Mechanistic Biomarkers in Acute Liver Injury

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ABSTRACT:

Acute liver failure continues to be a major medical problem. There are many underlying causes of acute liver failure, but drug induced liver injury is the most common. However, ischemic injury secondary to either liver transplantation or hypoxic hepatitis are also commonly encountered clinically. While the pathogenesis of some etiologies of liver failure are well known due to appropriate animal and cell culture models (i.e. acetaminophen toxicity), that of ischemic injury is not as well documented. A major reason for this is the lack of appropriate animal models available to recapitulate these conditions in humans. Furthermore, obtaining multiple liver biopsies to study these conditions at the cellular level is generally not possible owing, in part, to the invasive nature of obtaining the sample, but also to the fact that liver biopsies are contraindicated in acute liver injury patients. Thus, alternative methods which can help diagnose and study liver injury are being explored and refined. Among these methods are the use of circulating biomarkers, which are currently being extensively explored in the field of hepatology. Because biologic specimens in which these biomarkers are being measured can be easily obtained and are non-invasive, they offer a promising means by which to study liver injury, particularly for prolonged periods of time. Indeed, a series of blood collections can provide vital information into various injury-specific aspects of liver pathophysiology including mode of cell death, mitochondrial involvement, degree of liver injury, and presence or absence of a sterile inflammatory component to the injurious process.

Here, we use a well-established set of circulating plasma biomarkers to study the pathophysiology of both warm and cold ischemia to better characterize the cellular events which take place during these conditions. Data obtained demonstrates that during both warm and cold ischemia, the majority of injury occurs early in the reperfusion period and that necrosis, rather than apoptosis

predominates. Furthermore, we identified the mitochondria as critical mediators of liver injury following ischemia. However, we were unable to find evidence of an inflammatory component of ischemic injury. Furthermore, we conclude that due to advances in surgical technique and organ preservation strategies, future efforts to study injury secondary to liver transplantation should focus on the biliary system and the formation of biliary strictures rather than ischemic injury.

HepaRG cells are a human hepatoma cell line which is commonly used in the laboratory. Unlike other liver cell lines, HepaRG cells have a full complement of drug metabolizing enzymes, making them ideal for the study of drug induced liver injury. However, growth, maintenance, and differentiation of conventional HepaRG cells is a timely process. Recently, this lengthy process has been dramatically shortened with the advent of pre-differentiated cryopreserved HepaRG cells. Due to the frequency of acetaminophen toxicity, combined with the fact that liver injury is the most common cause of drug failure and market withdrawal, we set out to compare these two preparations of HepaRG cells. Using acetaminophen as a test substrate, we found both preparations of HepaRG to be similar in all aspects of acetaminophen metabolism. This finding will help advance the study of acetaminophen, as well as help identify idiosyncratic adverse drug reactions earlier in the drug development process.

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'Twenty years from now you will be more disappointed by the things that you didn't do than by the ones you did do. So throw off the bowlines. Sail away from the safe harbor. Catch the trade winds in your sails. Explore. Dream. Discover.'

Samuel Langhorne Clemens (Mark Twain)

'I don't need to fight to prove I'm right; I don't need to be forgiven'

Pete Townshend and Roger Daltry, The Who (Baba O'Riley)

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1. INTRODUCTION

1.1 ACUTE LIVER INJURY

1.1.1 Introduction

Acute liver injury (ALI) and acute liver failure (ALF) are clinical syndromes marked by severe hepatic injury in the absence of pre-existing liver disease. Acute liver injury can develop over a period of 6 months but often progresses much more rapidly (Lee, 2012). The difference between ALI and ALF is that with acute liver injury, liver function is maintained while in acute liver failure, it is not. Consequently, clinical measurements of liver function can easily be used to differentiate between acute liver injury and acute liver failure. In ALF patients, there is often coagulopathy, icterus, and altered mentation as a result of compromised liver function (Thawley, 2017; Trotter, 2009). The most common cause of acute liver failure in the United States is acetaminophen toxicity. Other common causes include ischemic injury secondary to liver transplantation and hypoxic hepatitis. Regardless of the cause, if left untreated, ALF can be fatal.

1.1.2 Clinical Symptoms of ALF

The liver is the largest organ in the body and serves multiple functions including bile synthesis, host defense, protein synthesis, biotransformation/detoxification, and metabolic homeostasis. Therefore, patients with advanced liver failure are at risk for complications resulting from the inability of the liver to function, most notably coagulopathies and hepatic encephalopathy. Coagulopathies secondary to liver injury result from disturbances in the synthesis of pro-coagulant proteins, particularly Factors V and VII (Munoz et al., 2009; Northup and Caldwell, 2013). Because of the short half-lives of these pro-coagulants, an increase in pro-thrombin test time is often one of the first clinical signs of acute liver failure (Munoz et al., 2009). Hepatic encephalopathy results from the injured liver's inability to convert ammonia into urea (Kodali and

