortality, 37% in the treated group versus 59% in control group (Margulis *et al.*, 1989).

Demetriou et al also used porcine hepatocytes in hollow fibre plasmapherisis in ten patients with AHF, eight of which survived to transplantation (Demetriou *et al.*, 1995). The same group subsequently entered their system into phase I clinical trials in patients with AHF (16/18 bridged to transplantation), primary non-function, (3/3 bridged to retransplantation) and acutely decompensated

chronic liver disease (2/10 recovered)(Watanabe *et al.*, 1997). The refined system was subsequently named the HepatAssist® system and is in commercial use.

The HepatAssist® system (Circe Biomedical) developed Demetriou's system and utilizes primary porcine hepatocytes cryopreserved in liquid nitrogen. The cells can be thawed and are available to treat patients within three hours. Patients undergo standard plasmapheresis with the plasma directed toward the HepatAssist® system where it circulates through a charcoal column, an oxygenator, and the hollow fibre cartridge containing the freshly thawed, viable porcine hepatocytes. The largest controlled trial included 171 patients with severe acute liver failure who were randomly assigned to the HepatAssist® system or usual care (Demetriou *et al.*, 2004b). No significant difference in survival at 30 days was observed between the two groups overall (71 versus 62 percent) or after adjusting for other known covariables. A statistically significant survival benefit was observed in a subgroup of patients with fulminant/subfulminant hepatic failure (RR 0.56, p=0.048)

Several other systems have undergone large clinical trials.

The Bioartificial Extra-corporeal Liver Support (BELS) system is a also a porcine cell-based extracorporeal liver assist device constructed with four separate capillary systems that mimic hepatic architecture (Gerlach, 1997). The system has successfully bridged eight out of eight patients with acetaminophen-induced acute liver failure to either transplantation or recovery (Detry *et al.*, 1999) .

The Extra-corporeal Assist Device (ELAD™, Hepatix Corporation, now Vital Therapies Inc) utilises a human hepotoblastoma cell line (HepG2 subclone

C3A) in its bioreactor. An initial open labelled study of 11 patients with FHF demonstrated that ELAD was associated with improvement in encephalopathy and liver function tests; survival was 70 percent at 30 days (Sussman *et al.*, 1994). A subsequent controlled trial included 24 patients (17 of whom were considered to have potentially recoverable causes of liver failure and 7 of whom were listed for liver transplantation). Patients were randomized within these groups to conventional therapy or conventional therapy plus ELAD therapy; survival was not significantly different within the two groups (recoverable group survival 78% versus contols 75%, transplant listed group 33% versus 25% in controls) (Ellis *et al.*, 1996b).

Whilst biological systems perform more of the livers endogenous functions, no bio-artificial device has been shown to reduce mortality in acute liver failure. Clinical trials are small and therefore often underpowered making demonstration of survival benefit unlikely. Larger trials are difficult due to the relative rarity of the condition, the heterogeneity of the pathogenesis and temporal differences with regard to the precipitating insult and presentation, and the issue of consenting patients who lack capacity into trials where best interest is unknown. There are also unique considerations with systems that contain live hepatocytes. Identifying hepatocyte sources that are biocompatible, readily available in large numbers, perform all the required detoxificatory and metabolic functions, that are pose no safety concerns has thus far remained elusive and so attention again shifted back to the use of mechanical detoxifying systems that would be highly selective, have a high capacity and be biocompatible.

Charcoal haemoperfusion had shown some promise in improvement of encephalopathy and blood pressure, and activated charcoal had a greater

potential binding capacity. The difficulties in biocompatibility and poor selectivity were overcome by keeping the patients blood from the charcoal and anion exchange resins separate from blood using a dialysis membrane. The Hemotherapies liver dialysis unit, and Prometheus System both work on this principal termed haemodiabsorption, a hybrid between hemodialysis and charcoal/resin hemoperfusion.

The HemoTherapies Unit™ — The HemoTherapies liver dialysis unit (www.hemotherapies.com) is based on the **BioLogic-DT®** System. A plate dialyzer (membrane 5000kDa molecular weight cut-off) separates the blood side from a suspension of sorbents (charcoal and cation exchangers), with biocompatibility similar to that of haemodialysis, but with chemical selectivity much greater than in hemodialysis/ hemoperfusion. The chemical capacity for adsorption of compounds by charcoal in hemodiabsorption is also greater than hemoperfusion columns. A blood treatment rate of 200 to 250 mL/min is achieved but instead of a dialysate solution, the liver dialysis system contains a two-litre suspension of powdered sorbents, including 140 grams of powdered activated charcoal (300,000 square meters) and 80 grams of the cation exchanger polystyrene sulfonate sodium (PSS, 80 meq binding capacity).

Effectively suction draws plasma water and substances with a molecular weight below the cut off of the cellulose membrane from the blood into the suspension of sorbent components where protein bound hydrophobic and organic substances are transferred to the powdered charcoal and ammonium and other cations are removed by the cation exchanger. The addition of plasmapheresis capability across the membrane allowed increased clearance of albumin bound substances (Ash *et al.*, 1998). Small controlled trials demonstrated reduction in albumin

bound substances and improvement in encephalopathy score and blood pressure, however overall improved survival in either acute or chronic liver disease has not been demonstrated (Ash *et al.*, 2000, Wilkinson *et al.*, 1998). Certain subgroups of patients with decompensated cirrhosis with grade III/IV encephalopathy, many with renal or pulmonary failure showed improved survival with treatment (71 versus 36 percent, $p<0.05$) (Ash, 2000).

Prometheus® — The Prometheus® is another extracorporeal device based on haemodiabsorption, it uses high-flux hemodialysis against a special adsorber to remove albumin-bound substances from the blood. A pilot study of 11 patients suggested improvement in laboratory tests after only two treatments (Rifai *et al.*, 2003). However the results of a much larger trial (HELIOS) presented recently in abstract at EASL 2010 confirmed no significant survival benefit.

Albumin dialysis – Another way to selectively remove albumin bound substances is by single-pass haemodialysis against an albumin solution. This technique removed albumin bound substances (Peszynski *et al.*, 2002) but would require a large volume of albumin with a high binding capacity. Thus systems were developed that would 'clean' the albumin which had become saturated with albumin bound substances. Since it was known that activated charcoal and anion exchange resins had a high capacity for such substances the passage of used albumin across such sorbent columns could be used to regenerate albumin. A hybrid system was developed, allowing the selectivity and biocompatibility of albumin dialysis with the clearance capability of haemodiabsorption, the albumin dialsylate effectively acting as a carrier molecule between charcoal and adsorbent columns and dialysate membranes. The molecular adsorbents

recirculating system (MARS) is such a system and is currently the main liver support system used in the U.K. Chapter 5 of this thesis reports the experience of MARS use at the Royal Free Hospital and investigates some of the mechanisms of its effects.

5.2 MOLECULAR ADSORBENS RECIRCULATING SYSTEM (MARS)

Introduction

The pathophysiology of liver failure is poorly understood (Sen *et al.*, 2002b). Ammonia, aromatic amino-acids, tryptophan, indoles, endogenous benzodiazepines and digoxin-like substances have all been implicated in the pathophysiology of encephalopathy; nitric oxide, prostanoids and other cytokines have been implicated in the pathogenesis of circulatory and renal dysfunction; other cytokines, bile acids and oxidative stress are known to be hepatotoxic and the vast majority of these substances are water-insoluble and therefore transported bound to albumin.

Biochemistry and physiology of Albumin

Human albumin, a major component of human plasma, produced by the liver, it is a 66kDa molecule with a strong net negative charge which binds weakly and reversibly to both anions and cations acting as a transporter/scavenger as well as being largely responsible for plasma's high oncotic pressure (Evans, 2002). In addition to its binding capacity a cysteine residue at position 31 has an exposed reduced sulphydryl group (thiol) and this acts as a scavenger of reactive oxygen and nitrogen species, especially superoxide, hydroxyl and peroxynitrite radicals. This gives albumin the capacity to directly reduce oxidative stress and is indeed the major extracellular source of reduced sulphydryl groups. In addition to

directly buffering oxidative stress albumin can bind free Cu^{2+} which is known to be a catalyst in the production of free radicals, thus also indirectly reducing oxidative stress. Since oxidative stress is known to be able to induce cell necrosis and apoptosis the reduction of oxidative stress may be another of albumin's beneficial effects. Albumin's effects on oxidative stress are known to be able to effect transcriptional events by inducing nuclear factor kappa B (NF – κβ) (Cantin *et al.*, 2000). Patients with liver disease have reduced quantities of albumin, and increased demand for its binding properties, albumin may also undergo qualitative changes decreasing its binding capacity (Bertucci *et al.*, 2001).

The possible beneficial effects of albumin as a scavenger/anti-oxidant in liver disease (Gines *et al.*, 2002) is suggested by the increased survival in patients with liver disease and Spontaneous Bacterial Peritonitis (Sort *et al.*, 1999) and the reduction in mortality seen when used together with glypressin in the treatment of type1 hepatorenal syndrome (Moreau *et al.*, 2002, Uriz *et al.*, 2000) when treated intravenously. These benefits cannot be accounted for by the plasma expanding effects of albumin alone as head to head trials of albumin versus other colloids have always demonstrated superiority of albumin (Fernandez *et al.*, 2005, Garcia-Compean *et al.*, 2002, Ortega *et al.*, 2002).

Since many of the toxins postulated to have a role in the pathogenesis of liver failure are albumin bound, and intravenous albumin is of benefit in liver disease, it has been suggested that systems which remove these toxic molecules, effectively "cleaning" the patients' own albumin, may improve outcome.

Function of MARS

The molecular adsorbents recirculating system (MARS) is an extracorporeal albumin dialysis system which allows removal of albumin bound substances. In brief via a special membrane the system allows transfer of water soluble and albumin bound toxins with molecular weight less than 50kDa from blood into a dialysate solution containing 20% human albumin; this albumin solution is then "cleaned" by passing it over a charcoal filter, resin adsorbents and a haemofilter/haemodialyser before recirculating it (Stange *et al.*, 1993).

The patient's venous blood is drawn off a large bore central venous double lumen catheter by a mechanical pump at a rate of up to 200mls/min via sterile heparin primed polyurathane dialysis tubing. The blood passes through a hollow fibre cartridge which is composed of an albumin-impregnated polysulfone membrane with a pore size of 50kDa. On the outside of the hollow fibre, in a counter current direction, flows 20% human albumin solution and is then returned to the patient via the central venous catheter. Albumin has been shown to have a higher affinity for toxins when bound to polymers (Hughes, Ton, Langley, Davies, Hanid, Mellon, Silk, & Williams, 1979), toxins therefore dissociate from the patient's albumin and bind to the albumin impregnated membrane. Since there is a gradient between the concentration of toxic molecules bound to the membrane and the albumin in the 20% dialysate solution, the toxins are effectively removed by dialysis. Since single pass dialysis would require massive quantities of albumin, the albumin in the diasylate's binding capacity is regenerated. After the 20% albumin solution has passed across the dialysis membrane (it being on the outside of the hollow fibres, contained within the dialysis cartridge), it is pumped through sterile

polyurethane tubing across a conventional haemofiltration/haemodialysis filter where water soluble substances are removed by either by passing a dialysate solution in a counter-current direction (if dialysis is chosen) or by ultrafiltration (if haemofiltration is required) (Sen *et al.*, 2002a). The albumin is then perfused through a column of activated charcoal and a subsequent anion-exchange resin which removes the albumin adsorbed substances. The 'clean' albumin is then recycled through the initial cartridge in a countercurrent direction to the patients blood. The flow rate in the albumin circuit is 200ml/min.

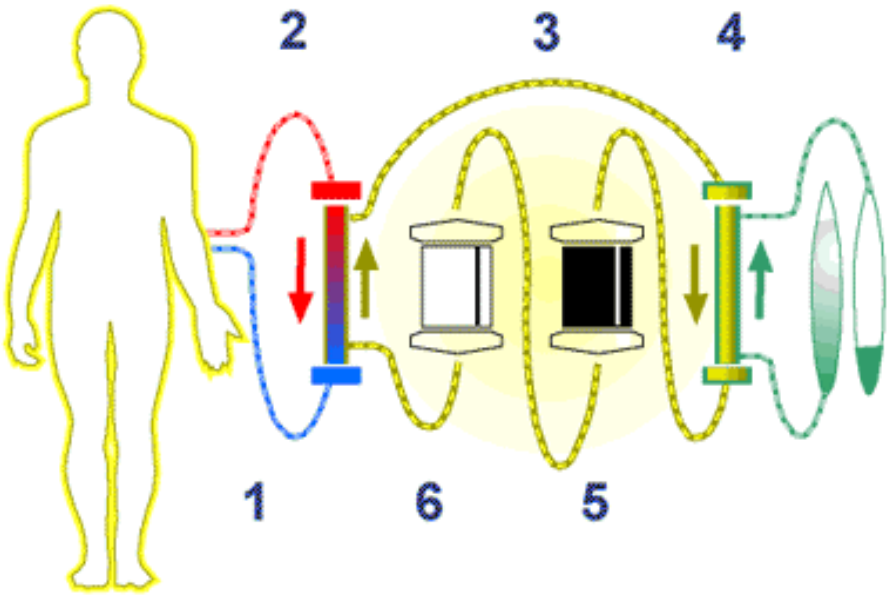


Figure 52. Schematic representation of MARS circuit *(courtesy of Teraklin UK)*

1 = Return of "detoxified" whole blood to the circulation via venous vascular catheter having passed through blood/20% albumin dialysis membrane cartridge.

2 = Removal of whole venous blood via venous vascular catheter using mechanical pump on haemodialysis/haemofiltration machine.

3 = Passage of 20% "dirty" human albumin dialsylate via albumin pump on MARS monitor to haemodialysis/haemofiltration membrane.

4 = Removal of water soluble molecules via haemodialysis/haemofiltration membrane (pumps & lines part of haemodialysis/haemofiltration machine).

5 = Passage of 20% human albumin across activated charcoal cartridge.

6 = Passage of 20% human albumin across anion exchange resin cartridge.

Table 10. Substances removed by MARS

Substance	**References**
Ammonia	(Awad *et al.*, 2001, Seige *et al.*, 1999, Stange *et al* 1999)
Bilirubin	(Awad *et al.*, 1997, Mitzner *et al.*, 1996)
Bile Acids	Stange *et al.*, 2000)
Aromatic amino acids (increase Fischer ratio)	(Loock *et al.*, 1997)
Middle and short chain fatty acids	(Mitzner *et al*, 2001)
Tryptophan	(Stange *et al.*, 2002)
Copper	(Kreymann *et al.*, 1999, Manz *et al.*, 2001)
Urea	(Sorkine *et al.*, 2001,)
Creatinine	Mitzner *et al*, 2000)
Cytokines – TNF-α, Interleukin 6	(Ambrosino *et al.*, 2003, Awad *et al.*, 1999)
Diazepam	(Majcher-Peszynska j *et al.*, 1999)
Nitric Oxide	(Guo L-M, 2003, Kurtovic *et al.*, 2004)
Drugs e.g. Phenytoin	(Sen *et al.*, 2003, Sen *et al.*, 2004)

Physiological effects of MARS

Treatment with MARS has been shown to have a number of beneficial physiological effects:

Cardiovascular system	Increased systemic vascular resistance (Schmidt *et al.*, 2001)
	Increased mean arterial blood pressure (Mitzner et al, 2000)
	Reduces portal pressure (Sen, 2005)
Cerebral function	Decreased hepatic encephalopathy (Awad et al, Mitzner *et al.*, 2002)
	Decreased intra-cranial pressure/brain oedema (Ben Abraham *et al.*, 2001)
	Increased cerebral blood flow
Kidney function	Increase in urine output (Mitzner *et al.*, 2001)
	Decreased serum creatinine (Steiner & Mitzner, 2002)
Liver function	Indocyanine green excretion increased
Quality of life	Decreased pruritus (Macia *et al.*, 2003)
	Decreased fatigue

Clinical benefits of MARS

In small trials MARS has shown a survival advantage in acute-on-chronic liver failure (Heemann *et al,* 2002), Alcoholic Hepatitis (Jalan *et al.*, 2003) and Hepato-Renal Syndrome (Mitzner et al, 2000). Despite improvement in several

parameters, particularly encephalopathy, MARS therapy has yet to be shown to produce any survival benefit in acute liver failure, although small case series describe positive results largely using MARS as a bridge to transplantation (Novelli et al, 2002), or in primary non-graft function as a bridge to recovery. A larger study (The RELIEF study) on 189 patients was presented at EASL 2010 again showed no significant survival benefit in patients with acutely compensated liver disease. A recent metanalysis of MARS therapy failed to show any benefit in either acute liver failure or acutely decompensated chronic liver disease.

Recent meta-analysis of all liver support devices (including MARS) compared with standard medical therapy has failed to show any significant reduction in mortality (RR 0.86, 95% CI 0.65-1.12). However stratified meta-analysis has shown that in acutely decompensated chronic liver disease mortality is reduced by 33%, whereas mortality in acute liver failure shows no reduction {Liu, 2002 20 /id}. Despite the paucity of large randomised controlled trials, more than 4500 patients have been treated with the MARS® Liver Support Therapy to date

Possible mechanism of action of MARS

The exact mechanism of the possibly improved clinical outcome remains unknown. The removal of albumin bound mediators that have direct toxic effects on organ function has been suggested as the primary mechanism of action of MARS. This assumes that this/these molecules are directly toxic, albumin bound and less than 50kDa in size so as to be able to cross the primary dialysis membrane and are efficiently removed by the dialysis process, and/or the charcoal/anion exchange resin.

An alternative explanation could be that by removing albumin bound substances from the patients endogenous albumin (these may not necessarily be toxic) the specific binding capacity of the patient's albumin (Klammt *et al.*, 2001) is increased and that *in-vivo* toxins are therefore bound, decreasing their toxic effects. This would mean that the toxic molecules need not be <50kDa in size or removed by the dialysis process.

Finally, the "toxic" molecules removed by MARS itself or made less toxic by MARS therapy by allowing increased albumin binding need not be directly toxic to cells. Indeed many of the improvements in end-organ function could be explained by improved organ perfusion due to improved haemodynamics (increased blood pressure and increased systemic vascular resistance) due to the removal/increased binding of mediators that have direct effects in the vasculature rather that the removal of directly organ toxic molecules.

Since albumin may also bind beneficial substances, the removal of which may have deleterious effects on hepatocytes and other cells, MARS therapy may in fact decrease the likelihood of organ regeneration.

Plasma from patients taken before and after MARS therapy could therefore be utilised to differentiate between some of these possibilities and could provide useful information for the development/improvement of artificial liver support devices and other therapeutic strategies. In this chapter, using the assays developed to look at the effect of human liver failure plasma on primary human hepatocytes described in the previous chapters we investigate the effects of MARS therapy both on patient physiology, particularly renal function, and outcome and on qualitative effects on plasma.

5.3 USE OF MARS AT THE ROYAL FREE HOSPITAL

Patients who reached inclusion criteria, had no exclusion criteria and gave informed consent (or assent of relatives) were enrolled on a trial of MARS therapy (see consent form appendix). Blood samples were obtained before and after each session of MARS therapy. Ethical approval for this trial was been obtained from the local ethics committee (see appendix).