McGuire, 2015). Under normal circumstances, gut-derived ammonia is taken up by hepatocytes and converted to urea. A small amount is also converted to glutamine by the enzyme glutamine synthetase (Aldridge et al., 2015). During acute liver failure, the ability of the liver to detoxify ammonia is compromised. As a consequence, ammonia levels raise within the serum and are able to cross the blood-brain barrier (Kodali and McGuire, 2015). Once in the brain, ammonia is converted to glutamine by astrocytes, which causes an osmotic pull of fluid from blood vessels into the extracellular space (Butterworth, 2015; Kodali and McGuire, 2015; Scott et al., 2013). At lower concentrations, ammonia also has direct effects on both inhibitory and excitatory neurons leading to altered patient mentation (Butterworth, 2015). Higher levels of ammonia can lead to cerebral edema, increased intracranial pressure, brain swelling, coma, and death.

There are many biochemical assays commonly used to assess liver injury and function. The most common markers of injury include alanine and aspartate aminotransferase (ALT and AST, respectively), cytosolic enzymes which are released upon hepatocyte death. The most common marker of liver function is bilirubin levels. It is important to remember that markers of liver injury may remain normal despite significantly decreased function. Conversely, liver function may remain normal in the face of severe hepatic injury, provided that the number of healthy hepatocytes are sufficient to carry out normal function. Thus, evaluation of liver injury and function should not rely on a single marker. Furthermore, these enzymes often provide little information as to the mechanisms which are occurring at the cellular level. When this information is lacking, development of additional therapeutics for liver disease cannot be identified. As such, scientists within the hepatology field have begun to focus their efforts on identification of other markers of cellular injury and death which may provide additional information regarding mode and mechanisms of cell death during acute liver injury and failure.

1.1.3 Biomarkers in Hepatology

1.1.3.1 Introduction to biomarkers

Some of the first biomarkers of liver injury were the aminotransferases – ALT and AST in 1955 (Karmen et al., 1955). Gamma-glutamyl transferase (GGT) was discovered and adopted into clinical practice in 1961 (Szczeklik et al., 1961) but since then, very few advances have been made in the identification of additional biomarkers of liver injury. However, in the previous decade, much research has been conducted to identify additional biomarkers of organ pathology. Broad categories of this biomarker research include mechanistic biomarkers, biomarkers of injury, biomarkers of inflammation, biomarkers of regeneration, and extracellular RNA based biomarkers specific to the organ in question (McGill, 2016). Drug induced liver injury, viral hepatitis, hepatocellular carcinoma, and hepatic steatosis appear to be among the most commonly studied conditions in the hepatology field, but applications in other fields such as transplantation, hypoxic hepatitis, and biliary diseases have been studied as well. Regardless, it is hypothesized that soon, the use of these biomarkers in the clinic will become as normal as the use of ALT, either in conjunction with, or instead of, currently used markers of injury (McGill, 2016). Regardless, there has been a tremendous amount of useful information gained from this research, particularly regarding their usefulness in the diagnosis, treatment, management, and prognosis of various causes of ALF, regardless of the etiology.

The most commonly used method of assessing liver injury in the clinic is ALT levels. However, ALT is not specific to the liver as it is also found in other tissues such as skeletal muscle and kidney. Thus, even a moderate increase in ALT may not indicate an injurious process specific to the liver. Furthermore, measurement of ALT provides little information as to the cellular mechanism of cell death. This is important because in addition to treatment of the underlying

cause, an important approach to treating ALF patients would be to prevent continued hepatocyte injury and death. Thus, if hepatocytes are dying via necrosis, necrostatins may be used to minimize cell death. Similarly, if apoptosis predominates, caspase inhibitors could be used as the optimal treatment modality.

Recent advances in the field of hepatology have identified a reliable set of circulating biomarkers which can be used to help establish both mode and mechanism of cell death following hepatic injury. In general, biomarkers can be classified as those of exposure, effect, or susceptibility. For the purpose of this dissertation, the research described subsequently focuses on biomarkers of effect – either the effect of a condition (ischemia-reperfusion injury) or the effect of a toxin (acetaminophen). These biomarkers, discussed in detail below, represent a promising and convenient method of assessing liver injury in humans following a variety of insults to the liver when invasive methods (ie: biopsy) are either unavailable or contraindicated. The greatest benefit to the use of these biomarkers is that they exist in the general circulation, and thus can be evaluated in peripheral blood.

The bulk of this dissertation will focus on the use of the following mechanistic biomarkers to aid in the description of the cellular events leading up to liver injury following ischemia-reperfusion injury secondary to orthotopic liver transplantation (OLT) and hypoxic hepatitis (HH).