Inclusion Criteria

Hepatic decompensation as evidenced by two out of three of the following:

Severe jaundice (Bilirubin >205μmol)

Hepatic encephalopathy

Hepatorenal syndrome

Exclusion criteria

Age <16 or>75

Lack of consent/assent of next of kin

Viral Hepatitis

Contraindications to MARS therapy: Disseminated Intravascular Coagulation

Active/recent haemorrhage

Significant Hypotension (systolic <90mmHg)

Table 11. Summary of Patients treated with MARS therapy at Royal Free Hospital

Patient	Age	Sex	Diagnosis	MELD Score Pre-Treatment
1. SD	40	M	Alcoholic Hepatitis Cirrhotic Renal impairment	20
2. FA	42	F	Fulminant Wilsons disease Encephalopathy Renal impairment	23
3. JW	34	M	Benign Recurrent Intrahepatic Cholestasis Renal impairment	16
4. SR	54	F	Alcoholic Hepatitis Renal impairment	17
5. AG	57	M	Decompensated Cirrhosis Obstructive jaundice Renal Impairment	19
6. TN	33	F	Decompensated Alcoholic Cirrhosis Encephalopathy Renal impairment	17
7. AM	60	M	Erythropoitic protoporphyria Acute liver failure previous transplant Renal failure Encephalopathy	13
8. NS	33	F	Alcoholic Hepatitis Renal impairment	17
9. BM	62	M	Primary graft non-function Renal Failure Multi-organ Failure	19
10. KB	40	F	Alcoholic Hepatitis Renal impairment	20
11.MR	56	F	Alcoholic Hepatitis Cholestatic drug reaction Cirrhosis	16
12.DW	31	F	Alcoholic Hepatitis Cirrhosis	20
13.DS	65	M	Hepato-Renal Syndrome Alcoholic Cirrhosis	23

Procedure

A double lumen vascular catheter was inserted into the femoral vein (FFP or platelets were given as prophylaxis if necessary) under local anaesthetic using aseptic technique.

The MARS monitor and haemofiltration (Baxter) or haemodialyisis machine (Baxter) were prepared and primed according to the manufacturer's instructions. In brief both haemodialysis and MARS circuits were primed using 1L of sterile normal saline containing 5000 iu of Heparin, ensuring there were no airlocks/bubbles in the circuit. The MARS circuit was then loaded with 600ml of sterile 20% salt-poor human albumin (Zenalb) which was circulated around the albumin circuit for 15 minutes prior to attachment to the patient to allow removal of stabilisers in the 20% human albumin and thus increase its albumin binding capacity. The patient's vascular catheter was then connected to the blood dialysis circuit and dialysis commenced initially at a blood flow rate of 50ml/min rapidly increasing to 200ml/min as haemodynamic status would allow.

Patients were treated with the MARS circuit at a flow rate of 200ml/min with haemofiltration at 2L exchange/hour. Dialysis fluid was returned to the circuit in the post filter circuit (post-dilution). The amount of potassium put in the dialysate bags and fluid balance over treatment duration was decided based on the individual needs of the patient. The patient's blood circuit was anticoagulated with Heparin 100units/hour to prevent clotting in lines or dialysis filter unless contraindicated. Patients were treated for 6 hours on 2-3 consecutive days. Blood samples were taken for full blood count, clotting, and routine biochemistry before and after each treatment. Serum was also taken,

aliquoted and stored at -20 ^{o}C for measurement of Cystatin C, a measure of renal function (Hoek *et al.*, 2003, Lamb *et al.*, 2003) and heparinised blood was taken and plasma separated by centrifugation as described in previous chapter) and plasma stored at -20 ^{o}C for assessment of in-vitro toxicity assays. Clinical parameters including encephalopathy score (where possible to assess), blood pressure and pulse, were also assessed before and after treatment. Full blood count, clotting and routine biochemistry (Urea, Creatinine, Sodium, Potassium, Total Bilirubin, Alanine transaminase, Aspartanine transaminase, serum albumin, total protein, Alkaline Phosphatase, Calcium and Phosphate were measured using standard labatory methods by the routine clinical laboratory.
MELD score pre-treatment was calculated using haematology and biochemistry parameters prior to treatment or transfusion of clotting factors for line insertion.

Cystatin C was measured by Cystatin C Immunoparticles kit (Dakocytomation cat no:LX002) according to manufacturers instructions by a laboratory experienced in this technique (Biochemistry Department, St Hellier Hospital, Carshalton, UK). A polyclonal rabbit antibody to cystatin C covalently coupled to polystyrene particles when mixed with heparinised plasma results in the formation of immune complexes that can be measured by turbidometry. A calibration curve is produced using DakoCytomation Cystatin C Calibrator (X0974), the Cystatin C Control Set (X0973) was used as a control.

Statistical analysis

Results are expressed as mean values ± SEM. Results were tested for normality using Gaussian distribution, parametric data was analyzed using two-tailed paired student t-test, non-parametric paired data was analyzed using Wilcoxon matched pairs test. $P<0.05$ was used to identify statistical significance. Statistical analysis was performed with Graphpad Prism version 3.02.

5.4 RESULTS I – Clinical outcome

Mortality

Previous data has shown that six-month actuarial survival while on the transplant waiting list is 90% with a MELD <17, whereas it decreases progressively to 40% at 6 months for those with a score of >16 (Adler *et al.*, 2005). Thirty day mortality in a comparable subgroup to ours is scarce but a previous trial of renal support in patients with hepatorenal syndrome treated with renal support (Adler M et al) showed patients' 30-day survival was 8/30 (median survival time 21 days)(Witzke *et al.*, 2004). Another trial for patients with alcoholic hepatitis showed the mean MELD score at admission for survivors at 30-days was 4.3, while for non-survivors was 18.7. Using a cut-off MELD score of greater than 11, the sensitivity and specificity of the MELD score for prediction of 30-day mortality was 86% and 82% respectively (Sheth *et al.*, 2002). Our patients had a mean MELD score of 18.1 before their first treatment (range 16-23, median 18) indicating a group of patients with a poor prognosis. Survival at 30 days was 8/13 (62%). One patient received a successful liver transplant within 30 days of treatment. Of the patients who died mean survival post treatment was 9.5 days (day 1=first day of treatment). Cause of death was

pneumonia in two patients (day 7 and day 30), asystolic cardiac arrest (day 2), GI haemorrhage (day 10), cerebral oedema (day 6) and haemothorax (day 2).

Summary of Patients treated with MARS therapy at Royal Free Hospital

Patient	Age	Sex	Diagnosis	MELD Score Pre-Treatment	Outcome Day 30
1. SD	40	M	Alcoholic Hepatitis Cirrhotic Renal impairment	20	Died
2. FA	42	F	Fulminant Wilsons disease Encephalopathy Renal impairment	23	Died
3. JW	34	M	Benign Recurrent Intrahepatic Cholestasis Renal impairment	16	Alive
4. SR	54	F	Alcoholic Hepatitis Renal impairment	17	Alive
5. AG	57	M	Decompensated Cirrhosis Obstructive jaundice Renal Impairment	19	Died
6. TN	33	F	Decompensated Alcoholic Cirrhosis Encephalopathy Renal impairment	17	Died
7. AM	60	M	Erythropoitic protoporphyria Acute liver failure previous transplant Renal failure Encephalopathy	13	Alive
8. NS	33	F	Alcoholic Hepatitis Renal impairment	17	Alive
9. BM	62	M	Primary graft non-function Renal Failure Multi-organ Failure	19	Alive
10. KB	40	F	Alcoholic Hepatitis Renal impairment	20	Alive
11.MR	56	F	Alcoholic Hepatitis Cholestatic drug reaction? Cirrhosis	16	Alive Liver transplant
12.DW	31	F	Alcoholic Hepatitis Cirrhosis	20	Died
13.DS	65	M	Hepato-Renal Syndrome Alcoholic Cirrhosis	23	Died

Haemodynamic status

There was a statistically significant increase in mean arterial blood pressure (MAP) by an average of 3.8mmHg per 6 hour MARS treatment, from a mean of 81.6 (sd=11.3) mmHg pre-treatment to 85.4 (sd=10.6) mmHg post-treatment (p=0.044) (95% confidence interval -7.519 to -0.111).

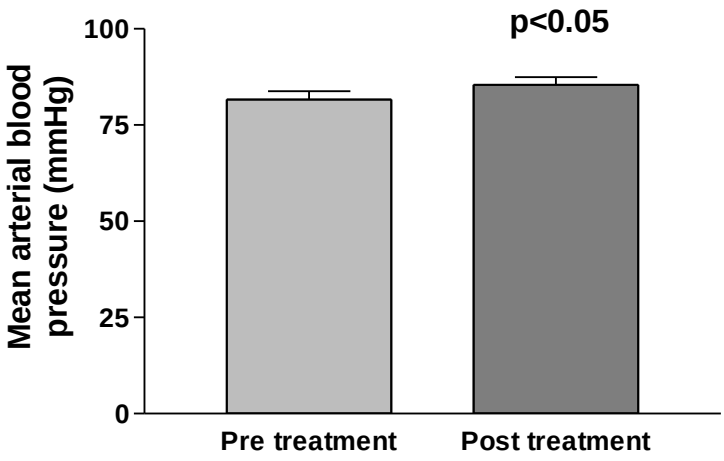


Figure 53. Mean arterial blood pressure (mmHg) (n=27) immediately before and after each treatment with MARS therapy.

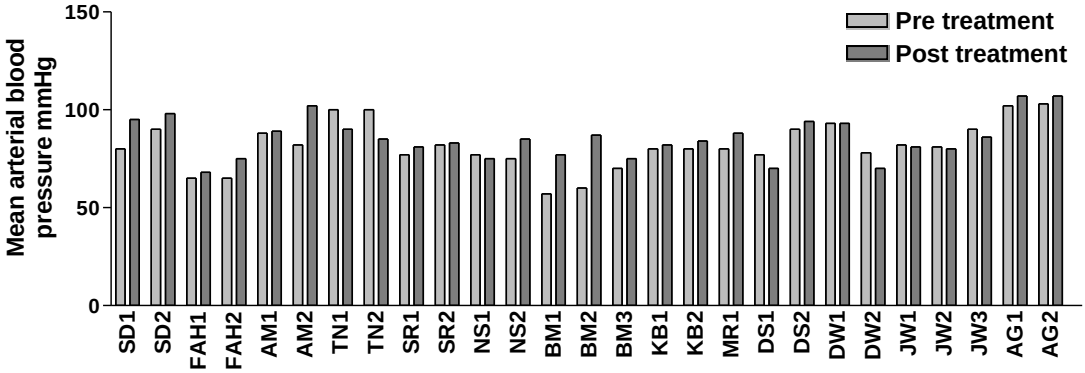


Figure 54. Mean arterial blood pressure for each individual before and after each treatment with MARS therapy.

Encephalopathy

Patients' degree of encephalopathy was scored before and after each treatment by clinical examination and graded accordingly.

Grade	Clinical findings
O	Normal
1	Mild confusion, shortened attention span, mood disturbance, reversal of sleep wake pattern. Hepatic flapping tremor (asterexis) usually absent.
2	Drowsiness, lethargy, gross deficits in ability to perform mental tasks, obvious personality changes, intermittent disorientation, lack of sphincter control. Hepatic flapping tremor (asterexis) usually present.
3	Somnolent but rousable, persistent disorientation in time and place, pronounced confusion, unable to perform mental tasks. Hepatic flapping tremor (asterexis) usually present.
4	Coma Hepatic flapping tremor (asterexis) usually absent.

In those patients where encephalopathy score could be assessed, (19 treatments), there was a significant improvement in mean encephalopathy score from 1.5 pre-treatment to 0.9 post-treatment p=0.003. Eight treatments were undertaken in patients who were paralysed and ventilated such that encephalopathy score could not be assessed.

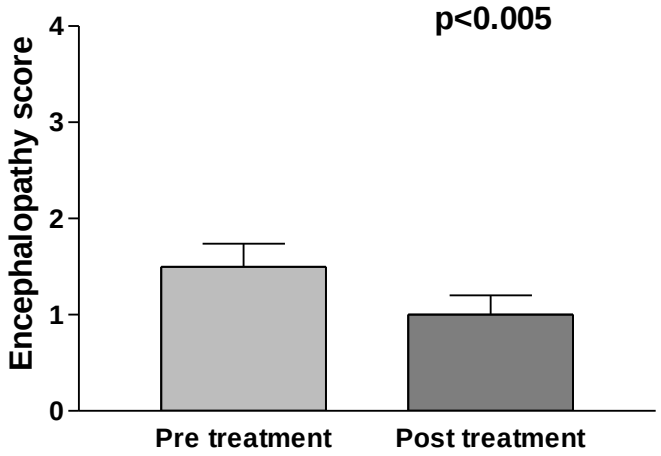


Figure 55. Mean encephalopathy score immediately before and after each treatment with MARS therapy.

5.5 ADVERSE CLINICAL EVENTS

Serious adverse effects which could possible be a result of the trial/MARS therapy occurred in three of the thirteen patients treated.

The original trial format included the measurement of Iohexol clearance as a measure of renal function before and after treatment with MARS therapy. Iohexol is a small molecule commonly used as radio-opaque contrast medium which is filtered by the glomerulus and not reabsorbed by the more distal parts of the nephron. Thus its elimination from the circulation after a single bolus intravenous injection provides a direct measurement of Glomerular Filtration Rate, which is both accurate and avoids the need for complex continuous infusions (as in the measurement of inulin clearance). The first two patients treated with MARS therapy were done according to this protocol. Patient 2 F.A. a 42 year old female with fulminant hepatic failure due to Wilson's disease suffered an asystolic cardiac arrest 5 minutes after injection of 5ml Iohexol 300 via an internal jugular line given after completion of the patients' second session of treatment with MARS therapy. The previous injections of Iohexol and the MARS therapy itself had been uneventful and there was no hypotension prior to the arrest or history of Iodine allergy. Basic cardiopulmonary resuscitation was instituted and Atropine 3mg, adrenaline 1mg and calcium chloride were given to the patient who transiently returned to sinus rhythm before suffering a further refractory asystolic arrest. The patient's past medical history revealed she had moderate mitral valve regurgitation but otherwise no cardiac history. Possible explanations for the patient's demise included the use of Iohexol which has an incidence of 2 in 10^6 of serious arrythmias and in case reports in animals may cause asytole. Alternative explanations included lyposomal Amphotericin

which can also cause asystole, undiagnosed cardiac Wilson 's disease which can cause serious arrythmias, or asystole as a terminal event in a septic patient with fulminant liver failure whose condition had been progressively deteriorating over the previous days despite appropriate medical therapy. In view of the possibility that Iohexol had contributed to this patient's death the use of MARS was temporarily halted and the ethics committee was informed of the adverse event. Whilst it was felt unlikely that Iohexol was the cause of death we removed Iohexol as a measurement of renal function from the protocol and replaced it with serum Cystatin C analysis. Cystatin C is a small, 13 kDa, non-glycosylated basic protein belonging to the cystatin super-family of cysteine protease inhibitors. Cystatin C is produced by virtually all nucleated cells, and is present in all body fluids investigated. The production rate is constant and is unaltered in inflammatory conditions. In the normal kidney, cystatin C is almost freely filtered through the glomerular membrane and then nearly completely reabsorbed and degraded by the proximal tubular cells. Therefore, the plasma concentration of cystatin C is almost exclusively determined by the glomerular filtration rate (GFR), making cystatin C an excellent indicator of GFR. Moreover, the cystatin C plasma concentration is uninfluenced by gender and muscle mass. Numerous studies and a meta-analysis incorporating 4492 subject samples have shown that serum cystatin C is clearly superior to serum creatinine as a marker for GFR. Measurement of serum Cystatin C was therefore performed pre- and post- each treatment of MARS therapy.

The amendment from Iohexol clearance to Cystatin C analysis as a measure of renal function was cleared by chairman's action of the ethics committee prior to recommencement of the trial. Stored samples were used for retrospective

measurement of Cystatin C for the initial two patients treated by the alternative methodology.

The other two complications of MARS therapy were the development of a bleeding diathesis whilst on treatment. Patient 10. K.B. developed bleeding spontaneously from a spider naevus over the right clavicle, which had bled after minor trauma two days earlier, in the last 10 minutes of her second session of MARS therapy. Approximately 200 mls of blood was lost, the patient remained haemodynamically stable. Clotting results showed large derangements in APTT (Pre treatment 51.6s post treatment >350s) and INR (Pre treatment 2.2, post treatment >8) and although the patients platelets did not fall (pre-treatment 126, post-treatment 161) it was suspected that the patient had developed a low grade DIC. (the patients Hb fell by 1.2g (pre-treatment 9.8g/dL, post treatment 8.6g/dL. Bleeding was controlled by direct pressure of the bleeding point and cessation of MARS therapy.

The second complication occurred in patient 11; in the last hour of treatment the patient developed cyanosis and shortness of breath, and developed a large haematoma (20cm diameter) on the right side of the neck, the site where a transjugular biopsy sheath had been removed 2 days earlier. Significant blood loss into a haematoma/haemolysis resulted in drop in Hb of 2g/dL. Blood results (PT 54.2, INR 4.8, APTT >250, TT 22, Fibrinogen 1, Platelets 30, Hb 8.4) which suggested the patient had developed disseminated intravascular coagulation. The patient was haemodynamically stable throughout. Local pressure was applied to the haematoma site and the patient was transfused with fresh frozen plasma 1500ml, Platelets 1 pool and packed red cells 2 units.

Complications were reported to Teraklin AG as part of their ongoing safety monitoring. All patient data was recorded in the international MARS registry.

5.6 RESULTS II - Biochemical parameters

Renal function

There was no statistically significant fall in serum urea before and after each six hour MARS treatment. Paired student t-test p=0.052, (n=26, (95% confidence interval 0.014 – 2.914).

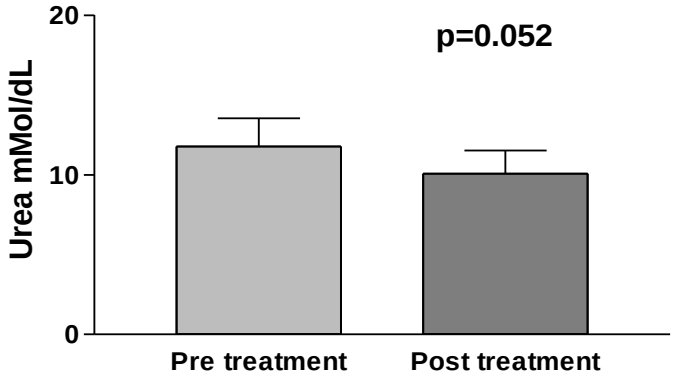


Figure 56. Mean Urea (mMol/dL) immediately before (pre-treatment) and after (post-treatment) a single six hour MARS treatment.

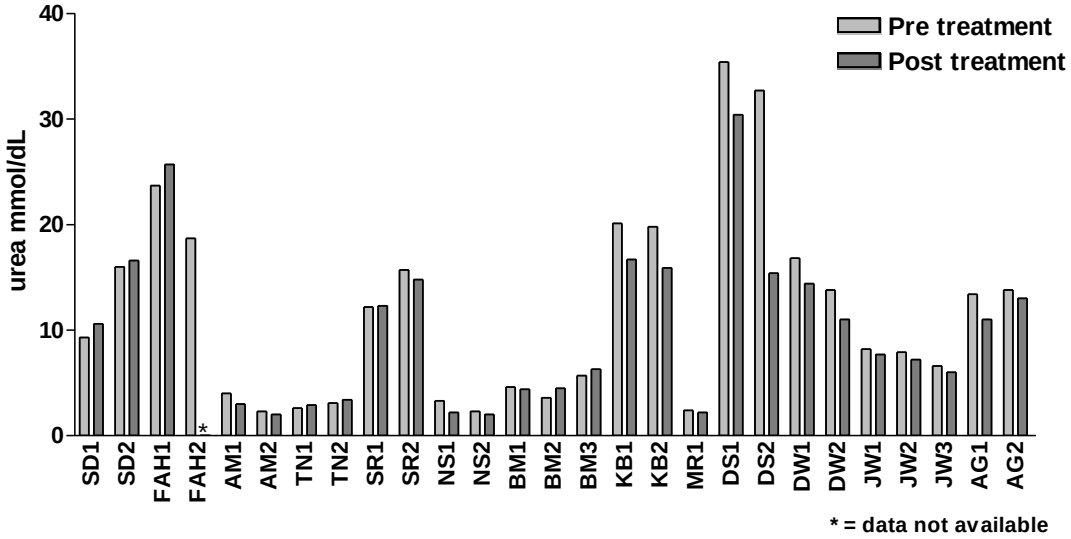


Figure 57. Individual Urea (mMol/dL) immediately before (pre treatment) and after (post treatment) each six hour MARS treatment.

Mean serum creatinine fell by 20% from a mean pre-treatment of 201 uM/dL to 161uMol/dL with each six hour MARS treatment session. This decrease was statistically significant. Paired student t-test $p<0.005$, (n=26, 95% confidence interval 15.2-59.9).

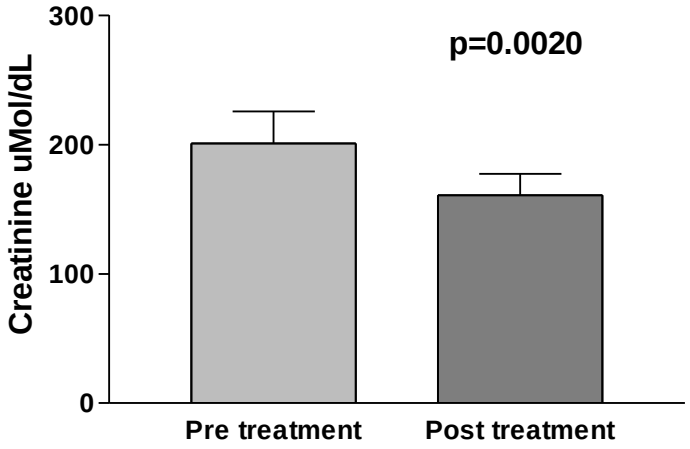


Figure 58. Mean serum creatinine (uMol/dL) before (pre treatment) and after (post treatment) a single six hour MARS treatment.

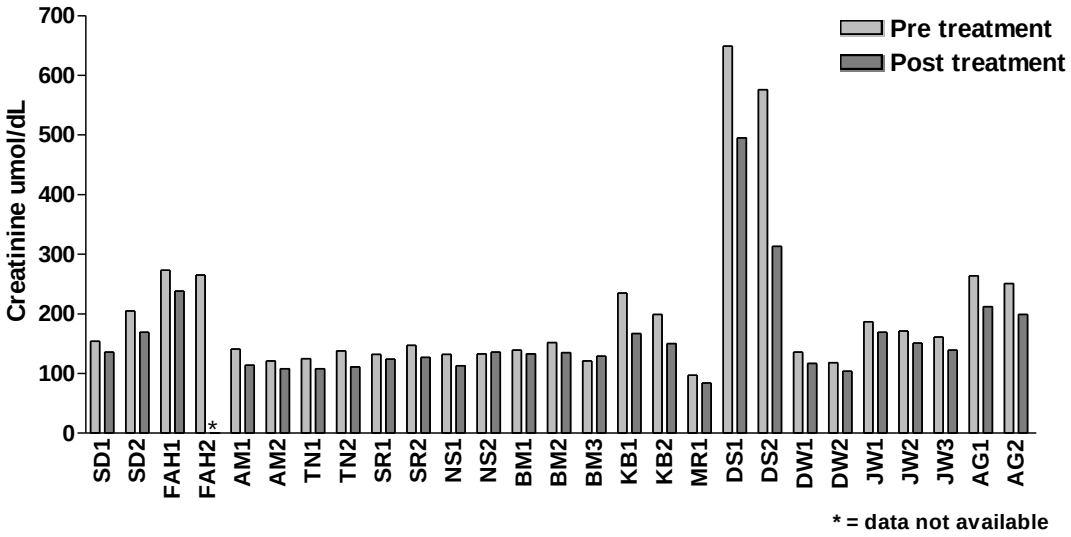


Figure 59. Individual Creatinine (uMol/dL) immediately before (pre treatment) and after (post treatment) each six hour MARS treatment.

Mean plasma Cystatin C concentration fell by 10.4% from a mean pre treatment of 2.33 IU/L to 2.09 IU/L with each six hour MARS treatment session. This decrease was statistically significant. Paired student t-test $p<0.05$, $n=26$, (95% confidence interval 0.074 - 0.410).

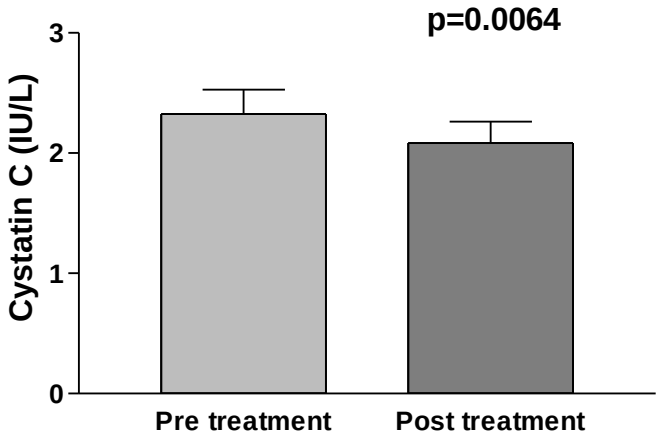


Figure 60. Mean plasma Cystatin C (IU/L) before (pre-treatment) and after (post-treatment) a single six hour MARS treatment.

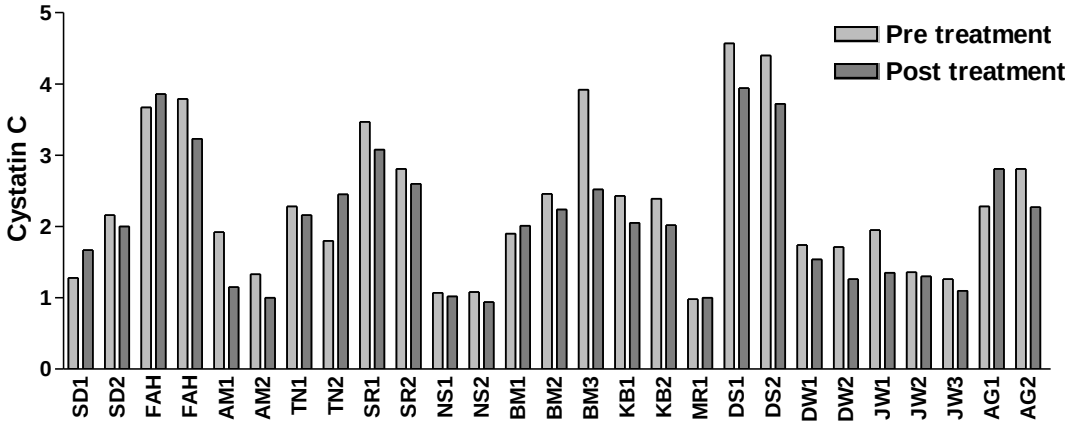


Figure 61. Individual Cystatin C (IU/L) immediately before (pre treatment) and after (post treatment) each six hour MARS treatment.

Liver function

Mean plasma Bilirubin concentration fell by 26.6% from a mean pre treatment of 380uMol/dL to 279uMol/dL with each six hour MARS treatment session. This decrease was statistically significant. Paired student t-test $p<0.001$, n=26, (95% confidence interval 51.9-134).

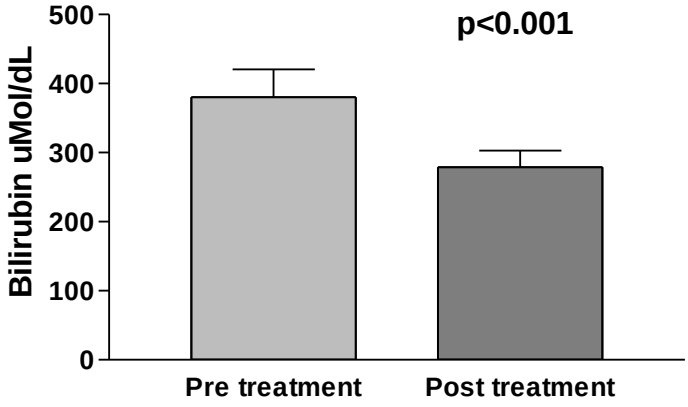


Figure 62. Mean plasma Bilirubin (µMol/dL) before (pre-treatment) and after (post-treatment) a single six hour MARS treatment.

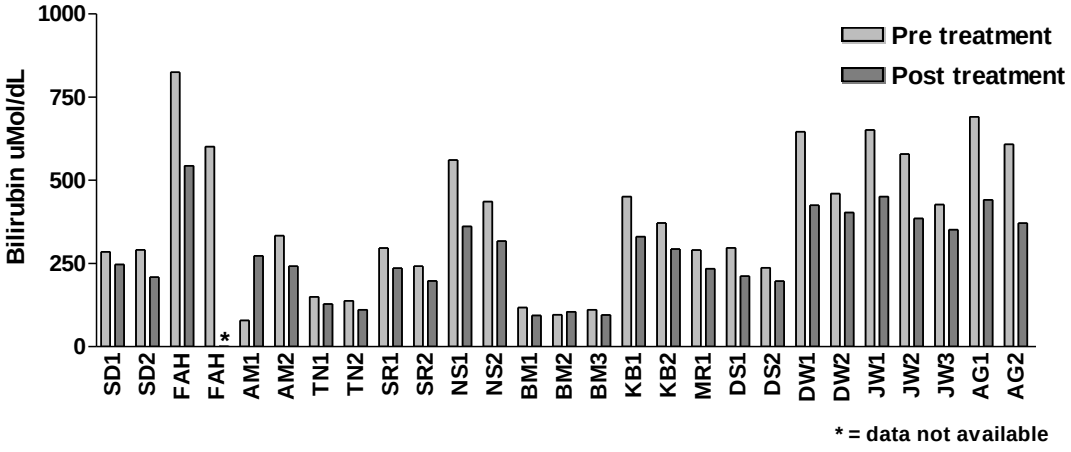


Figure 63. Individual Bilirubin uMol/dL immediately before (pre treatment) and after (post treatment) each six hour MARS treatment.

Mean plasma AST (IU/L) concentration fell by 37% from a mean pre-treatment of 428IU/L to 270IU/L with each six hour MARS treatment session. This decrease was statistically significant. Data was not normally distributed therefore was analysed using Wilcoxon ranked pairs test $p<0.01$, $n=26$.

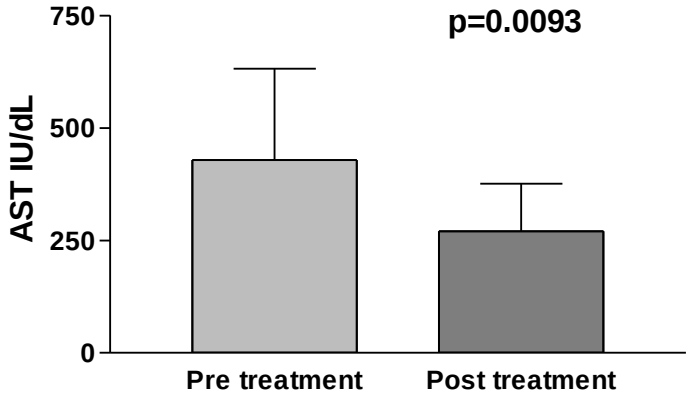


Figure 64. Mean AST (IU/L) before (pre treatment) and after (post treatment) a single six hour MARS treatment.

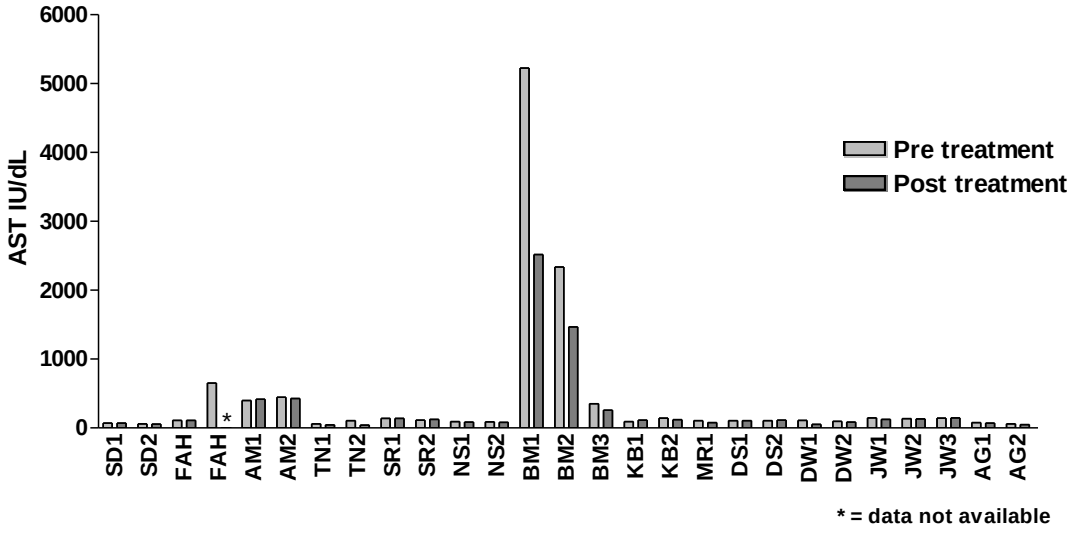


Figure 65. Individual serum AST levels (IU/L) immediately before (pre-treatment) and after (post-treatment) each six hour MARS treatment.

Mean plasma ALT (IU/L) concentration fell by 20% from a mean pre-treatment of 485IU/L to 386IU/L with each six hour MARS treatment session. This decrease was statistically significant. Data was not normally distributed therefore was analysed using Wilcoxen ranked pairs test $p<0.05$, $n=23$.

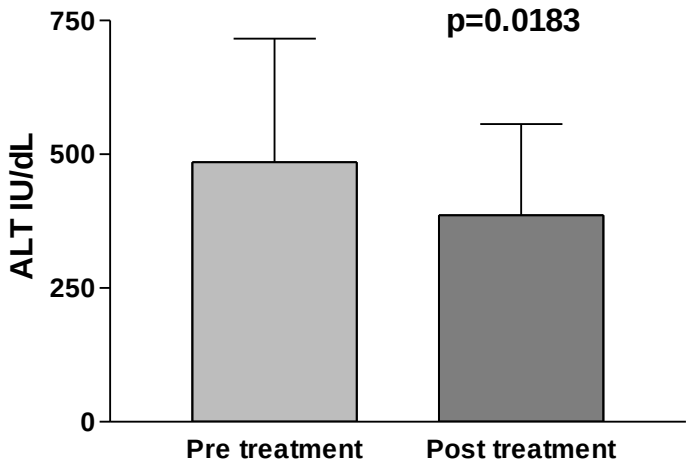


Figure 66. Mean ALT (IU/L) before (pre treatment) and after (post treatment) a single six hour MARS treatment.

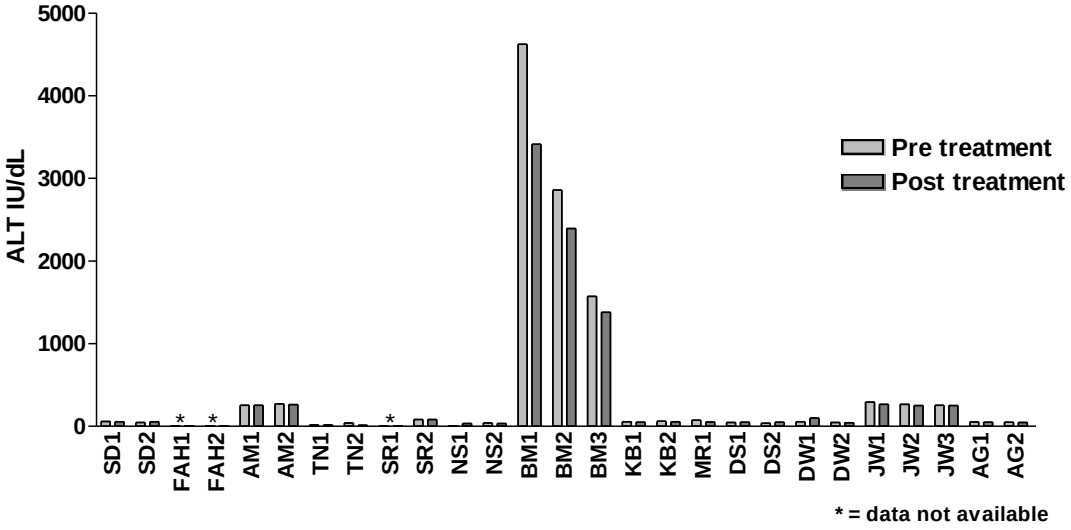


Figure 67. Individual serum ALT levels (IU/L) immediately before (pre treatment) and after (post treatment) each six hour MARS treatment.

Mean plasma ALP (IU/L) concentration fell by 5% from a mean pre-treatment of 202IU/L to 193IU/L with each six hour MARS treatment session. This decrease was statistically significant. Paired student t-test $p<0.05$, n=26 (95% confidence interval 2.23-27.3).

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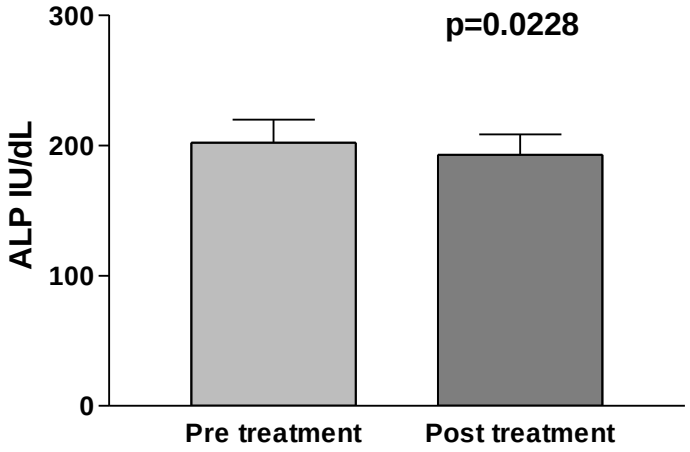

Figure 68. Mean ALP (IU/L) before (pre treatment) and after (post treatment) a single six hour MARS treatment.