1.1.3.2 Biomarkers of Liver Injury

ALT is responsible for the transfer of an amino group from alanine to α-ketoglutarate to form pyruvate and glutamate and is found in the cytosol of hepatocytes. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are cytosolic enzymes which catalyze the transfer of alanine, or aspartate, to α-ketoglutarate to form pyruvate and glutamate or oxaloacetate and

glutamate, respectively. Though they exist in multiple tissues such as the muscle, kidney, brain, and red blood cells, their highest concentration is in the liver (Steven Stockham and Michael Scott, 2002). Upon cell death, these cytosolic enzymes are released into the sinusoids and can easily be measured in the blood. In fact, ALT is considered the gold standard for the measurement of liver injury clinically (Steven Stockham and Michael Scott, 2002). Despite the sensitivity for liver injury, ALT and AST have limited specificity for diagnosis of liver injury, particularly at low levels. In contrast, microRNA-122 (miRNA-122) is liver specific. MicroRNAs are small non-coding RNAs thought to be formed by the cell as a means to regulate protein expression at a post-transcriptional level. Importantly, it has been shown that miRNA-122 is a more sensitive marker for liver injury than ALT, becoming elevated earlier and to a greater degree than ALT (Wang et al., 2009a). A faster identification of injury following transplantation would allow for a more rapid response and treatment. MicroRNAs will be discussed in more detail below.

1.1.3.3 Biomarkers of cell death modality

There are many forms of cell death, but the most common are apoptosis and necrosis. Recently, research has been conducted into the use of circulating biomarkers to differentiate between these two forms with the need for invasive biopsy procedures. Cytokeratin-18 is one such biomarker. Cytokeratin-18 is a type 1 intermediate filament protein which is ubiquitous in the cytoplasm of cells (Omary et al., 2009). Following membrane rupture from oncotic necrosis, cytokeratin-18 is released in its full-length form (FK18). During apoptosis, however, activated effector caspases cause cleavage of cytokeratin-18 at aspartic acid #397 (Asp397) along the protein, cleaving it into to smaller caspase-cleaved fragment (ccK18) and creating a neo-epitope (Leers et al., 1999; Linder et al., 2010; Omary et al., 2009). Commercially available kits containing antibodies to full-length

and caspase-cleaved cytokeratin-18 (ccK18) allowing for the easy quantification of FK18 and ccK18. In this way, by comparing the ratio of FK18 to ccK18 it is possible to determine which mode of cell death is predominating at any given point in time. In fact, after subjecting mice to 45 minutes of ischemia followed by various periods of reperfusion, up to 24hr, we found a time dependent increase in full length cytokeratin-18 which closely correlated with degree of liver necrosis, as determined histologically, suggesting the primary mode of cell death after IRI is necrosis (Yang et al., 2014). A study evaluating cytokeratin-18 in humans undergoing liver transplantation shows that there is an increase in FK18 following transplantation, suggesting that necrosis predominates following OLT (Ulukaya et al., 2010). However, this study compares the differences in living donors versus cadaveric donors, the livers of which undergo different procurement procedures, as well as surgical procedures which can affect liver viability following transplantation (Jassem et al., 2003; Oliveros et al., 2005). In addition, no measurement of ccK18 is shown here, which is crucial since we have also shown a slight increase in ccK18 at 3 hours post-reperfusion, when no relevant amount of apoptosis is present. This is because the corresponding increase in FK18 is >150-fold greater (Yang et al., 2014). Thus, it is the ratio of FK18 to ccK18 that is necessary to make conclusions about mode of cell death.

In addition to helping differentiate between apoptosis and necrosis, cytokeratin-18 has a number of other practical diagnostic and prognostic applications for a variety of liver disorders. For malignancies, much use of cytokeratins comes from immunohistochemical staining, which necessitates biopsy. Therefore, an in-depth discussion of cytokeratins for this purpose would be beyond the scope of this dissertation. Nevertheless, malformations in keratin organization have been shown to predispose individuals to certain conditions such as copper storage disease and non-alcoholic steatohepatitis (Ku et al., 2007; Strnad et al., 2012; Zatloukal et al., 2007). The thought

is that these malformed keratin structures contribute to hepatocyte ballooning in these conditions (Guy et al., 2012; Lackner, 2011). Furthermore, overexpression of cytokeratin variants have been useful in differentiating between various tumor types. For instance, HCC can be differentiated from cholangiocarcinoma by the overexpression of K19 relative to K18 because hepatocytes only contain cytokeratin-8 and -18 whereas cholangiocytes contain K8, 18, and 19 (Moll et al., 2008; Omary et al., 2009).

More relevant to the field of circulating biomarkers is that elevated levels of caspase-cleaved cytokeratin is present in patients suffering from NASH and can help not only differentiate NASH from simple steatosis, but may also be correlative with the degree of severity (Alkhouri et al., 2011; Molnar et al., 2011; Musso et al., 2011; Wieckowska et al., 2006). This is also true for chronic HBV and fibrotic injury associated with HCV (Bantel et al., 2004; Papatheodoridis et al., 2008). In fact, circulating cytokeratins are the only non-invasive marker currently being used for the diagnosis of NASH. A recent study demonstrated that cytokeratin-18 fragments were able to predict the presence of NASH in patients with a sensitivity of 0.78, specificity of 0.87, and area under ROC of 0.82 (Musso et al., 2011). Similarly, in cases of acute liver failure, an increase in caspase-cleaved cytokeratin-18 was also associated with favorable prognosis and elevated full length cytokeratin-18 indicated more significant liver injury and a poor outcome (Bechmann et al., 2010; Volkmann et al., 2008). However, there is conflicting data suggesting that outcome is more dependent on etiology than on M65 levels; in one study of APAP toxicity, evaluation of keratin fragmentation did not appear to offer additional benefit in predicting outcome relative to the currently used ALF criteria (Craig et al., 2011). On the other hand, in a study of patients who presented to the ER for APAP overdose, elevated full length cytokeratin-18 levels were shown to be highly predictive of which patients would go on to develop liver injury (Antoine et al., 2013).