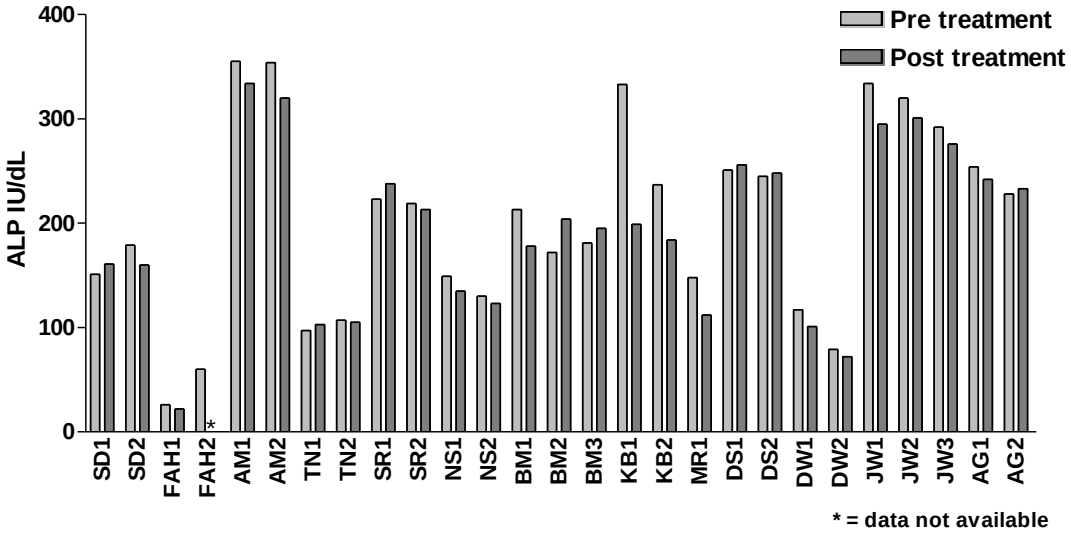

Figure 69. Individual serum ALP levels (IU/L) immediately before (pre treatment) and after (post treatment) each six hour MARS treatment.

Mean plasma albumin (g/dL) concentration was unchanged by treatment mean: pre-treatment of 29g/dL to 30g/dL by each six hour MARS treatment session. Paired student t-test p=0.34, n=26 (95% confidence interval -1.32 to 0.471)..

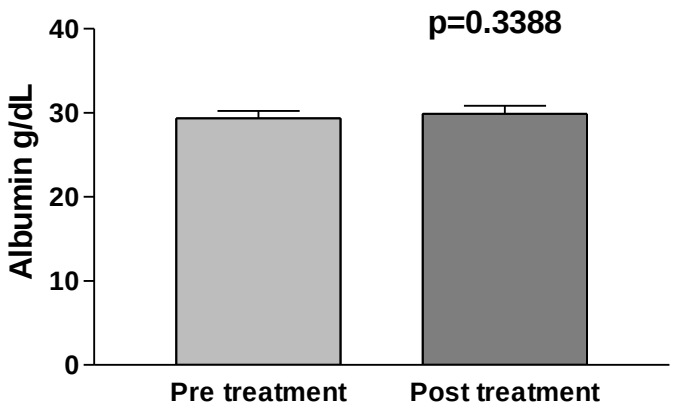

Figure 70. Mean serum albumin (g/dL) before (pre treatment) and after (post treatment) a single six hour MARS treatment.

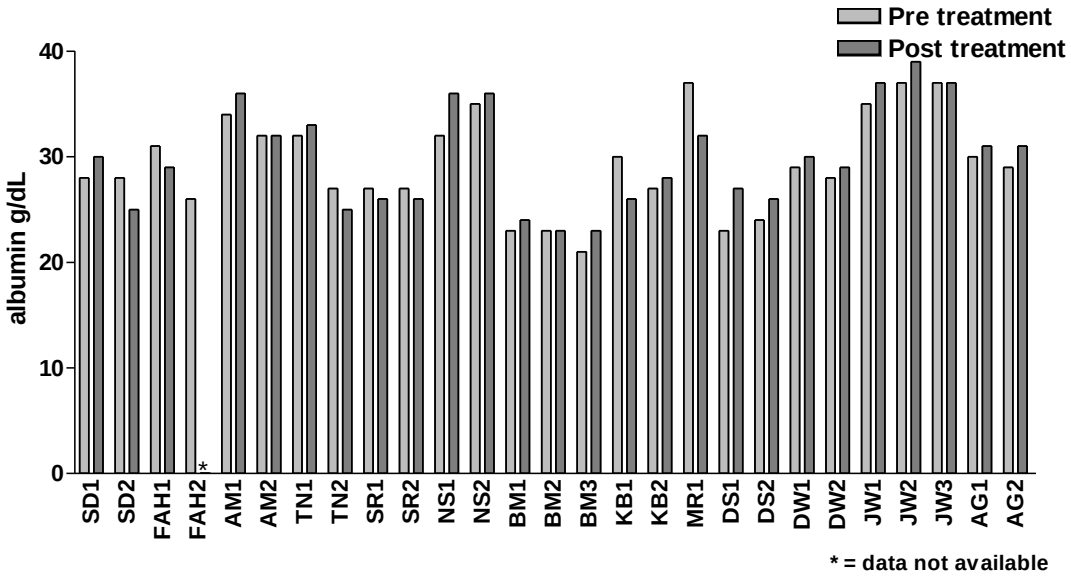

Figure 71. Individual serum albumin levels (g/dL) immediately before (pre treatment) and after (post treatment) each six hour MARS treatment.

5.7 RESULTS III – Apoptosis and cell viability

The quantity of apoptosis, measured by percentage cells TUNEL positive, induced in primary human hepatocytes exposed to patients' plasma samples taken immediately before and after treatment with MARS therapy showed no reduction in the amount of apoptosis induced by plasma. Paired student t-test p=0.134, n=27.

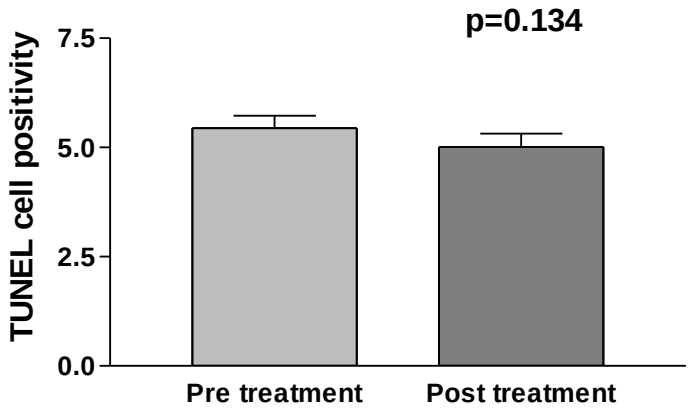

Figure 72. Mean TUNEL cell positivity induced in primary human hepatocytes exposed to plasma taken before (pre treatment) and after (post treatment) a single six hour MARS treatment (95% confidence interval -0.417 to 1.006).

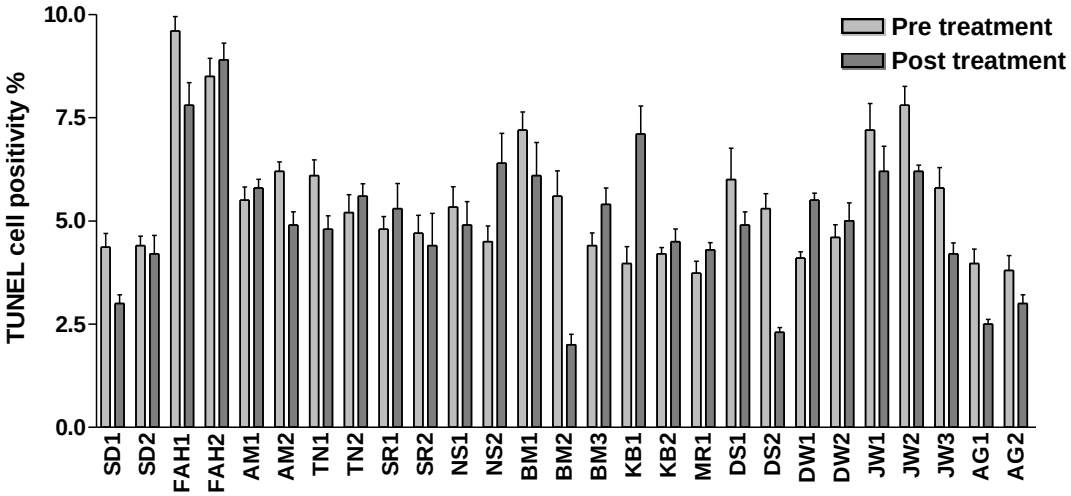

Figure 73. TUNEL cell positivity (%) induced in primary human hepatocytes exposed to plasma taken before (pre treatment) and after (post treatment) individual six hour MARS treatment sessions.

The quantity of apoptosis, measured by caspase 3 activity, induced in primary human hepatocytes exposed to patients' plasma samples taken immediately before and after treatment with MARS therapy showed no reduction in the amount of apoptosis induced by plasma. Paired student t-test p=0.078, n=27 (95% confidence interval -35.67 to 626.2).

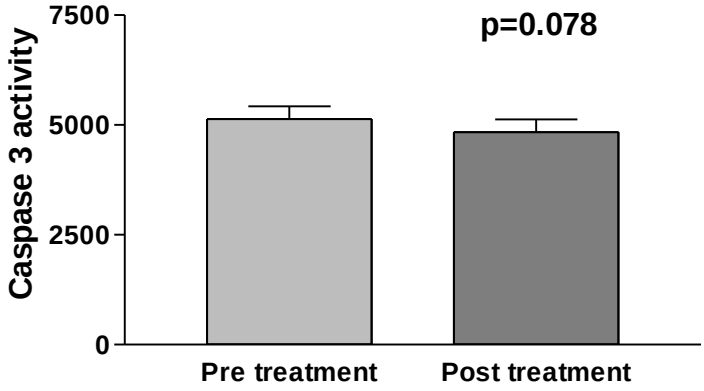

Figure 74. Mean Caspase 3 activity induced in primary human hepatocytes exposed to plasma taken before (pre treatment) and after (post treatment) a single six hour MARS treatment.

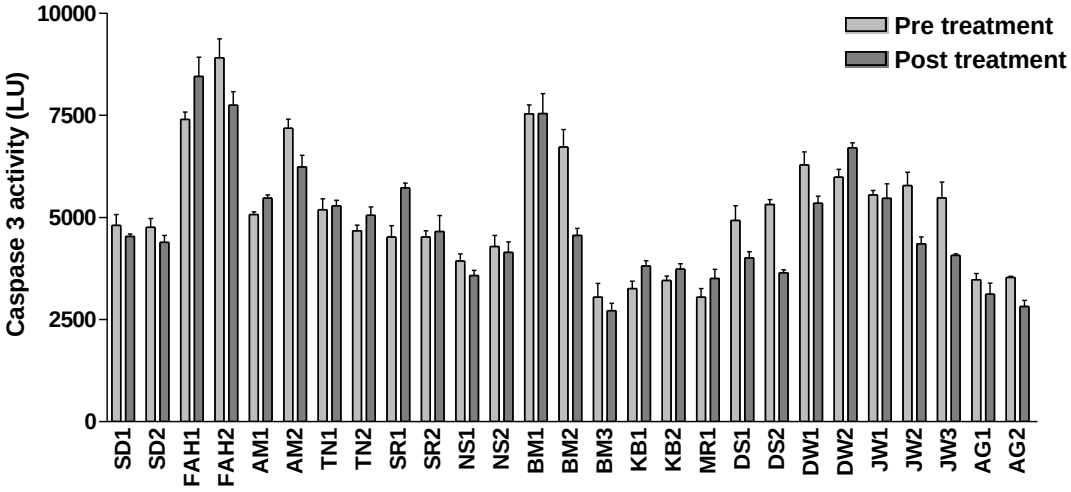

Figure 75. Caspase 3 activity induced in primary human hepatocytes exposed to plasma taken before (pre treatment) and after (post treatment) individual six hour MARS treatment sessions.

Cell viability assessed by MTT activity measured in primary human hepatocytes exposed to patients' plasma samples taken immediately before and after treatment with MARS therapy showed no reduction in the cell viability induced by plasma. Paired student t-test p=0.568, (n=27, 95% CI -0.0491 to 0.028).

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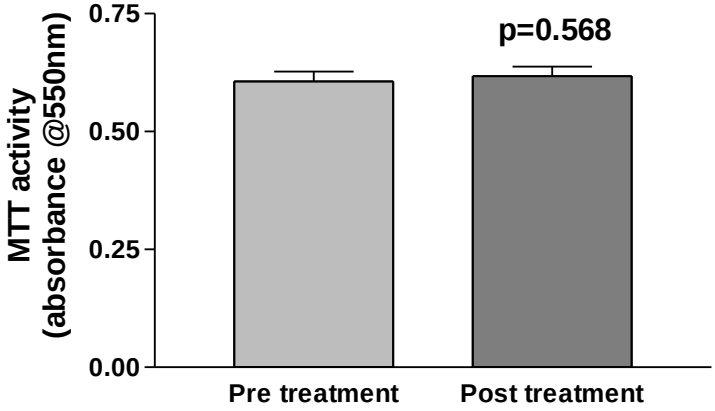

Figure 76. MTT activity in primary human hepatocytes exposed to plasma taken before (pre treatment) and after (post treatment) a single six hour MARS treatment.

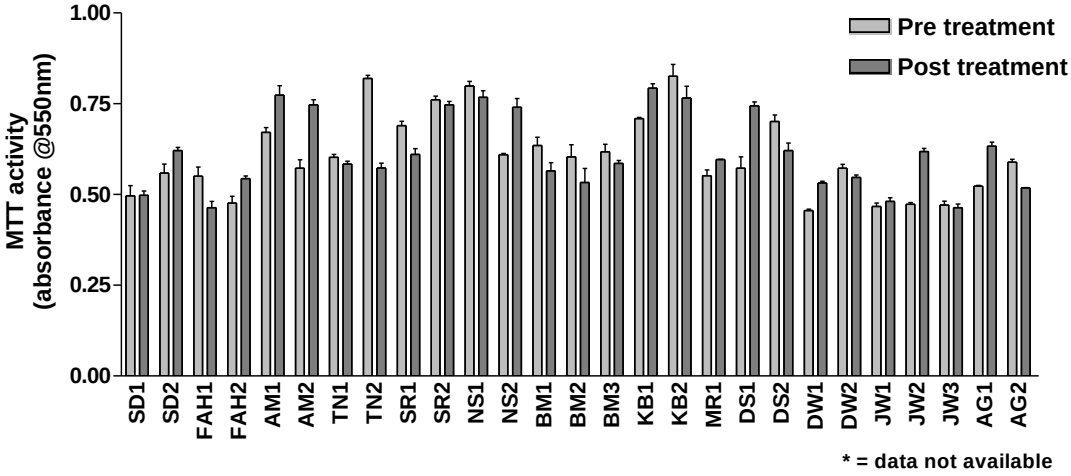

Figure 77. MTT activity in primary human hepatocytes exposed to plasma taken before (pre treatment) and after (post treatment) individual six hour MARS treatment sessions.

Correlation

Measurement of Caspase 3 activity (LU) showed a strong positive correlation to TUNEL cell positivity as a measure of apoptosis. Pearson r +0.6851, n=54, p<0.001.

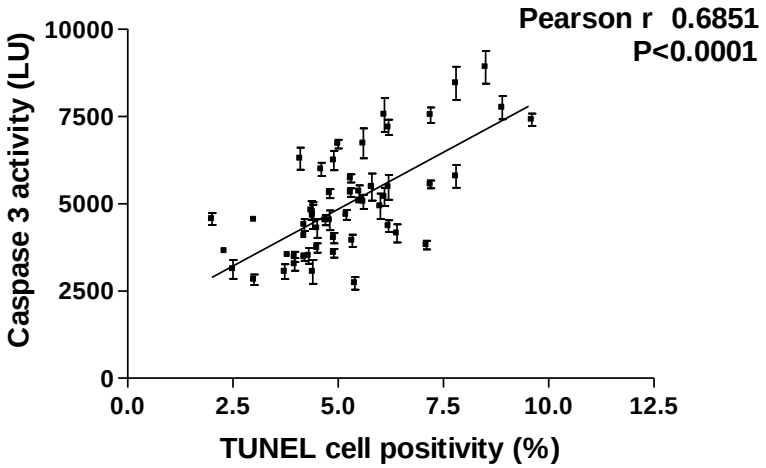

Figure 78. Correlation between Caspase 3 activity and TUNEL cell positivity for primary human hepatocytes incubated in plasma samples of patients treated with MARS.

Measurement of Caspase 3 activity (LU) as a measure of apoptosis showed a week negative correlation to cell viability measure by MTT activity. Pearson r -0.3582, n=54, p<0.01.

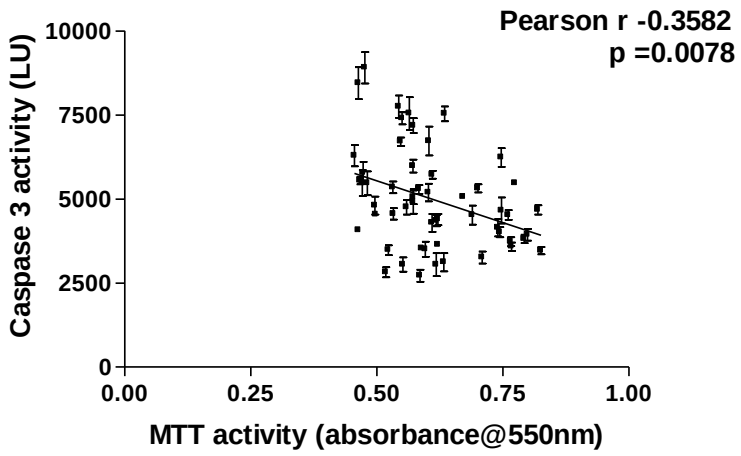

Figure 79. Correlation between Caspase 3 activity and MTT activity for primary human hepatocytes incubated in plasma samples of patients treated with MARS.

Measurement of TUNEL cell positivity (%) as a measure of apoptosis showed no significant correlation to cell viability measure by MTT activity. Pearson r -0.1268, n=54, p=0.361

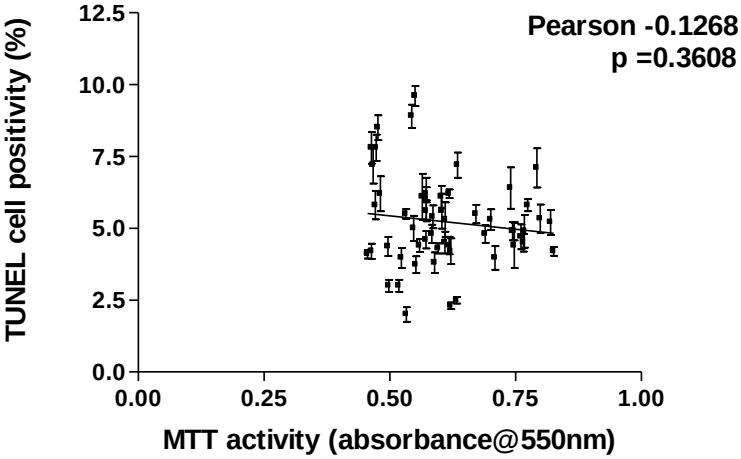

Figure 80. Correlation between TUNEL cell positivity and MTT activity for primary human hepatocytes incubated in plasma samples of patients treated with MARS.