While these numbers may not be ideal, they are much better than the use of ALT levels at the time of presentation for the prediction of development of liver injury. Finally, keratin-18 may be able to predict which patients will respond to anti-HCV therapy (Volkmann et al., 2008). Further research into cytokeratins will provide more information into their use as both predictive as well as prognostic biomarkers and will make them a more valuable clinical resource, particular in conjunction with other markers of injury.

HMGB-1 is a nuclear protein which sits in the minor groove of DNA and acts as a transcription factor for a variety of proteins. HMGB-1 exists in two forms, a hypo-acetylated (HMGB-1) form and a hyper-acetylated form (acHMGB-1) (Antoine et al., 2009; van Golen et al., 2012). In its hyper-acetylated form, acHMGB-1 is actively secreted by macrophages and represents a pro-inflammatory biomarker (Antoine et al., 2009; Bonaldi et al., 2003). However, during necrosis, hypoacetylated HMGB-1 is passively released into sinusoids and acts on macrophages through toll-like receptors to produce cytokines (Tsung et al., 2005; Yang et al., 2010). Studies conducted by our laboratory, and others, have shown that increased levels of total HMGB-1 correlate with degree of necrosis, particularly during earlier time points (Tsung et al., 2005; Yang et al., 2014). Oxidation status of non-acetylated HMGB1 can also differentiate between necrosis and apoptosis and whether or not an immune response may be generated. Isoforms of HMGB1 which contain reduced residues are generally associated with necrosis and facilitate chemotaxis and cytokine release from innate immune cells. The opposite is true of isoforms of HMGB1 which contain fully oxidized residues and are associated with apoptosis and the lack of an innate immune response (Tang et al., 2010, 2012) Thus, much like cleaved and full-length cytokeratin, the ratio of oxidized to reduced isoforms of HGMB1 can be used to differentiate between necrosis and apoptosis. Furthermore, correlations between HMGB1 and outcome have been identified following acetaminophen toxicity in

humans (Antoine et al., 2013). Finally, caspase activity and cleaved caspase protein (particularly caspase-3) can be used as markers of apoptotic cell death, but more research needs to be conducted before their application in the clinical setting can be assessed (McGill et al., 2012; Woolbright et al., 2015)

1.1.3.4 Biomarkers of Mitochondrial Injury

The premise with all mechanistic biomarkers of cellular injury and death is that they are only released into circulation as a result of cellular injury. For instance, mitochondrial DNA (mtDNA) exists within the mitochondrial matrix and injury to the mitochondria would lead to release of mtDNA into the cellular matrix. However, this alone would not be expected to cause an increase in circulating mtDNA, provided, of course, that the injury to the mitochondria was not sufficient to cause cellular death.

Drug hepatotoxicity often involves mitochondrial damage and dysfunction (Pessayre et al., 2012). Indeed, during APAP toxicity, which is the major cause of DILI, mitochondrial injury is a critical feature of liver injury (Jaeschke et al., 2012a). As a result, several biomarkers of mitochondrial injury have been identified.

Glutamate dehydrogenase (GDH) is an enzyme situated within the mitochondrial matrix. Using NAD+ as a cofactor, GDH catalyzes the conversion of glutamate to oxoglutamate – forming ammonia and NADH. Critically, mitochondrial injury must occur for GDH to exist in measurable amounts in the plasma. This is exemplified by studies comparing hepatotoxic drugs which have different mechanisms of action. For instance, both acetaminophen and furosemide cause hepatocyte injury but only APAP toxicity leads to mitochondrial injury. In studies comparing APAP to furosemide, measurements of GDH following acetaminophen toxicity are elevated while

those following furosemide toxicity are not (McGill et al., 2012). This is because if mitochondrial rupture does not precede necrosis, in-tact mitochondria can be removed from plasma prior to the measurement of GDH. Previous studies from our lab have shown definitively that mitochondria play a critical role in hepatocyte necrosis following acetaminophen toxicity (McGill et al., 2012) and data using rodent models of ischemia suggests this may be true in humans following OLT (Yang et al., 2014).

In conjunction with GDH, measurement of mitochondrial DNA within the plasma can be used to identify mitochondrial injury. During the measurement of mtDNA, total DNA is isolated from the plasma, then subjected to PCR, using primers for genes encoded specifically by mitochondrial specific DNA, such as NADH dehydrogenase or Cytochrome C oxidase subunit 3. In studies detailed in this dissertation as well as those of acetaminophen metabolism, mtDNA is not only elevated in patients with liver injury relative to those without liver injury, but preliminary data from our lab suggests that elevations in GDH and mtDNA may even slightly precede elevations in ALT, underscoring the critical role of mitochondrial injury in cell death under these conditions. Although mores studies are needed to confirm this, it would seem to indicate that therapies targeted towards the prevention of mitochondrial injury would have a tremendous impact on the progression of liver injury in these conditions.

In addition to these matrix macromolecules, Bajt and co-workers have shown that damage to the mitochondria also results in release of apoptosis inducing factor (AIF) and endonuclease G (Bajt et al., 2006). Endonuclease G then translocates to the nucleus where it begins cleaving nuclear DNA into fragments, ultimately leading to necrosis (Bajt et al., 2006, 2008, 2011). Therefore, measurements of nuclear DNA fragmentation following OLT in humans may be indicative of

mitochondrial involvement. Indeed, in rodent models of IRI, there is an increase in both biomarkers, suggesting a role of mitochondrial involvement (Yang et al., 2014).

1.1.3.5 Nucleic Acid Biomarkers

Regardless of whether cell death mode is necrosis or apoptosis, the final step in the death process involves DNA fragmentation. Therefore, it is not surprising that identification of methods to measure DNA fragmentation have been explored as a biomarker of cell death. The biggest drawback to the use of older tests such as gel electrophoresis and the TUNEL assay is that they require invasive means for acquisition. Nevertheless, when liver tissue can be obtained, these tests can provide valuable information regarding both the degree and mode of cell death. This is because the extent of DNA fragmentation correlates with the degree of cellular injury, and the pattern of fragmentation varies due to differences in the cellular pathways for apoptosis and necrosis. During apoptotic cell death, activated effector caspases (ie: caspase-3) cleave the inhibitor of caspase activated DNAse (iCAD) protein, allowing CAD to cleave DNA. Once activated, CAD cleaves DNA at regular intervals of about 180-200 bp, or multiples thereof. In contrast, during necrosis, DNA fragmentation is random, leading to DNA fragments of random size. The resulting electrophoresis pattern following apoptosis would therefore show up as a ‘ladder’ of bands as a result of numerous fragments of similar size but would appear as a ‘smear’ with no distinct identifiable band following necrosis. While gel electrophoresis can provide valuable information into the mode of cell death, it provides little information into the extent of injury. The opposite is true for the TUNEL assay, which based on the degree of staining can show extent of injury relative to another test compound, or contro group (Duan et al., 2016). Although this assay is not specific for apoptotic cell death, the pattern of staining can still provide information as to whether apoptosis

or necrosis predominates. This is because during apoptosis, TUNEL staining will appear as punctate areas in shrunken cells which have been pulled away from neighboring cells. In contrast, during necrosis, the TUNEL staining often encompasses large areas of tissue as a result of membrane rupture and spillage of DNA fragments into the surrounding area (Yang et al., 2014).

Recently, methods have been developed which allow for the measurement of nuclear DNA fragmentation in plasma following liver injury. This assay is commercially available and utilizes the principle of the ELISA assay and uses a primary capture antibody against histones. Following incubation with secondary antibody, a colorimetric reaction occurs and the intensity of this color change can be compared between different injury groups as well as healthy volunteers. Nuclear DNA fragmentation has been assessed in a variety of liver conditions such as acetaminophen toxicity, liver transplantation, and hypoxic hepatitis (Bajt et al., 2006; McGill et al., 2012; Weemhoff et al., 2017). While this can provide information into the extent of injury, it does not provide any information into the mode of cell death. In fact, one of the biggest drawbacks to this assay is that if the DNA fragment is long enough, it may actually fold back onto one or more additional primary antibodies, thus over estimating the amount of injury.

One of the most rapidly growing topics in the field of biomarker research is micro-RNAs, or miRNA. Micro-RNAs are short, non-coding RNA sequences which regulate gene expression of numerous proteins by inhibiting translation of mRNA (Bala et al., 2009; Cortez and Calin, 2009). Since they were first identified in 1993, much research has been conducted on the role of miRNAs in pathways such as cell death, differentiation, proliferation, and the pathogenesis of infectious and neoplastic diseases (Bala et al., 2009; Lee et al., 1993; Voinnet, 2005; Zamore and Haley, 2005). Micro-RNAs are being used to not only assess extent of cell death, but also to help

differentiate between underlying etiology. In fact, the vast majority of biomarker research has been conducted in this particular field and the future of the use miRNA as a clinical tool is promising.