5.8 DISCUSSION

Hepatocyte apoptosis increases in chronic but particularly in acute liver disease. There are multiple mechanisms potentially leading to hepatocyte apoptosis, and this may be initiated by soluble or cellular factors at the cell surface, through direct or indirect interactions with death receptors, and through the intrinsic pathway affecting mitochondrial energetics leading to cytochrome c release. It is a reasonable hypothesis that factors in the plasma in acute liver disease initiating apoptosis through the death receptors may be responsible for a significant proportion of the liver dysfunction present, although additional contributions via direct cell-to-cell contact or through the intracellular pathway may also play a significant role.

It has been demonstrated in subgroups of meta-analysis that MARS therapy decreases mortality in acutely-decompensated chronic liver disease and hepatorenal syndrome. The exact mechanism of this improvement is unknown but the removal of substances that are toxic to hepatocytes (and other organs) is suggested. The identity of these substances are unknown, and difficulties in isolating and identifying them have been described in previous chapters. The clinical context of a series of patients who had been exposed to MARS dialysis provided the opportunity to evaluate its clinical effectiveness, and in addition to determine whether this dialysis system – which involves the depletion of both albumin bound molecules and those low molecular substances mobilised in a conventional haemodialysis system – would remove toxic factors which induce apoptosis and cell death. The hypothesis was that by improving the milieu in which endogenous hepatocytes are bathed would promote endogenous liver

recovery, this would be confirmed if apoptosis was reduced by MARS treatment.

Thirteen patients with either acute or acute on chronic liver failure were treated with between 1 and 3 treatments of 6 hours of MARS therapy. MARS treatment was generally well tolerated in our patient cohort with 2 patients suffering significant side effects during therapy, both of which responded to conservative management.

Significant removal of albumin bound toxins was achieved; using bilirubin as a marker for other albumin bound substances, there was a 27% reduction in bilirubin per 6 hour treatment.

Our patients were not part of a randomised trial therefore there is no control group with which to compare mortality. However from the patients' pre-treatment MELD scores (18.1) it can be seen that they constituted a group with a particularly poor prognosis. The 30 day survival rate of 62%, with only one patient undergoing transplantation in this time, would therefore suggest a favourable response to MARS therapy. Patients had a favourable haemodynamic response with a small but significant increase in mean arterial blood pressure. Patients also had an improvement in encephalopathy grade.

Renal function as assessed by a reduction in serum creatinine improved, however since MARS therapy is known to remove creatinine it may be a poor measure of intrinsic renal function. An alternative measure of renal function unaffected by MARS therapy was required. Cystatin C (an alternative measure of GFR) was also reduced after treatment with MARS therapy, again suggesting improvement in renal function. There are several possible explanations for this. The first is that the removal of substances from plasma that are directly

nephrotoxic is responsible for the improvement. This theory could be tested by in-vitro experiment using renal cells in a similar way to the methods we have used with hepatocytes. A second explanation is that the improvement in renal function could be secondary to the improvement in liver function; this hypothesis could be tested by in-vivo animal studies using MARS in hepatectomised and non-hepatectomised animals. A third explanation is that the improvement in renal function was a consequence of improved mean arterial blood pressure; this again could be tested in animal experiments. Finally Cystatin C could also be removed by MARS therapy. This could be tested by testing Cystatin C levels in the albumin dialysate in the MARS circuit or by spiking a volume of normal plasma with Cystatin and perfusing through the blood circuit in the MARS dialysis circuit, measuring Cystatin C levels before and after treatment. Since renal impairment is an indicator of poor prognosis in liver disease, improvement in renal function may be responsible for improved outcome in patients treated with MARS therapy.

As expected MARS therapy removed significant amounts of albumin bound substances, as demonstrated by a significant fall in the concentration of Bilirubin from pre- to post-treatment levels. Bilirubin is not itself toxic to hepatocytes (indeed biliprotein is thought to be a growth factor), but its removal is used as a marker of removal of other putative toxic albumin bound molecules. The significant reduction in bilirubin during treatment of these patients thus showed that MARS therapy had performed effective “detoxicatory” function.

Serum transaminases also fell during treatment, this is in accordance with findings from other groups. There are several possible explanations for these findings. Firstly the transaminases could be being removed by MARS therapy

itself. Secondly since serum transaminases represent leak from damaged hepatocytes it could suggest that hepatocyte damage in-vivo was less as a result of treatment. This could be via two possible mechanisms, either the direct removal of hepatotoxic substances by MARS therapy or due to improved perfusion of the native liver via improved arterial blood pressure, improved regional perfusion, improved delivery of oxygen or a combination of these factors . These effects occur via removal of albumin bound vaso-active intermediates by MARS therapy, for example nitrous oxide.

Alkaline phosphatase also fell during treatment and the same arguments (direct removal of ALP via MARS, removal of substances that are directly toxic to cholangiocytes or reduce cholangiocyte function or indirect effects) as outlined above could be postulated for the improvement in biliary function for which ALP is a marker.

Changes in concentrations of substances within plasma could also be explained by dilution if significant volumes of fluid were given during treatment. All patients were in fact kept at neutral fluid balance during treatment and this is confirmed by there being no change in serum albumin pre- and post-treatment. Finally falling liver enzymes could be the result of spontaneous improvement in the patient's general condition. This would seem unlikely since up to the institution of MARS therapy all the patient's condition (measured by increasing Bilirubin) had been deteriorating.

Previous trials of MARS therapy have not been shown to directly remove liver enzymes, therefore improvements in liver enzymes by direct removal by MARS seems unlikely.

Despite significant reduction in albumin bound substances, improvement in clinical parameters in our patients, and the improvement in many biochemical parameters used as markers of renal and liver function, MARS dialysis did not effect the cytotoxicity of plasma.

Comparison of the extent of apoptosis induced in primary human hepatocytes by 100% heparinised plasma, taken before or after the dialysis, showed no change in apoptosis measured either by TUNEL cell positivity or the induction of Caspase 3 activity. Total hepatocyte viability measured by MTT assay also showed no improvement before and after therapy. This is highly suggestive that improvements in liver function are not the result of removal of albumin bound toxins that are directly hepatotoxic.

Looking at hepatocyte death is however only half the equation, improvements in liver function post MARS therapy could be the result of improved liver regeneration. This theory could be tested either in-vitro using hepatoma cell lines or organ bath experiments to assess removal of directly inhibitory substances or in-vivo in animal models performing liver histology (BRDU staining) before and after therapy to assess both direct and indirect effects.

Further studies need to be performed on a larger group of patients, particularly those with acute liver failure. In the previous chapter we have demonstrated that there are humoral factors that increase hepatocyte apoptosis in acute liver failure, but that these are not present in acutely-decompensated chronic liver failure. It therefore seems unlikely that MARS therapy detoxifying plasma in acutely decompensated chronic liver disease will remove humoral factors directly improving hepatocyte viability by reducing apoptosis. Patients with acute liver failure represent a very different pathophysiology and having

demonstrated humoral factors that induce apoptosis it remains possible that MARS therapy does remove directly toxic substances. Our patient cohort contains only 2 patients with acute liver failure, too few for subgroup analysis.

In summary whether or not this dialysis system will turn out to be of significant clinical value, an issue still under investigation, it does not however appear to offer benefit through alteration of the apoptotic capacity/cytotoxicity of plasma in acutely-decompensated chronic liver disease. It seems likely that improved outcome in these patients is a result of improved arterial blood pressure, alterations in regional perfusion, or indirect effects by improving other organ function, probably by removal of albumin bound cytokines and vaso-active mediators e.g Nitric oxide. This observation is particularly important in the development of future therapies for liver failure; it calls into question the use of therapies aimed at detoxifying plasma as a means of promoting liver recovery, particularly in acutely-decompensated chronic liver disease, and suggests that alternative approaches particularly antagonising vascular and cytokine effects may be beneficial.

5.9 CASE REPORTS

Two patients treated with MARS offered unique insights into the possible pathogenesis of Erythropietic Protoporphyria (EPP) associated liver disease and Benign Recurrent Intrahepatic Cholestasis (BRIC) and offered new therapeutic strategies; as such both were studied in more depth and are submitted as case reports (see appendix).

CHAPTER 6: CONCLUSIONS

Hepatocyte cell death with resultant loss of functional liver cell mass, and the release of inflammatory mediators from the failing liver are the cause of the syndrome of acute liver failure. Strategies to treat this syndrome need to address the issue of a reduction of functional liver mass by decreasing the amount of hepatocyte loss and increasing liver cell regeneration. One of the mechanisms of cell loss is apoptosis, which leads to loss of cell number both directly, and indirectly by generating secondary necrosis. Understanding the factors that lead to, and the pathways by which, apoptosis is executed in hepatocytes is essential in developing strategies to protect hepatocytes. Some of these toxic factors are present in plasma in liver failure.

The liver is known to have a tremendous capacity for regeneration and this has led to the aim of developing an artificial liver that would act as a bridge to transplantation or recovery. Mechanical detoxifying systems such as MARS do this to some extent, however due to the complexity of liver function it is likely that any successful "artificial liver" would contain a bio-reactor full of liver cells. These cells would be exposed to the plasma of patients with liver failure plasma. The induction of apoptosis in these cells by liver failure plasma would result in loss of function of the bio-artificial liver.

Another alternative strategy is the transplantation of hepatocytes or stem cells. These cells are again however exposed to liver failure plasma and any toxicity is therefore likely to have deleterious results.

For the above reasons it can be seen that the identification of toxins and identifying there mechanism of action is crucial to the development of strategies for the treatment of acute liver failure.

The aim of this project was to characterise the toxic molecules in liver failure plasma, their mechanism of action and possible ways to antagonise their effects, or remove them. At the outset of this project the aim was to identify these toxins. Identification of any substance initially requires purification and by using the biological assay of MTT as a measure of cell number in HepG2 cell lines as our measure of toxicity we had hoped to "track" the toxic fraction through various processes and ultimately identify these factors, then having identified the molecule/s ways of eliminating there effects could be logically developed. These attempts were hampered by difficulties with cellular adhesion and whilst the effects of liver failure plasma on cellular adhesion were of interest (indeed the paper by Newsome *et al* subsequently demonstrated acute liver failure plasma causes loss of adhesion) they were not the aim of study of this project.

Having eliminated the problems of cellular adhesion it became apparent that whilst there was variability in the toxicity of plasma that the effects where not as large as originally hoped. The toxic effect was not therefore significant enough to use a biological assay to "trace" the toxicity through purification steps. In many ways this was rather unsurprising firstly because HepG2 cells are tumour cell lines and due to their dysregulated cell growth and death pathways they are extremely resilient, and secondly because any toxin that could be significantly toxic to tumour cell lines over a short time period would be likely to have an even more pronounced effect *in vivo* to the far more fragile primary human hepatocyte. Essentially if acute liver failure plasma contained a substance that could cause cell death in, for example, 25% of tumour cell lines in 16 hours and

we assumed equal toxicity in primary hepatocytes there would be less than 10% of Hepatocytes remaining in the liver within 5 days.

Furthermore the processes involved in separating the soup that is plasma would almost certainly dilute and denature any toxin, finally the toxin is almost certainly there in minute quantities and large volumes of liver failure plasma would be required for purification, isolation and subsequent identification. A better system for the study of toxins in liver failure plasma was therefore required.

Using primary human hepatocytes on collagen coated slides a robust, reliable, quantifiable in-vitro system that could be easily manipulated was developed. This system can be used to test toxic molecules in liver failure plasma using a candidate molecule approach and can be used to assess factors that may be protective against apoptosis. Using this system we determined that Epidermal Growth Factor and not Hepatocyte Growth Factor protected cells against Fas-Ligand binding induced apoptosis. This system can also be used to assess strategies designed to reduce toxins in liver failure plasma. This has the advantage of not requiring the identity of the putative toxins to be known, and being able to assess the effect of multiple substances in combination. Whilst not perfect, the model was sensitive and looked at a specific biological pathway. The use of human hepatocytes eliminated any cross-species differences, important since it is known that apoptotic responses are species dependent with rat hepatocytes relatively insensitive to Fas-ligand induced apoptosis and cultured human hepatocytes being exquisitely sensitive. It also eliminated any changes in behaviour due to tumour cell line origins. The model was however

not without its drawbacks, firstly there is a very limited supply of hepatocytes and secondly the harvesting of cells may activate cell survival pathways which may alter the cells response. An example of this is demonstrated by the fact that mouse hepatocytes in culture do not undergo apoptosis when exposed to Fas-ligand, whereas massive liver injury occurs in-vivo and this is thought to be due to activation of survival pathways during harvest and culture. This effect can be overcome by blocking protein synthesis with Cyclohexamide, thus preventing synthesis of the gene products of these survival genes. Similar mechanisms may be activated by harvest of primary human hepatocytes. Interestingly even death receptor activation itself can activate cell survival pathways. Activation of the death receptor TNFR1 results in proteins (TRAF2 and RIP) activating Iκβ kinase (IKK) and Iκβ degradation, leading to activation and nuclear translocation of NFκβ . NFκβ induces expression of survival genes including $Bcl_{XL,}$ A1, XAIP, cFLIP and iNOS. Stimulation of these pathways inhibits apoptosis. This adds another level of complexity with activation of death receptors being able to kill or protect cells the response being unpredictable by any currently clearly defined factors.

Hepatocyte apoptosis is increased in chronic but particularly in acute liver disease and using our system we demonstrated that plasma taken from patients with acute liver failure induced apoptosis, whereas plasma from patients with acutely decompensated liver failure did not. This is despite similar increases in markers of liver insufficiency based on for example similar bilirubin. This supports the argument that acute liver failure is not predominantly due to insufficient metabolic mass causing accumulation of toxins which then further damage the liver, as similar effects would be seen in acutely decompensated

chronic liver disease if this were the case. It suggests that specific mechanisms, almost certainly involving inflammatory cytokines and possibly death receptors are at work. The clear differentiation between acute and chronic liver disease having very different pathophysiology is very important particularly when looking at the mechanisms of removal/antagonism of factors inducing apoptosis because if there is no demonstrable toxicity induced by chronic liver failure plasma then there is no point in further of this patient group.

There was also no correlation between apoptosis induced by the patients' plasma and any single parameter of liver or renal function, nor with aetiology nor prognosis. One simple hypothesis, was that if apoptosis was induced by toxins accumulating in plasma due to failure of liver excretion, markers of excretion/cholestasis (bilirubin, ALP, GGT) would correlate with apoptosis inducing capacity of liver failure plasma. If factors in plasma were inducing apoptosis, leading to secondary necrosis other markers of hepatocellular damage, e.g. AST, ALT, might positively correlate with the apoptosis- inducing ability of plasma. No such correlation could be demonstrated. If albumin binds hepatotoxins, a negative correlation between albumin concentration and apoptosis inducing ability of plasma might be expected, but no significant trend emerged.

The next stage was to use this in-vitro system to test strategies that may reduce toxicity of liver failure plasma e.g. MARS to assess if such treatments are liable to improve the chances of endogenous liver recovery in addition to providing temporary replacement of some liver functions. MARS therapy has been shown to improve outcome in Alcoholic Hepatitis and hepato-renal syndrome, it has not been shown to improve survival in acute hepatic failure. We had proposed

that the putative toxic molecules were removed by MARS therapy and this may reduce the pro-apoptotic effects of plasma. No such effect was identified despite other markers demonstrating significant reduction in albumin bound molecules. This could be because the toxic molecules are not significantly removed by MARS treatment, which is why no clinical benefit in terms of liver recovery is seen with MARS therapy. Since the majority, 9 of the 11, of the patients undergoing MARS therapy had acutely decompensated chronic liver disease, and we had previously failed to demonstrate increased plasma toxicity in this group, there was no pro-apoptotic tendency to reverse in the majority of patients treated. However it is exactly this patient group that have shown clinical benefit in terms of reduced mortality compared to acute liver failure patients who have not shown mortality benefit with MARS therapy. Further study on patients with acute liver failure plasma pre and post MARS therapy is required. A plausible explanation would be that both the in-vivo studies in acute liver failure and our in-vitro study pre-and post-MARS therapy were underpowered in terms of patient numbers to demonstrate a response. This is likely to always be the case as the ideal trial where patients were randomised to MARS therapy versus placebo (+/-renal support) with a primary outcome of mortality would be unethical, patients would almost certainly be near to reaching transplant criteria at enrolment and those who deteriorated would be transplanted as soon as an explants became available. If transplant was used as an endpoint then the results would be skewed by the availability of a graft and so other markers of outcome would be required, encephalopathy cannot be assessed in a patient once intubated, clotting would have to be corrected for line insertion and so could not be used and other biochemical parameters are affected by the MARS therapy

itself. Finally since MARS dialysis improved the clinical status in the patients in this series, and the expected improvement in biochemical parameters but left the degree of apoptosis induced by plasma entirely unchanged another possibility is that the removal of pro-inflammatory molecules by the dialysis process is matched, and therefore the beneficial effect cancelled, by simultaneous clearance of anti-apoptotic molecules. HGF for example, protective against apoptosis, and elevated in the plasma of patients with liver failure, is one candidate for such a substance.

A more rational approach would be to use our in-vitro system to test for toxicity in mechanical treatments which have been shown to improve outcome in acute liver failure. The ultimate plasma detoxifying treatment in acute liver failure is plasmapheresis, and this treatment has been shown to reduce mortality in fulminant hepatic failure, initial experiments using plasma samples pre and post plasmapheresis would therefore seem to be a good starting point for any further study investigating the toxic effects of acute liver failure plasma using our *in-vitro* model.

Since there appear to be substances in liver failure plasma which can induce apoptosis and the only detoxifying therapy available to us did not appear to remove this effect then alternative ways of antagonising the effect were investigated. We found that treatment of hepatocytes with either a Caspase 8 or a Caspase 3 inhibitor protected against the apoptosis induced by acute liver failure plasma *in-vitro*. This suggests that the apoptotic effect is dependent on pathways involving both these enzymes, which would suggest involvement of death receptor pathways. To study this in-vivo we used Caspase inhibitors in an animal model of acute liver failure.

Caspase inhibitors have been proposed in the treatment of a number of different diseases. Since caspase inhibition may prevent deletion of auto-reacting clones of lymphocytes, and other abnormal cells, their use in chronic disease processes is likely to be limited, due to the risk of both of generating auto-immune disease and malignancy. Acute insults such as stroke or acute liver failure may be diseases where preventing apoptosis would be of value. Animal studies in models of acute liver failure by induction of apoptosis using known death receptor pathways, for example the agonistic jo-1 antibody which trimerises and activates death receptors, have been shown to be attenuated by treatment with caspase inhibition.

We chose a toxic liver injury model of acute liver failure because this is a model not previously explored with inhibition by caspase inhibitors, and since paracetamol toxicity remains the commonest cause of acute liver failure, would be a disease in which there is the greatest utility for a therapeutic option. Finally this model of toxic liver injury was developed and licensed for use in our laboratory.

We initially needed to investigate if apoptosis was implicated in induction of liver injury by Thioacetamide. Apoptosis was demonstrated by showing increased TUNEL staining, and the time course of this histological event was documented. Treatment with caspase inhibitors, including caspase 8 and caspase 3 inhibition, which we had previously shown reduced apoptosis by plasma in primary human hepatocytes in our in-vitro model were used in the animal model. The caspase inhibitors did not reduce mortality in this animal model. This effect was at least in-part due to the lower than expected mortality in the control group of animals. Further animal studies of caspase inhibitors are

required using a larger number of animals and an animal model with a reproducible mortality.

Caspase inhibition may be a fruitful area in the treatment of acute liver failure although there are two potential barriers over this approach being of clinical benefit, firstly survival of cells is not the be all and end all of such interventions the cells must be functionally viable and lack of functionality in "saved" cells will not help, as demonstrated by the use if an apoptosis inhibitor in a model of Parkinson's disease, benzyloxycarbonyl-Val-Ala-Asp-(O-methyl)fluoromethylketone (zVAD-fmk), which protected dopaminergic neurons in-vitro but did not prevent loss of neuritis and decrease in dopamine uptake (von Coelln *et al.*, 2001). Also the beneficial effects of Caspase inhibitors in the acute setting as Caspases are necessary for proliferation of some cells, including t-cells.(Kennedy *et al.*, 1999), and increased apoptosis and Caspase-3-like protease activity is seen in regeneration after partial hepatectomy with bursts of apoptosis correlated with DNA synthesis taking place when a large body a cells are in S-phase, this may be mediated by cytokines for example TGF-β and may be important in terminating proliferation (Hayami *et al.*, 2000). Thus interference with apoptosis may also effect liver regeneration.

The preliminary work in this study developed and validates an in-vitro model for apoptosis assays relevant to human hepatocytes. It shows toxic molecules is liver acute liver failure plasma but not chronic liver failure plasma, highlighting the differences in pathophysiology of the two disease processes. The inhibition of this pro-apoptotic effect by direct inhibition of Caspase 3 and of Caspase 8 point the way to alternative novel therapeutic approaches in the treatment of acute liver failure. Non-toxic caspase inhibitors are now emerging in

experimental studies and in clinical trials. The failure of benefit in removal of toxic molecules by treatments such as MARS must question the validity of these treatments and time will prove whether removal strategies such as this will improve outcome in acute liver failure.

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APPENDIX

MATERIALS AND METHODS

Materials

Reagents were from Sigma Chemicals or BDH, and of biochemical or analytic grade. Reagents from other sources are noted.

Cell isolation and culture reagents including cell culture media, Fetal Calf Serum (FCS), penicillin-streptomycin, L-glutamine, Dexamethasone, Insulin, EGTA tetrasodium salt, Bovine Serum Albumin (BSA), Collagen, Collagenase type IV and DNAseI were purchased from Sigma UK . PBS, HBSS (containing Calcium and Magnesium) and HEPES were purchased from Invitrogen. Trypsin/EDTA solutions were from Gibco BRL (Life Sciences, Paisley, Scotland)

Consumables – Tissue culture plates, dishes and flasks were all obtained from Nunc (Nunclon, Nunc A/S, Roskilde Denmark) supplied by Gibco. Membrane filters (0.2µM) were obtained from Sartorius, Goettingen, Germany.

Batches of foetal calf serum (FCS) were tested for relative hepato-toxicity and batches held on reserve at Gibco. FCS was prepared before use by heating to 56ºC for 30 minutes to inactivate complement and aliquots stored at -20ºC until use

Generalised Cell Culture - HepG2 cells

HepG2 cells were kept in continuous culture in alpha modified Eagles minimal essential medium (α-MEM) with nucleosides, supplemented with 10% v/v FCS with the following additives; L-Glutamine 2mM, Penicillin (200U/ml) and Streptomycin (200IU/ml), Fungizone 2.5ug/ml, Insulin 0.01ug/ml,

Dexamethasone 10^{-8}M, Selenic Acid 10^{-7}M, Linoleic Acid/BSA (0.5ug/ml,0,05mg/ml), Thyrotropin releasing hormone 10^{-6}M

This is hereafter referred to as Complete α-MEM medium. Medium was refreshed every 48 hours.

Passaging cell cultures

Cell cultures were kept in continuous culture and regularly passaged when required (70-90% confluence). Cells were incubated at 37ºC in a humidified atmosphere of air and 5%CO_2

Medium was removed and cell monolayers washed twice with warm HBSS without calcium and magnesium to remove dead cells and residual protein. Cells were detached by incubating with trypsin/EDTA solution (filter sterilised) for 3-4minutes. The cells were dislodged by gently tapping the flasks and transferred into fresh FCS containing medium to quench trypsin activity. Cells were centrifuged (200g for 5 minutes), and resuspended in medium. Clumps were dispersed by gentle aspiration through a 21G needle.

Viability was assessed by trypan blue exclusion, cells were counted and seeded at densities as required in complete α-MEM medium.

HepG2 cells were plated at 15000/well in 96-well slides and 33000/well in 8-chamber slides in complete alpha-MEM. Cells were allowed to attach for 12 hours in 5%CO_2 at 37ºC before washing with HBSS to remove unattached cells and cellular debris before exposure to test substance, for example plasma.

Trypan Blue Assay - Estimating cell viability

Trypan Blue 2% solution in PBS was stirred overnight then centrifuged (14,000rpm for 30 minutes in a micro-centrifuge). Supernatant removed, filtered (0.2uM filter, ministart, Sartorius) and stored as aliquots at -20ºC.

A 20uL aliquot of cells suspended in serum free medium was mixed with 160uL of HBSS and 20uL of trypan blue solution and then mixed gently by flicking. After 3 minutes the cells were assessed using a haemocytometer chamber. The intact membrane of viable cells excluding trypan blue and remaining uncoloured whereas dead cells appear blue because dye leaks through their damaged membrane. From this an estimate of yield and cell viability could be obtained. HepG2 cells with viability >95% were used for experimental purposes.

Primary human hepatocytes

Isolation of primary human hepatocytes

Liver tissue removed as part of the resection specimens was used as the source of hepatocytes. Tissue donors: patients undergoing clinically indicated hepatic resections for the treatment of secondary colonic tumours; gave informed consent for experimental use of tissue. Primary human hepatocytes were prepared by Dr Clare Selden by a modification of the Seglen method (Seglen, 1971) using collagenase perfusion of segments of resected normal human liver.

Wedges of human liver taken from partial hepatectomy specimens were placed on a sterile dish in a pool of phosphate buffered saline (PBS) on ice. Using a catheter sheath, 3 suitable branches of the portal vein were identified and cannulated. Veins were flushed with ice cold PBS to remove blood and then

perfused for 30 minutes with chelation buffer (PBS, HEPES buffer 20mM, EGTA 0.5mM) at 37C followed by 30 minutes with perfusion buffer (PBS, HEPES buffer 20mM), followed by 30 minutes with digestion buffer (HBSS, 0.5% BSA, Ascorbic Acid 50ug/ml, Insulin 4ug/ml, Collagenase Type IV 0.05% w/v, DNAseI 0.01% w/v)(Seglen, 1972). The cells were released by cutting into the digested areas and gently agitating in ice-cold dispersal buffer (Williams E medium, FCS 10%v/v, DNAseI 0.01%w/v). The resultant slurry was then passed successively through 200μM and 100μM nylon filters and centrifuged at 32g for 6 minutes at 4 ^{o}C. Cell pellets were pooled, resuspended in dispersal buffer and, centrifuged as before. Finally cell pellets were washed with ice-cold Williams E medium centrifuged as before and resuspended in complete Williams E medium. Cell viability was assessed using trypan blue and only preparations with viability >80% were used.

Culture of primary human hepatocytes

Primary human hepatocytes were cultured in Williams E medium containing the following additives; FCS 10% v/v, L-glutamine 2mM, Penicillin/Streptomycin 100U/100Ug/ml, Fungizone 2.5ug/ml, Insulin 0.01ug/ml, Dexamethasone 10^{-8}M. Hereafter known as complete Williams E medium. Primary human hepatocytes were plated on collagen coated receptacles at 50000/well in 96-well plates and 250000/well in 8-chamber glass slides. Cells were allowed to attach for 12 hours in 5%CO_2 at 37ºC before washing with HBSS to remove unattached cells and cellular debris and replacing with fresh medium prior to experimental use.

Preparation of rat-tail collagen

Primary human hepatocytes require a suitable matrix on which to attach and survive in culture. An extract rich in Collagen type 1 was prepared from rat tails, by sequentially fracturing the tail and removing the inner central tendons. The tendons were dissolved in 0.01M acetic acid at 1g of tendon per 300ml. The solution was stirred for 2-3days at 4ºC and centrifuged at 800g for 2hours. Aliquots were stored at 4ºC.

Collagen Coating plates

Tissue culture plates/slides were coated with rat-tail collagen solution (0.2ml/well for 96 well micro-titre plates) and incubated for ten minutes at room temperature in a laminar flow hood. Excess collagen was "flicked off" and the plate washed twice with 1 volume of HBSS and filled with 0.9%saline. Plates were UV-irradiated with short wave UV (UVG-L Mineralight lamp, Gabriel Ca, USA) for 15 minutes at a distance of 7cm and stored for a maximum of 24hours at 4ºC.

MTT Assay

The MTT assay utilises the conversion of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma) to the purple formazan product which is quantified by optical density at 550nM.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma) in PBS (0.75mg/ml) aliquots were stored at -20ºC and protected from light. A working stock was stable for 1 week stored at 4ºC. Acidified isopropanol equates to 0.04M HCl in isopropanol.

Medium was removed from cells in 96 well plate and 100uL of MTT solution 0.75mg/ml PBS) was added per well and incubated at 37ºC for 3 hours. After incubation excess MTT was removed and 100uL of acidified iso-propanol was added to dissolve the formazan product. Cells were wrapped in aluminium foil to prevent evaporation and photo-degradation, and shaken on an orbital shaker (Labline Instrument Inc, III USA). After 30 minutes the optical density was read at 550nm in an automated ELISA plate reader (Anthos htIII, Anthos Labtec Instruments, Salzberg, Austria).

Preparation and storage of plasma samples

Large volumes of liver failure plasma (LFP 1-4) were kindly donated by Fin Larsen from the Department of Hepatology, University of Copenhagen, Denmark . This plasma had been collected from patients with acute liver failure and grade IV encephalopathy at the onset of therapeutic plasmapheresis as part of a clinical trial with consent.

Heparinised plasma patients acute liver failure due to various aetiologies (Table 1) and 4 normal controls was acquired with informed consent. Samples from patients fulfilling criteria for MARS treatment was collected before and after each treatment.

Briefly blood was collected through a 16 gauge needle into a heparinised blood tubes. The plasma was rapidly separated by centrifugation at 2500g for 20minutes at 4^{o}C. Plasma was gently aspirated from cellular debris and stored at -20ºC until further use.

Routine biochemical and haematological analysis was sent to the diagnostics laboratory.

dialysis and after the second dialysis, rapidly separated and kept at – 20ºC until used in the apoptosis assay as described above.

Measurement of protein concentration (The Bradford Method (Bradford, 1976))

Coomassie dye binds protein in an acidic medium, an immediate absorbance shift occurs from 465nm to 595nm with a simultaneous colour change from green/brown to blue. This reagent gives a characteristic linear response curve within a given range (100-1,500µg/ml). Coomassie Plus-200 Protein assay Reagent (Pierce, Rockford, USA); containing the Coomassie G-250 dye, methanol, phosphoric acid and solubilising agents in water was stored at 4ºC until use.

Diluted Bovine Serum Albumin (BSA) standards were prepared by diluting the 2.0mg/ml BSA standard to the following concentrations 0µg/ml, 1µg/ml, 2µg/ml, 5µg/ml, 10µg/ml, 15µg/ml, 20µg/ml. Having allowed the Coomassie plus reagent to come to room temperature, the reagent was mixed by gently inverting the bottle several times. 150µL of each standard or unknown sample was placed in wells of a 96 well plate in triplicate. Three samples of the diluent alone were also used as blanks. 150uL of the Coomassie plus reagent was added to each well, and the plate mixed well on a plate shaker for 30 seconds. Absorbance was measured at 595nm on a plate reader. The average 595nm reading for the blanks was subtracted from each reading or standard. A standard curve was prepared by plotting the average reading for each of the standards minus the blank. Readings from unknown samples were then converted to protein concentrations using the standard dilution curve.

Silver Staining of protein Gels

Gels were carefully removed from casting apparatus, wearing gloves to avoid protein contamination, and soaked in 300ml of fixer (10% ethanol: 5% glacial acetic acid) for 40 minutes, fixer being refreshed every 10 minutes. Fixer was removed by aspiration and replaced with 50% Methanol and washed for 1 hour, refreshing the solution every 20 minutes. The gel was then washed three times each for 10 minutes in distilled water.

As the last wash was finished the stain solution was made up by adding solution A (0.8g of silver nitrate dissolved in 4 mls of distilled water) to solution B (21 ml of 0.36% NaOH (0.18 g in 50 ml water), added to 1.4 ml of 14.8M Ammonium hydroxide) drop wise with constant shaking. The solution was added slowly to avoid precipitation of silver salts and then made up to 100ml with distilled water.

The gel was soaked in stain solution for 10 minutes and then rinsed twice with distilled water to remove stain solution. Developer (2.5 ml of 1% citric acid (0.2g per 20 ml) and 0.25 ml of 38% formaldehyde (stock usually 38-40%). Made up to 500 ml with distilled water) was added to the gel and gently shaken over a light box. Bands were seen to appear within minutes. Once a suitable band intensity was reached, the developer was quickly removed and stopper (45% methanol: 10% acetic acid) was added and left to soak for one hour. Gels were stored complete in polythene containing water to prevent dehydration.

Caspase3 Staining of cells with Caspatag™

The CaspaTag™ Caspase Activity Kit (Serologicals Corporation USA) detects active caspases in living cells through the use of a carboxyfluorescein labelled caspase inhibitor. The inhibitor irreversibly binds to active caspases, they are cell permeable and non-cytotoxic Caspase, positive cells (+) are distinguished by fluorescence microscopy. FAM-DEVD-FMK is a carboxyfluorescein analogue of benzyloxy-carbonylaspartyl-glutamylvalylaspartic acid fluoromethyl ketone (zDEVD-FMK), potent inhibitor of caspase-3 and caspase-3 like caspases. FAM-DEVD-FMK enters the cell and irreversibly binds to activated caspase-3 > caspase-8 >caspase-7 > caspase-10 > caspase-6 in the order of decreasing binding affinity.

The kit was stored at 4ºC until first use. Lyophilized Peptide-FMK was reconstituted with 50 µL DMSO resulting in a150X concentration and the contents mixed at room temperature until completely dissolved. Aliquots were made and stored frozen at -20ºC until ready to use protected from light and moisture to avoid multiple freeze-thaw cycles. Prior to use 30X Working Dilutions of FAM labelled inhibitors in were made by diluting in PBS. FAM labelled inhibitors were protected from light at all times.

10X Wash Buffer wash incubate at 37ºC for 30 minutes to redissolve precipitated protein and buffer salts. The wash buffer was diluted by adding 10 mL 10X Wash Buffer to 90 mL deionized H_2O and mixed thoroughly.

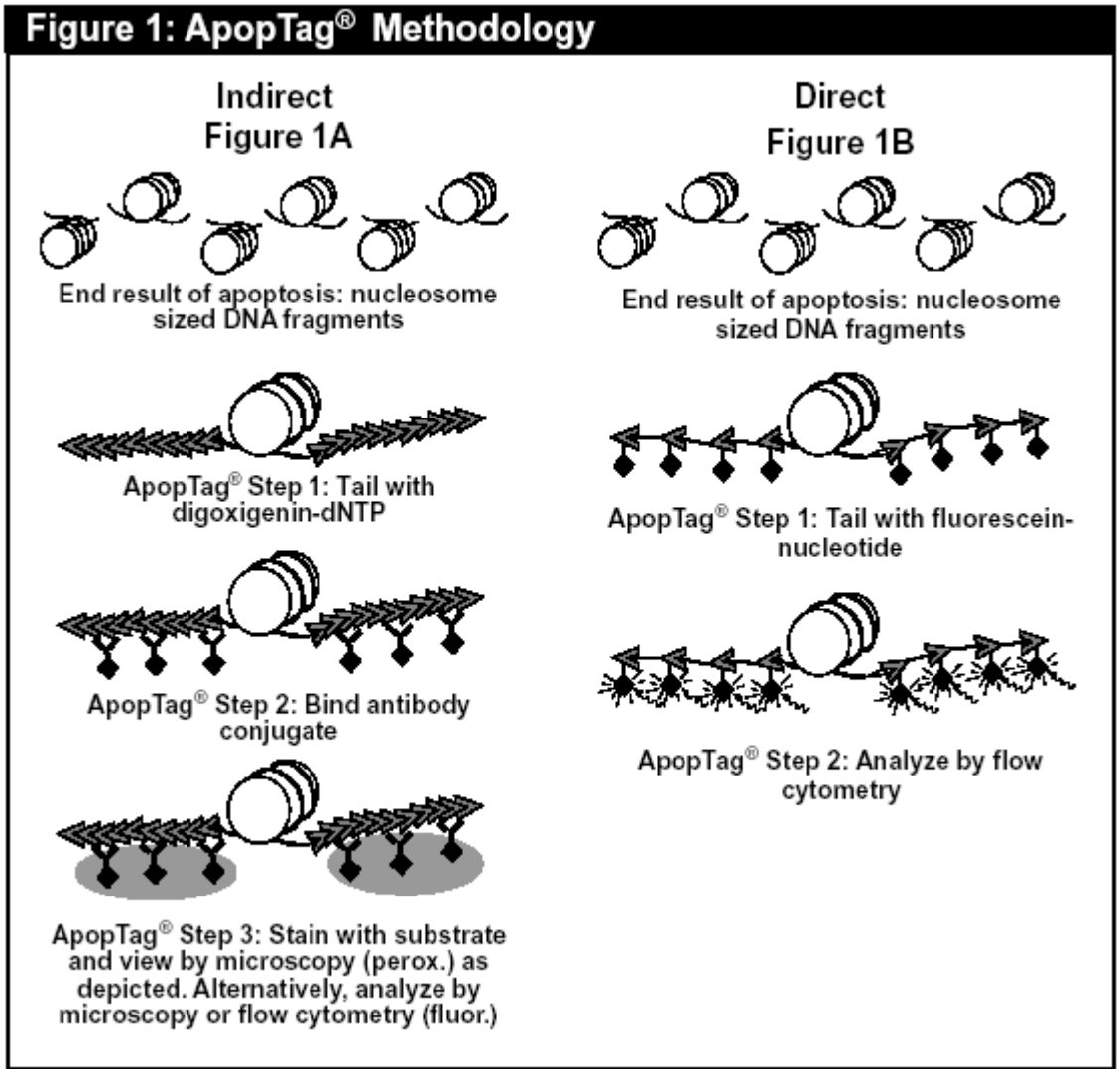
Cells having previously been seeded onto sterile collagen coated 8-chamber slides were exposed to plasma or test solution for 4 hours. Test solution was gently removed and slides washed three times with complete Williams E medium to remove any remaining plasma or debris. 10 µL of 30X Working

Dilution FAM-Peptide-FMK was added to 290 µL medium and mixed, 300uL was added to each chamber of the eight chamber slide and incubated for 1 hour at 37ºC under 5% CO2, protected from light. Nuclei were labelled by the addition of 1.5 µL Hoechst Stain per 300 µl medium and incubated for a further 5 minutes. The medium was removed and cells and washed cells twice with 0.5 mL 1X Working Dilution Wash Buffer. The plastic frame of the chamber slide was removed and cells fixed by placing slides in a 1% paraformaldehyde solution in PBS) and incubated for 15 minutes at room temperature. Slides were then washed twice with PBS followed by mounting onto a microscope slide in anti-fade mounting medium.