The use of miRNA, particularly miRNA-122, in the field of hepatology is of benefit because this miRNA-122 is specific to the liver. Thus, unlike other markers of liver injury such as ALT, elevated miRNA can only be attributed to liver injury. The correlation between liver injury and increases of miRNA have been shown in numerous studies (Ward et al., 2014; Weemhoff et al., 2017). In fact, miRNA-122 levels may be a more sensitive marker of liver injury than ALT as numerous studies have shown it to become elevated prior to ALT (Dear et al., 2014; Wang et al., 2009b; Ward et al., 2014).

While the studies detailed in this dissertation have focused only on miRNA-122, many other microRNAs have been studied and described in the context of liver injury. Other miRNAs such as miR-192 and miR-125b, are elevated in plasma or serum after acetaminophen toxicity in humans and in mice (Krauskopf et al., 2015; McGill and Jaeschke, 2015; Ward et al., 2014; Yang et al., 2015). Furthermore, some studies of the liver specific miRNA-122 have not only been shown to be predictive of the development of liver injury in early-presenting acetaminophen overdose patients, but is also associated with a poor outcome (Antoine et al., 2012, 2013).

The use of miRNAs as biomarkers is multifaceted and goes well beyond the measurement of cell death and prediction of injury and outcome following acetaminophen toxicity. Indeed, circulating miRNA profiles could be beneficial in differentiating the underlying cause of injury. In a 2014 study, expression profiles of various liver specific miRNAs were used to help differentiate between APAP toxicity and hypoxic hepatitis (Ward et al., 2014). Additionally, specific changes in miRNA expression profiles have been associated with specific liver diseases such as non-alcoholic fatty liver disease, alcoholic liver disease, primary biliary cirrhosis, and hepatocellular carcinoma

(Dolganiuc et al., 2009; Jin et al., 2009; Ladeiro et al., 2008; Li et al., 2009; Murakami et al., 2006; Padgett et al., 2009). Using the knowledge gained from miRNA profiles in these types of diseases, research has been carried out to explore the possibility of miRNA as a therapeutic mechanism to counteract aberrant expression of miRNA during disease processes. In one example therapeutic silencing of miRNA-122 lead to a significant decrease in HCV levels in chimpanzees (Lanford et al., 2010). In another study, HCC progression was reversed following miRNA-26a administration (Kota et al., 2009). Finally, overexpression of miRNA-150 and 194 leads to decreased stellate cell activation, potentially playing a critical role in the therapy of liver fibrosis (Antoine et al., 2015).

1.1.3.6 Other Biomarkers

In the rapidly growing field of biomarker research, new markers of injury are constantly being identified and investigated. In some cases, markers of other organ systems have been investigated for their use as markers of liver injury. For instance, kidney injury molecule-1 can be a sensitive predictor of outcome following APAP overdose (Antoine et al., 2015). The relationship between KIM-1 and liver injury is of particular importance for determining the urgency of liver transplantation in these cases.

In addition to plasma biomarkers of liver injury, several studies have identified a number of changes in urine composition as a method to assess liver injury (Amacher, 2002). For instance, urinary biomarker profiles following exposure to several hepatotoxicants were used to differentiate between administered toxins (Beckwith-Hall et al., 1998).

1.2 ISCHEMIA-REPERFUSION INJURY

1.2.1 INTRODUCTION

Ischemia-Reperfusion Injury (IRI) is the process by which reintroduction of oxygen to a previously ischemic organ leads to exacerbation of injury to that organ. IRI has been described for decades and observed in a number of organs, including the heart (Hausenloy and Yellon, 2013), liver (Jaeschke, 1991; Marubayashi et al., 1986), and kidneys (Chatauret et al., 2011). Clinically, IRI can occur during veno-occlusive disease, severe hypotension, or hemorrhagic shock (Eltzschig and Eckle, 2011). It can also be introduced iatrogenically during the Pringle Maneuver, when blood supply to an organ is intentionally occluded to prevent blood loss during prolonged surgical procedures. In the context of the liver, this can occur during lobectomy, mass removal, or transplantation.

After decades of research in rodent models of IRI, much has been learned about the mechanisms of injury following an ischemic insult to the liver (Jaeschke, 2003). Despite these advances, little is known about the mechanisms of reperfusion injury in humans. Even in rodent models there is considerable debate about the mechanisms of injury, though recent studies by our laboratory have conclusively demonstrated that necrotic rather than apoptotic cell death predominates (Gujral et al., 2001; Yang et al., 2014). Determination of the type of cell death is important for the design of therapeutic agents intended to minimize injury following ischemia.

One difficulty in characterizing the mechanisms of ischemic injury in humans, particularly over longer periods of time, is that multiple biopsies in human patients during liver injury is not possible. Thus, histologic evaluation to determine mode of cell death and neutrophil infiltration cannot be used. Therefore, we sought to use the specific circulating biomarkers discussed above to characterize mode of cell death, the role of mitochondria in cell death, and the role of

inflammation leading up to, and following, cell death following ischemia-reperfusion injury. Importantly, these same biomarkers provide useful insight into prognosis of patients suffering from other clinical conditions such as acetaminophen toxicity and cholestasis.

1.2.2 INFLAMMATION DURING ISCHEMIA-REPERFUSION INJURY

In rodent IRI, Kupffer cells and neutrophils play a significant role in the initiation and propagation of injury, respectively (Jaeschke and Farhood, 1991a; Jaeschke et al., 1992, 1993). Through the activation of Kupffer cells and the subsequent release of cytokines, neutrophils are recruited to the area and mediate the later phase of injury. Studies have demonstrated that antibody-mediated depletion of neutrophils affords substantial protection following IRI in rodents (Jaeschke et al., 1990). In addition to pro-inflammatory, anti-inflammatory, and regenerative mediators are also released (Lentsch, 2012). Thus, the balance between injury and repair is dependent on the balance of these cytokines. Indeed, increased pro-inflammatory and decreased anti-inflammatory chemokines are associated with increased injury and risk of graft rejection (Camargo et al., 1997; Friedman et al., 2012; Tomiyama et al., 2008; Zhai et al., 2008).

As mentioned previously, HMGB-1 can be a marker of necrosis, but can also serve as a marker of inflammation. A study evaluating the presence of HMGB-1 following OLT found a measurable amount of HMGB-1 in the early stages of reperfusion but not in the later stages, suggesting necrosis occurs during the early phase of injury (Ilmakunnas et al., 2008), which is in agreement with our rodent studies (Yang et al., 2014). This study concludes there is no correlation between HMGB-1 and inflammation, as measured by TNF-α and IL-6 (Ilmakunnas et al., 2008). However, these conclusions were based on total HMGB-1, which is released passively during necrosis, rather than hyper-acetylated HMGB-1, which is released actively and serves to initiate an immune

response. Studies from our laboratory demonstrate an increase in HMGB-1 corresponding to necrosis at the earlier time points, and an increase in acHMGB-1 corresponding to inflammation at later time points, underscoring the importance of measuring both forms of HMGB1 (Yang et al., 2014).

In previous studies using the rodent model of IRI, an increase in neutrophil priming and activation (CD11b expression) was observed. This correlated with degree of injury at later time points following ischemia (Jaeschke et al., 1992, 1993) and confirmed the importance of neutrophils in the late stage of injury. Neutrophils are also capable of phagocytosis which can both help stop the inflammatory process by removing inflammatory debris, and it can also prepare the tissue for regeneration. In fact, neutrophil infiltration is crucial for regeneration following acetaminophen overdose (Williams et al., 2014). A study examining the role of neutrophils in human OLT injury concluded that despite early (5 minutes post-reperfusion) activation of neutrophils, there was no effect on graft function, suggesting neutrophil activation does not exacerbate tissue injury (Ilmakunnas et al., 2009). However, this study failed to examine neutrophil involvement at later time points (>6h) which has been shown in the rodent model to be the time at which neutrophils have extravasated and begin to propagate injury in the mouse model (Jaeschke and Smith, 1997; Jaeschke et al., 1990).

1.2.3 ISCHEMIC INJURY FOLLOWING LIVER TRANSPLANTATION

1.2.3.1 Introduction

Liver transplantation remains the only therapeutic option for end-stage liver disease of any etiology. Although liver transplantation has become a routine therapy, there are significant post-operative risks associated with the procedure, such as reperfusion injury and the formation of

biliary strictures, which can affect graft survival, morbidity and mortality, and long-term outcome. Furthermore, methods to predict which patient will develop these complications are lacking.

During donor liver procurement and transplantation, the organ experiences no-flow ischemia upon procurement, and is stored in a preservative such as University of Wisconsin (UW) solution until a recipient is identified (El-Wahsh, 2007). Thus, there is a varying degree of time during which the donor liver experiences no-flow ischemia. Unfortunately, this period of ischemia can predispose parenchymal and non-parenchymal cells to injury (reperfusion injury) upon warm reperfusion, leading to increased risk of graft injury and primary graft failure. Research has shown that longer ischemic times lead to greater injury to the liver and an increased risk of complications, such as primary graft failure (Marsman et al., 1996; Perez-Daga et al., 2006; van der Vliet and Warlé, 2013). Additionally, certain donor liver factors, such as increased levels of steatosis, predispose the allograft to increased injury and failure. As a result, these marginal quality livers are not frequently used in transplantation, limiting the number of organs available for the life-saving procedure. Indeed, according to the Organ Procurement and Transplantation Network (OPTN), there are currently over 14,000 individuals on a waiting list to receive a transplant despite the fact that more than 26,000 transplants have been performed thus far in 2017. Moreover, approximately 4,100 people have died while waiting for a transplant. In light of these statistics, it is crucial to determine the mechanisms leading to reperfusion injury and biliary stricture following human liver transplantation.