Cells were observed under a fluorescence microscope using a bandpass filter (excitation 490 nm, emission 520 nm) to view green fluorescence of caspase positive cells. For viewing Hoechst Stain, a UV filter (excitation 365 nm, emission 480 nm) was used.

TUNEL staining of cells

TUNEL staining was performed using ApopTag® Red In Situ Apoptosis Detection Kit (Serologicals Corporation USA) using the indirect method (figure 1. courtesy of Serologicals Corporation USA)



The kit was stored at -20ºC until first use, thereafter the TdT Enzyme was aliquoted into suitable sized aliquots and stored at -20ºC to avoid multiple freeze thaw cycles. the remaining components were stored at 4ºC. Fluorescent reagents were protected from unnecessary exposure to light throughout.

Eight-chamber slides having been exposed to test substances were washed three times in PBS and the chambers removed. Cells were fixed in 1% Paraformaldeyde in PBS pH7.4 in a Coplin jar for 10 minutes at room temperature. Excess liquid was drained off and slides washed in 2 washes of PBS each for 5 minutes

Cells were permeablised by post-fixing in pre-cooled ethanol:acetic acid 2:1 for 5 minutes at -20ºC in a Coplin jar, which was then drained and cells washed in 2 changes of PBS for 5 minutes, each wash.

Excess liquid was removed and the edge of the slide carefully blotted. 75ul of equilibration buffer was applied directly to the specimen and incubated for 10 seconds at room temperature, excess buffer was removed and 55uL of working strength TdT enzyme (66uL TdT enzyme + 154ul of reaction buffer, mixed by vortexing, kept on ice) was applied and the slides incubated in a humidified chamber at 37C for 1hour. The reaction was quenched by placing the specimen in a Coplin jar containing working strength stop/wash buffer (1ml stock stop/wash buffer +34ml ddH2O), agitated for 15 seconds, and incubated for 10 minutes at room temperature. An aliquot of antidigoxigenin conjugate sufficient to process the desired number of slides was removed from the stock vial and warmed to room temperature avoiding exposure to light. Working strength Rhodamine antidigoxigenin conjugate was prepared by adding 68uL of blocking solution to 62uL of Ant-digoxigenin Conjugate and vortexing, the solution was stored on ice and protected from light. Excess liquid was removed from slides and 65uL of conjugate which had been allowed to warm to room temperature was added to each slide. Slides were incubated in a humidified chamber for 30minutes at room temperature in the dark. Specimens were then washed in 3

changes of PBS in a Coplin jar for 2 minutes per wash. Nuclei were counterstained by the application of 1.5uL of Hoesch stain in 65uL of PBS and incubated at room temperature for 5 minutes in a humidified chamber. Finally slides were washed by 2 further washes in PBS before being mounted in anti-fade mountant. Slide edges were sealed with rubber cement and slides stored at -20C protected from light. Slides were viewed with a fluorescence microscopy with a filter of wavelength suitable for viewing of Rhodamine (red) fluorescence.

TUNEL staining tissue samples

Liver tissue harvested from experimental animals was snap frozen in liquid nitrogen at -80C. Liver tissue was fixed in formalin overnight and later placed in Paraffin blocks. Paraffin embedded tissue sections were cut by Miss Sherri-Anne Chalmers, placed on APES coated slides and allowed to dry

Tissue was deparafinise tissue by washing in 3 changes of xylene for 5 mins each wash, followed by 2 changes of absolute ethanol for 5mins each wash. Followed by rehydating by washing in 95% ethanol for 3 minutes; followed by washing in 70% ethanol for 3 mins; followed by washing in PBS for 5 mins.

Proteinase k solution used to pre-treat the tissue was reconstituted according to manufacturers (R&D) instructions by mixing 1uL Proteinase k solution in 50uL dH20. The solution was place on ice until use. 50uL of Proteinase k solution was added onto each sample, covered with a coverslip and incubated at room temp for 20 minutes. Slides were washed twice 2 minutes per wash in water.

A positive control was made by adding TACS nuclease 1uL in 50uL TACS nuclease buffer to liver tissue and incubating at 37C for 30 mins. The reaction was stopped reaction by washing in PBS.

Excess liquid was removed by gently tapping off blotting around specimens. 75uL of equilibration buffer was applied directly onto specimens and incubated for 10 secs at room temp. Working strength TdT enzyme was made up by mixing 33uL TdT enzyme with 77uL of reaction buffer and stored on ice . 55uL of working strength TdT enzyme was added to each specimen and incubated at 37C for 2 hours in protected from light.

Stop/wash buffer was made up by adding 1ml of stop wash buffer to 34ml dH20 and slides were placed in stop wash buffer, agitated for 15 secs and then incubated for 10 mins room temp. Slides were then treated with Antidigoxigenin conjugate and counterstained with Hoescht stain and mounted as described in the above method.

Primary human hepatocytes or HepG2 cells were plated onto collagen coated glass 8- chamber slides or collagen coated white sided, optical bottomed 96 well, tissue culture plates.

Caspase 3 activation measurement in live cells (Caspse-Glo^{tm} 3/7 Assay)

Apoptosis inducible by liver failure plasma was confirmed by the measurement of Caspase 3 activation. Caspase-Glo^{tm} is a homogeneous luminescent assay that measures Caspase 3 activity by providing a proluminescent Caspase 3 substrate which contains the tetrapeptide sequence DEVD in a substrate which lysis cells followed by Caspase cleavage of the substrate and generation of a "glow type" luminescent signal.

Hepatocytes were seeded on collagen coated sterile 96 well plates and having been allowed to attach for 12 hours and debris washed off as described earlier, were exposed to plasma for 4 hours (100uL/well) at 37ºC in a humidified incubator. Identical plates, with no cells added, were prepared for the addition of plasma samples for use as blanks.

Caspase-Glo buffer and lyophilized substrate which had been stored at-20C protected from light was allowed to warm to room temperature. The contents of the buffer bottle was poured into the amber bottle containing the Caspase-Glo substrate and mixed by swirling to thoroughly dissolve the reagent. An equal volume of mixed reagent was added to medium in a 1:1 ratio (100uL added to each well of a 96 well plate) and mixed using a plate shaker at 300-500rpm for 30 seconds. Plates were then incubated for 2 hours, having determined this as the optimal exposure time. 200uL was aspirated from each well and placed in white-sided clear bottomed 96 well luminometer plates. Luminescence was recorded and the luminescence of the appropriate blank subtracted. Each sample was prepared in triplicate.

Caspase inhibitors – Caspase inhibitors were purchased from R&D Systems (Minneapolis, Mn, USA) solubilised in DMSO and a 100x final concentration prepared in Williams E medium. Similarly prepared no-inhibitor dilutions were used to eliminate possible DMSO effects. Caspase inhibitor or control preparations were then diluted 1:100 with plasma, mixed thoroughly and applied to cell layers for 4 hours before TUNEL analysis as before. Caspase 3 inhibitor Z-VAD-FMK, Caspase 8 inhibitor Z-IETD-FMK, and Caspase 9 inhibitor Z-LEHD-FMK were all used at final concentrations 20 μMol. Each experiment was repeated on at least three separate occasions each time using hepatocytes prepared from a different liver resection. Results shown are representative of all experiments.

Caspase inhibitors for animal studies was supplied *gratis* by Vertex Pharmaceuticals, Didcot.

Royal Free Hampstead
Research Ethics Committee

NHS

Please reply to:

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20 November 2002

Professor Humphrey Hodgson
Centre of Hepatology (U3)
Royal Free Hampstead NHS Trust
Pond Street
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London
NW3 2QG

08 APR 2003

Dear Professor Hodgson

Studies on the presence of toxic molecules in the blood in liver failure, and techniques for their removal

Ethics Reference: LREC 5787 ***(Please quote on ALL correspondence)***

Further to your letter dated 15.11.2002 enclosing a revised application form, a revised patient information sheet and consent form to the above protocol, I am pleased to inform you that this was approved by Chairmans Action.

Meanwhile you are free to continue with your project.

Yours sincerely

Rosemary Brown
Ethics Committee Secretary
Royal Free Local Research Ethics Committee

cc: Dr A Davenport
cc: Dr R Saich

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INFORMATION SHEET & CONSENT FORM

Re: Clinical research project – Study on the presence of toxic molecules in the blood in liver failure, and techniques for their removal

You are being invited to take part in a research study. 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 and discuss it with friends' relatives or your GP 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. You will be given a copy of this information sheet and a copy of your signed consent form.

Consumers for Ethics in Research (CERES) publish a leaflet entitled 'Medical Research and you'. This leaflet gives more information about medical research and looks at some questions you may want to ask. A copy may be obtained from CERES, PO Box 1365, London N16 0BW.

Thank you for reading this.

What is the purpose of the study?
One of the functions of the livers is to remove toxins from the blood. If the liver fails to work these toxins build up in the body and can be harmful to other parts of the body particularly the brain and the kidneys.
There is a new machine called MARS (Molecular Absorbent Recirculation system) which has been designed to remove toxins from the blood. The doctors looking after you feel it would be beneficial to use this machine on you. The aim of this study is to see if, when the machine is used to removing the toxins from your blood of patients, your kidneys will work better.

What are the risks of taking part?
Occasionally being on the MARS machine may increase the risk of bleeding. .

What are the benefits of taking part?
The information we get from this study may help us to treat future patients with liver disease.

What if new information becomes available?
Sometimes during the course of a research project, new information becomes available about the treatment being studied. If this happens, your research doctor will tell you about it and discuss with you whether you wish to continue with the study. If you decide to withdraw your research doctor will make arrangements for your care to continue. If you decide to continue you will be asked to sign an updated consent form. Also on receiving the new information your study doctor might consider it to be in your best interests to withdraw you from the study. He/she will explain the reasons and arrange for your care to continue.

What happens when the research study stops?
The treatment will still be available when the trial stops.

What if something goes wrong?
The machine is only used by specially trained nurses in intensive care, high dependency units or in the renal dialysis unit, and if anything goes wrong they will immediately attend to the problem. Doctors are also continually available should you need help.
If taking part in this project harms you, there are no special compensation arrangements. If you are harmed due to someone's negligence, then you have grounds for a legal action but you may have to pay for it. Regardless of this, if you wish to complain about any aspect of the way you have been approached or treated during the course of this study the normal National Health Service complaint mechanisms may be available to you.

Will my taking part in the study be confidential?
All information, which is collected, about you during the course of the research will be kept strictly confidential. Any information which leaves the hospital will have your name and address removed so that you cannot be recognised from it.

What will happen to the results of the study?
The results will be published at the end of the study estimated to be in 18 months. A letter will be sent to you explaining the results.

Who is organising and funding the study?
The study is being organised by the Department of Medicine at the Royal Free Hospital. The doctors and nurses involved are paid by the NHS or the University of London. The equipment has been donated by the manufacture (Teraklin UK). Some of this research is funded by grants and awards made to the liver group from charities and trusts. The doctors are impartial and are not paid to include you in the study.

Who has reviewed the study?
The Royal Free Hospital and Medical School Local Research Ethics Committee have reviewed this study.

Contact for further information.
Prof. H.Hodgson,
Centre of Hepatology
Department of Medicine, Royal Free Hospital
Pond Street
London

Date *22/07/2003*

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Consent Form

Title of project: Study on the presence of toxic molecules in the blood in liver failure, and techniques for their removal

Name of Researcher: Prof. H.J.F.Hodgson

Please initial each box.

1. I confirm that I have read and understand the information sheet dated ..... for the above study and have had the opportunity to ask questions. ☐

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. ☐

3. I understand that sections of any of my medical notes may be looked at by individuals from Teraklin 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. ☐

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

Name of patient	Date	Signature
Name of person taking consent (If different from the researcher)	Date	Signature

Benign Recurrent Intrahepatic Cholestasis (BRIC) with secondary renal impairment treated with extracorporeal albumin dialysis (MARS).

Rebecca Saich, Peter Collins, Aftab Ala, Richard Standish & Humphrey Hodgson

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Running Head

BRIC with renal impairment treated with MARS

Sources of support

Dr.Rebecca Saich holds a Dunhill Medical Trust Research fellowship

Abstract

Benign recurrent intrahepatic cholestasis is a rare autosomal recessive condition characterised by intermittent episodes of pruritis and jaundice that may last days to months. Treatment is often ineffective and symptoms, particularly pruritis, can be severe. Extracorporeal albumin dialysis (MARS) is a novel treatment which removes albumin bound toxins including bilirubin and bile salts. We describe a case of a 34-year-old male with BRIC and secondary renal impairment who having failed standard medical therapy was treated with MARS. The treatment immediately improved his symptoms, renal and liver function tests and appeared to terminate the episode of cholestasis.

We conclude that MARS is a safe and effective treatment for BRIC with associated renal impairment**.**

Key words

Benign recurrent intrahepatic cholestasis (BRIC)

Extracorporeal albumin dialysis (MARS)

Acute renal failure

Cholestasis

Introduction

Benign recurrent intrahepatic cholestasis (BRIC) or Summerskill and Walshe syndrome, is a rare autosomal recessive condition. It is characterised by recurrent episodes of acute onset cholestasis separated by periods of complete physical and biochemical normality (1;2) . The precipitants to the episodes of cholestasis are unknown, after a virus-like prodromal illness patients develop pruritis and jaundice, usually with pale stools and dark urine. The episodes vary in duration lasting from weeks to months.

The mainstay of pharmacological treatment is corticosteroids and cholestyramine (3), with equivocal efficacy (4), which does not improve with the addition of phenobarbitone (5). More recently ursodeoxycholic acid has been used to reduce itch and diarrhoea(6) and rifampicin has been shown to improve itch and biochemical parameters of cholestasis (7). In severe or protracted disease plasma exchange can be beneficial and it is suggested that its use early in the course of the illness may shorten the duration of an attack (8;9). Extracorporeal albumin dialysis (MARS) is a new method of removing albumin bound toxins. A special membrane allows transfer of water soluble and albumin bound toxins with molecular weight less than 50kDa from blood into a dialysate solution containing 20% human albumin (10). MARS provides potential advantages over plasmapheresis since it is less expensive, has less theoretical risk of transmission of infection, is less immunogenic and is more selective leaving possibly beneficial growth factors and hormones in the circulation (11) MARS has been previously used in a single case of BRIC and was shown to improve cholestasis, reduce bile salts, change bile salt composition, and improve symptoms of diarrhoea and itching (12). We describe a case of BRIC with an

episode of progressively worsening cholestasis and the onset of secondary renal insufficiency despite standard pharmacological therapy. We proposed that a short course of treatment with MARS (3 daily treatments) would remove albumin bound toxins, including bile salts, and would result in improvement in both renal and liver function terminating the episode of cholestasis.

Patient

A thirty-four year old man with BRIC was transferred from his local hospital after a 2-month episode of cholestasis with increasing bilirubin and deteriorating renal function despite treatment.

He had first presented aged 15 years with jaundice, itching, lethargy and pale stools and dark urine, which was initially diagnosed as "infective hepatitis". Subsequently in view of negative viral serology, copper studies, ferritin, alpha-1-antitrypsin and in the light of his family history and liver biopsy the diagnosis was revised as BRIC.

His sister had previously been diagnosed with BRIC after she presented with cholestasis at the age of 6 months and had suffered from frequent episodes of cholestasis thereafter. His parents who were non-consanguineous, and his other sibling were well. His mother had two cousins both of whom had died in childhood of liver disease, one at 11 weeks of age, cause of death recorded as "?Infective Hepatitis. Obstruction of the bile duct" and one at 5 years of age cause of death recorded as "1. Pulmonary Oedema. 2(a). Hepatic Cirrhosis. (b). Erythroblastosis Foetalis".

The patients presenting episode was treated with corticosteroids and cholestyramine and he made a rapid and complete recovery. A year later he had acute pancreatitis and was found on ultrasound examination to have gallstones,

there was no cholodocholithiasis or duct dilatation on ERCP and he underwent cholecystectomy. Whilst awaiting surgery he had a further attack of cholestasis which, unlike his initial episode was slow to resolve, taking 3 months, despite treatment with prednisolone and cholestyramine. He had further episodes aged 22 and 24 years both of which were of short duration and he remained well with no further episodes of cholestasis until he represented on this occasion.

Two months prior to admission to the Royal Free Hospital he had suffered several days of pruritis which resolved spontaneously. One month later he became jaundiced, with pruritis and lethargy. He was commenced on cholestyramine 4g t.d.s., prednisolone 30mg o.d. multivitamins and calcichew. He failed to improve and was admitted to his local hospital where ursodeoxycholic acid 500mg b.d. was added to his treatment. Unfortunately his symptoms and liver function continued to deteriorate. Associated with the very high levels of bilirubin and bile salts, and despite appropriate i.v. fluid therapy, he developed acute renal impairment with a urea of 8.2 mmol/l and a creatinine of 187μmol/l having had previously normal renal function.

After transfer to this hospital he underwent a liver biopsy, which showed extensive severe intracellular and canalicular cholestasis, with some hepatocyte ballooning. Occasional portal tracts contained a mild mixed inflammatory cell infiltrate, and a few foci of parenchymal inflammation were also seen. However there was no evidence of portal tract fibrosis or ductopenia. The appearance was identical to those of his original biopsy nineteen years prior and was in keeping with the diagnosis of BRIC (figure 1). His serological screen for HIV, CMV, Hepatitis B and C, autoantibodies and immunoglobulins was negative.

In view of his failure to respond to standard medical therapy, increasing symptoms and onset of renal impairment he was commenced on MARS treatment. He was treated for 6 hours a day (flow rate 200ml/min) on three consecutive days via a dual lumen haemodialysis catheter in the right femoral vein. The patient gave informed consent in accordance with local ethics committee approval.

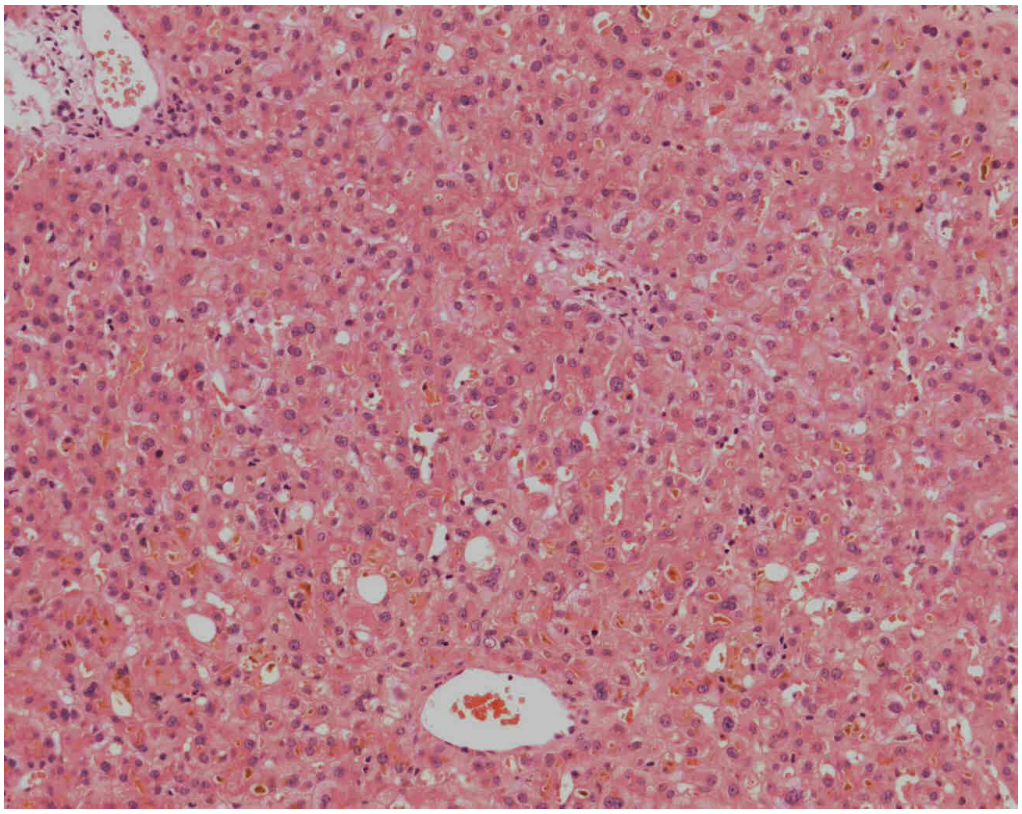

Figure 1. Photomicrograph of the current liver biopsy (x10 objective), showing extensive cholestasis. The portal tracts (top centre and left) show no evidence of fibrosis or ductopenia, and there is a minimal portal inflammatory cell infiltrate

Results

After treatment he was visibly less icteric, had less pruritis and lethargy and described himself as “feeling on top of the world”. His bile acids fell during each six hour treatment from a mean pre-treatment of 136.7μmol/L to a mean post-treatment of 75 μmol/L. His liver and renal function improved dramatically and he was discharged home 1 week later. His liver and renal function continued to recover and returned to normal 7 weeks later. He remains well with no further episodes of cholestasis.

Discussion

The exact mechanism of cholestasis in BRIC and many other cholestatic conditions is poorly understood. The gene responsible FIC1(ATP8B1) is a member of a family of P-type ATPases, and it is probably responsible for ATP dependent aminophospholipid transport. It is expressed in liver in the cholangiocyte and canalicular membrane (13;14), and is also expressed in the small intestine where it is likely to play a role in enterohepatic circulation of bile acids (7;15). Progressive Familial Intrahepatic Cholestasis (PFIC), or “Bylers” disease, is a more severe inherited form of cholestasis. It usually presents in infancy and unlike BRIC, causes progressive cholestasis leading to cirrhosis and death in childhood. Some forms of Progressive Familial Intrahepatic Cholestasis (PFIC 1)” map to the same locus as BRIC (16) and it has been suggested that a single gene is at fault in both BRIC and PFIC 1 and that the two are allelic diseases. In some rare cases BRIC may progress to PFIC (17). Interestingly, and not previously described, there appears to be both BRIC and PFIC phenotypes within the same family in this case.

The exact precipitants of the episodes of cholestasis are unknown but there is characteristic elevation of bile acids bilirubin and Alkaline Phosphatase (ALP) with mild elevation of transaminases, white cell count, erythrocyte sedimentation rate (ESR), with relatively normal gamma GT(4;18)

Unlike other cholestatic liver diseases in BRIC bile acid levels rise before bilirubin (19) suggesting a possible role for bile acids in the pathogenesis of this condition. The role of "toxic bile" is further supported by the efficacy of external biliary diversion and ursodeoxycholic acid in the treatment of PFIC(20). Bile acids are cholestatogenic(21) and pro-apoptotic to hepatocytes (22;23). Thus apoptosis and secondary necrosis may be responsible for the mild portal inflammation found in up to a third of patients with BRIC (9). It is therefore a reasonable hypothesis that by removing factors, which may perpetuate the disease process, that resolution of a cholestatic episode would be accelerated. MARS is known to remove a number of cholestatogenic and pro-apoptotic agents and appeared to resolve this patient's episode of protracted cholestasis, which had been resistant to standard medical therapy. MARS has also been shown to improve renal function in patients with hepato-renal syndrome, due to both removal of nephrotoxic substances and improved haemodynamics (24). MARS treatment also reversed this patients deteriorating renal function.

We confirm that MARS is a safe and efficacious treatment for BRIC and suggest it should be used to terminate episodes of cholestasis particularly when associated with secondary renal impairment. We also suggest that MARS may have therapeutic potential in other more common episodic cholestatic conditions e.g. cholestasis of pregnancy (25) and drug induced cholestasis, particularly when there is developing secondary renal impairment.

Abbreviations

BRIC Benign Recurrent Intrahepatic Cholestasis

MARS Molecular Adsorbent Recycling System

ERCP Endoscopic retrograde choledochopancreatography

PFIC Progressive Familial Intrahepatic Cholestasis

ALP Alkaline Phosphatase

ESR Erthrocyte Sedimentation Rate

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Case report
If only King George III had a MARS – A case of Erythropoietic

Protoporphyria treated with MARS.

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Running Head If only King George III had a MARS.

Word count - 1405 words

Abstract

Erythropoietic protoporphyria (EPP) causes photosensitivity, abdominal pain and in some patients severe liver dysfunction and confusion. The mechanism of liver injury is unknown but deposition of protoporphyrins in the liver has been implicated. Protoporphyrins are albumin bound and theoretically should be removed by extracorporeal albumin dialysis (MARS). This case study reports the effects of MARS therapy on plasma protoporphyrins, liver function and confusion in a patient with advanced EPP. A sixty year old male with EPP developed liver failure and a severe organic confusional state which continued to deteriorate despite full medical therapy, including plasmapheresis. He was therefore treated with MARS therapy alternating with plasmapheresis. Plasma protoporphyrins decreased by 9.1% per treatment with plasmapheresis (mean pre-treatment 4570nmol/L, mean post-treatment 4150nmol/L, P=0.33, normal range<200nmol/L), but there was no improvement in mental state. MARS therapy decreased plasma protoporphyrins by 21.4% per treatment (mean pre-treatment 4710nmol/L, mean post-treatment 3700nmol/L, P<0.01,) and dramatically improved liver function and mental state. *Conclusions:* This is the first reported use of MARS therapy in porphyria. It supports a direct toxic role for protoporphyrins in EPP associated liver failure and confusion and suggests MARS therapy may be useful in the treatment of porphyric disorders. (Word count abstract 195 words)

Keywords

Porphyria

Extracorporeal albumin dialysis

Plasmapheresis

Introduction: The porphyrias are a group of disorders caused by enzyme deficiencies in the heme biosynthetic pathway. Erythropoietic protoporphyria (EPP), first described by Professor Magnus in 1961, is caused by a partial deficiency of ferrochelatase, the terminal enzyme of heme biosynthesis whose substrate is protoporphyrin. Inherited as an autosomal dominant trait, EPP results in the accumulation of protoporphyrin in erythrocytes, liver, plasma and faeces (but not in urine as it is poorly water soluble). In most cases, patients present in childhood with cutaneous photosenstitivity. Characteristically, there is an immediate painful, burning or stinging sensation in skin on light exposure followed several hours later by erythema and oedema (1). Hepatic manifestations are diverse, with mildly disturbed liver enzymes in 20% and fatal hepatic failure in 5%-10% of cases. Black protoporphyrin can be identified in liver biopsy specimens. Protoporphyrin crystal deposition in hepatocytes, canaliculi, bile ductules and macrophages with formation of cytotoxic bile and disturbance of redox systems are suggested toxic mechanism. Abdominal pain, haemolysis and neurological dysfunction may complicate end-stage liver disease (2;3).

Treatments include beta-carotene, canthaxanthin, N-acetyl cysteine, phototherapy, blood transfusion and antihistamines as well as binding agents, charcoal and cholestyramine. Good evidence for efficacy has been difficult to assess due to the subjective nature of symptoms and high placebo response rates. Liver transplantation is sometimes required for end-stage liver disease. However, because the underlying enzyme defect in EPP is bone marrow derived there are high rates of recurrence of liver dysfunction post-transplant usually occurring after a number of years (4). The only curative treatment is bone

marrow transplantation. However, since only a minority of patients develop life threatening complications, and these cannot be predicted, selection of patients for elective transplantation of liver, bone marrow or both is difficult. Conversely patients may present suddenly and deteriorate rapidly making urgent transplantation hazardous.

Plasmapheresis (5) has been used to reduce levels of plasma and erythrocyte protoporphyin levels in patients both before liver transplantation (6), and after recurrence in the transplanted liver, with improvement in liver histological and biochemical abnormalities (7). Whilst this is suggestive of a directly hepatotoxic role for protoporphyrins, plasmapheresis is highly non-selective and removal of other humoral substances may be implicated.

Extracorporeal albumin dialysis with the Molecular Adsorbents Removal System (MARS) is a new method of removing albumin bound toxins. A special membrane allows transfer of water soluble and albumin bound toxins with molecular weight less than 50kDa from blood into a dialysate solution containing 20% human albumin (8). MARS provides potential advantages over plasmapheresis since it is less expensive, has potentially greater clearance of albumin bound substances, has less theoretical risk of transmission of infection, is less immunogenic and is more selective leaving more, possibly beneficial, growth factors and hormones in the circulation (9). Protoporphyrin is more than 90% albumin bound in plasma with a high affinity for albumin (KA = 3 X 10(9)M-1 in phosphate-buffered saline)(10) and should theoretically be removed by MARS therapy. We hypothesised that MARS would efficiently remove protoporphyrin resulting in improvement in liver failure associated with EPP and would therefore offer therapeutic potential.

Case History: A sixty-year-old man underwent cadaveric liver transplantation to treat end stage liver disease due to EPP (figure 1). He was diagnosed with EPP aged 53 years when he first presented with photosensitive skin rash and mildly abnormal liver function tests. After his liver transplant he was entirely asymptomatic. Six months

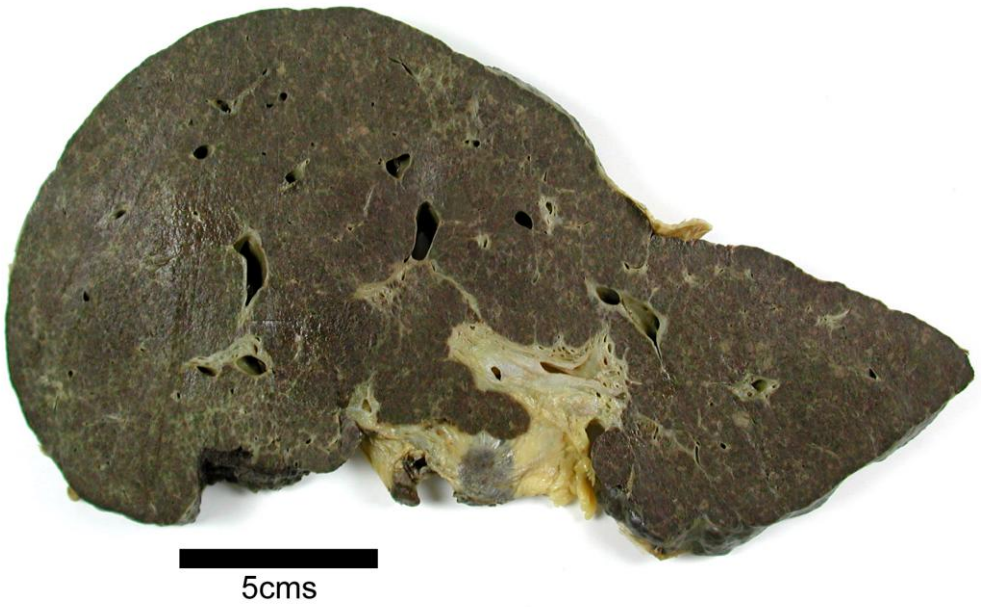


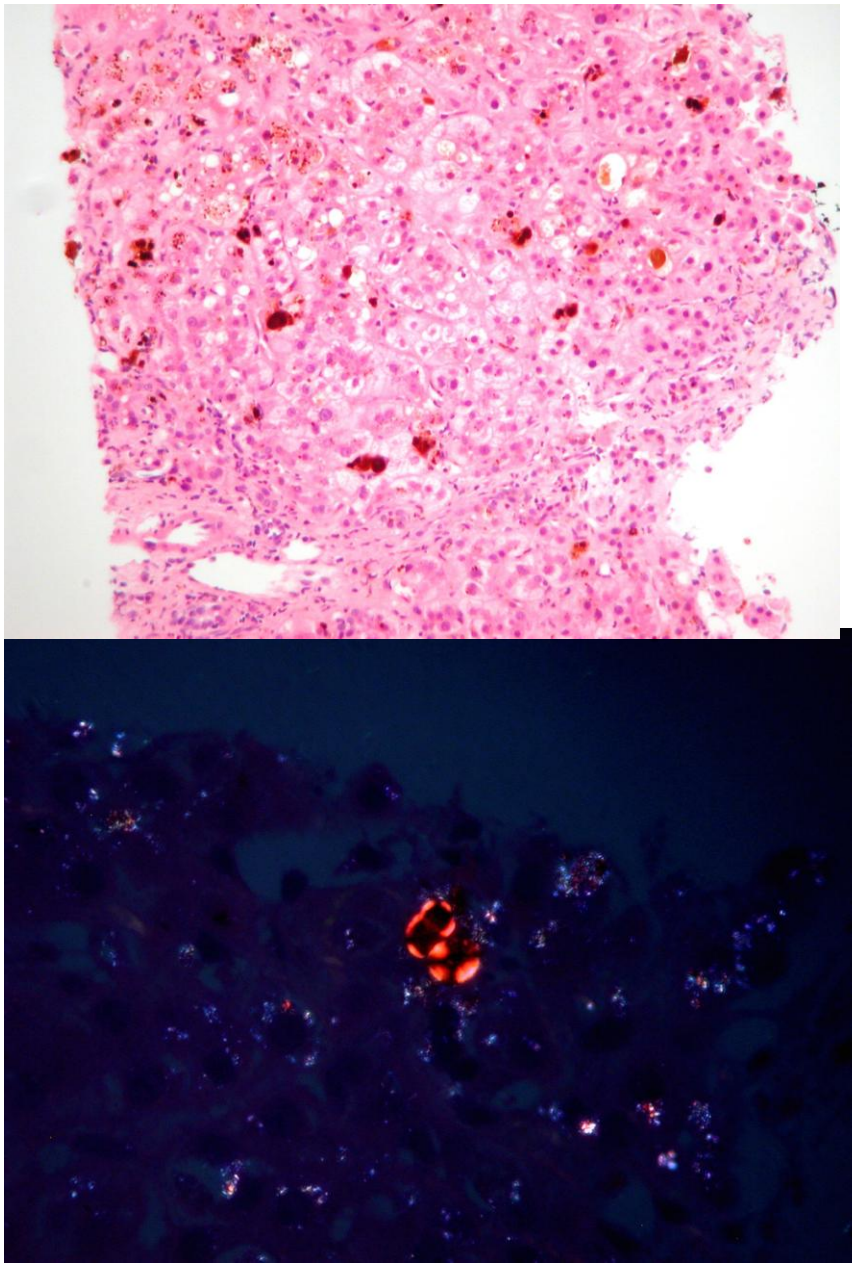
Figure 1. A formalin fixed slice of the explanted liver. The liver is almost blackened due to protoporphyrin deposition which partly obscures the fibrosis and nodularity of the cirrhosis which is also present.

after transplantation he became increasingly icteric and photosensitive. Despite treatment with cholestyramine he remained unwell and was admitted after a one week history of an acute confusion state and mild abdominal discomfort. On admission he was found to have markedly deranged liver function. Total bilirubin 391μMol/L (NR 5-17), alkaline phosphatase 288 IU/L (NR 35-130), aspartate transaminase 380 IU/L (NR 5-40), albumin 35g/dL (NR 35-50), INR 1.7 (NR 0.9-1.2). A transjugular liver biopsy taken 10 months after liver transplantation showed marked hepatic deposition of protoporphyrins (Figure 2). He was treated for three weeks with standard medical therapy including plasmapheresis, haemarginate and hypertransfusion. Unfortunately his condition continued to deteriorate with increasing confusion, and the onset of visual hallucinations. He underwent psychiatric assessment and an organic confusional state was diagnosed. In view of his relentless deterioration he was commenced on MARS therapy. In view of the experimental nature of this treatment he was also treated with plasmapheresis. He received two consecutive days of plasmapheresis followed by two days of MARS therapy, followed again by two days of plasmapheresis finishing with two days of MARS therapy. Plasma protoporphyrin levels were measured before and after each daily treatment and were analysed in a blinded fashion. All filters and lines were protected from light during treatment.

Figure 2a. (Top)

A low magnification x40 photomicrograph of a liver biopsy taken 10 months after liver transplantation and before MARS therapy. There is abundant dark brown pigment in hepatocytes, dilated canaliculae, and macrophages.

Figure 2b (bottom). The same biopsy as shown in Fig 2a, viewed with polarised light and at high magnification. The typical red birefringence of protoporphyrin is seen.



Results: After the second day of treatment with MARS therapy the patients' cognitive function returned to normal, as assessed by independent psychiatric assessment and the opinion of relatives. He had no memory of his confusional episode. His abdominal discomfort and lethargy also resolved and he noted return of his appetite and sense of humour. During treatment it was noted that the anion exchange resin in the MARS circuit had become noticeably blackened compared to treatment of equally icteric non-porphyric patients, presumably due to protoporphyrin deposition.

Plasma protoporphyrin levels fell by 9.1% per daily treatment with plasmapheresis (mean pre-treatment 4570nmol/L, mean post-treatment 4150nmol/L, P=0.33) and 21.4% per daily treatment with MARS therapy (mean pre-treatment 4710nmol/L, mean post-treatment 3700nmol/L, P<0.01, normal range for plasma protoporphyrin <200nmol/L). This demonstrates that MARS therapy removes protoporphyrins and may be more efficient in there removal than plasmapheresis. The patient had significant clinical improvement after treatment with MARS, his clinical condition having been deteriorating up to that point despite full medical treatment including plasmapheresis. Subsequently the patients liver function continued to improve and he was discharged home. He was briefly readmitted two months later again with worsening of his liver function and acute confusion that again completely resolved with MARS therapy. MARS therapy was electively continued monthly and four months after his original presentation the patients liver function tests were entirely normal.

Discussion: The normal route of excretion of protoporphyrins, as with many poorly water-soluble albumin bound substances, is in the bile after liver

metabolism. We propose that in excess protoporphyrins are directly hepatotoxic and cholestatic resulting in decreased liver function and decreased excretion of protoporphyrins. Thus a vicious circle of worsening liver function and accumulating protoporphyrin ensues. The removal of protoporphyrin by MARS therapy may allow improvement in liver function such that adequate endogenous excretion of porphyrins is re-established thus breaking this chain of events. Reducing plasma porphyrins and removing other toxins which are normally cleared by the liver may also account for the striking improvement in the mental state of this patient after MARS therapy. We conclude that MARS is an effective alternative to plasmapheresis in the treatment of EPP induced liver disease and can be used to treat life-threatening episodes of liver failure allowing subsequent elective curative treatment to be undertaken. MARS could also potentially be used prophylactically to prevent recurrence of protoporphyrin deposition in allograft livers. Plasmapheresis has been suggested to be of value in acute intermittent porphyria and porphyria cutanea tarda (11),(12) and these conditions could also potentially benefit from treatment with MARS. In summary MARS represents an exciting new tool in the treatment of porphyric disorders.

Abbreviations

Erythropoeitic Protoporphyria (EPP)

Molecular Adsorbents Recirculating System (MARS)

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