1.2.3.2 Cell death following LT

One of the most basic questions is whether cells die by necrosis or apoptosis following liver transplantation. These two modes of cell death differ both in intracellular events as well as

histological characteristics. Apoptosis involves activation of caspases and ultimately activation of caspase-activated DNAse, which leads to fragmentation of nuclear DNA. Histologically, apoptosis is characterized by cellular shrinking, formation of apoptotic bodies, nuclear condensation, and an intact cell membrane (Jaeschke and Lemasters, 2003). In contrast, necrosis can be recognized by cell swelling, karyorrhexis and karyolysis, and loss of membrane integrity (Jaeschke and Lemasters, 2003). Many studies arguing for a relevant impact of apoptosis in IRI rely on terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining (Kim et al., 2013; Rao et al., 2013). However, because both forms of cell death involve fragmentation of nuclear DNA, TUNEL staining alone cannot be used to differentiate the two. Interestingly, the pattern of TUNEL staining can give insight into which mode of cell death predominates. We have shown that in cells undergoing apoptosis, TUNEL-positive cells appear as punctate stains within the microscopic field. However, due to nuclear and cellular lysis that occurs during necrosis, DNA fragments diffuse into surrounding areas leading to large, irregularly shaped stained areas.(Jaeschke et al., 2011; Yang et al., 2014). Since each mode of cell death is carried out by two distinct processes, determination of the predominant form of cell death following OLT in humans is necessary to identify appropriate therapeutic targets. Furthermore, not much is currently known regarding downstream cellular events which lead to hepatocyte injury and death.

1.2.4 LIVER INJURY FOLLOWING HYPOXIC HEPATITIS

1.2.4.1 Introduction

Hypoxic hepatitis (HH), also called ischemic hepatitis, or ‘shock’ liver, is a clinical condition precipitated by prolonged periods of oxygen deprivation to the liver and can have several underlying causes. It is characterized by a sudden and rapid increase in ALT (~20X normal) in the

absence of any other causes of liver injury, such as viral hepatitis, alcoholic hepatitis, or drug-induced liver injury. Its prevalence in critically ill patients can reach upwards of 10%. Despite its prevalence, little is known about the mechanisms of injury.

1.2.4.2 Clinical Hypoxic Hepatitis

Typically, the inciting cause for HH involves an episode of cardiogenic, circulatory, or respiratory failure leading to decreased oxygen delivery to the liver (Henrion et al., 2003). Hypoxic hepatitis represents a serious source of morbidity and mortality, with a prevalence of approximately 10% in intensive care patients (Fuhrmann et al., 2010). Treatment of hypoxic hepatitis involves treatment of the underlying cause, but mortality can still be as high as 50% (Fuhrmann et al., 2010; Hawker, 1991; Horvatits et al., 2013).

In the laboratory, hypoxic hepatitis has been studied in a variety of ways including the hemorrhagic shock model. This model involves hemorrhage of the animal to a hypotensive state, thereby decreasing oxygen delivery to the liver. In another model, the cardiogenic shock model, a balloon catheter is placed in the coronary artery and inflated, leading to cardiogenic shock. A major downside to the use of animal models for the study of hypoxic hepatitis is that they are not often reproducible. In the most commonly used model, hemorrhagic shock, ALT values don't always reach the level expected during hypoxic hepatitis and even under similar conditions, the degree of injury is often vastly different between experiments. Furthermore, the number of underlying conditions that can precipitate hypoxic hepatitis in humans, such as respiratory failure/shock, aortic dissection, and obstructive sleep apnea (Alcorn and Miyai, 1992; French et al., 1984; Henrion et al., 1997, 1999; Leslie et al., 1989; Mathurin et al., 1995; Trilok et al., 2012). far outnumber the types of animal models developed for the study of the condition. Thus, subtle

differences in the pathophysiology may be missed by relying on animal models which do not recapitulate the condition in humans. Furthermore, most laboratory studies of hypoxic hepatitis are limited to a specific time point, rather than a complete clinical course. Thus, an alternative approach is necessary.

One possible method to study HH clinically is through the use of the same biomarkers of liver injury and death described above. Once a diagnosis of hypoxic hepatitis is made, these biomarkers can be measured for an extended time course. Furthermore, they could easily be catalogued and classified according to underlying etiology so that patterns in injury can be identified, regardless of the underlying cause.

1.3 DRUG-INDUCED LIVER INJURY

1.3.1 Introduction

Drug induced liver injury is the most common cause of acute liver failure in the US (Chen et al., 2015; Reuben et al., 2010). Drug induced liver injury can be classified as intrinsic or idiosyncratic; the basis for the classification being whether or not the mechanism of injury is intrinsic to the drug or not. For the drugs that fall under the 'intrinsic' category, either the drug itself or a metabolite, has a known deleterious effect on the liver. Thus, injury secondary to the use of these drugs, is both predictable and dose-dependent (Fisher et al., 2015). However, the vast majority of drugs responsible for DILI fall within the umbrella of idiosyncratic. Idiosyncratic drug induced liver injury remains a problem due to the fact that the basis for the injury in susceptible individuals remains unknown. There is not likely a single cause for the development of injury in these patients, but rather a combination of chemical, genetic, and immunologic factors for the individual which

leads to the reaction (Chen et al., 2015; Tailor et al., 2015). Thus, injury with these drugs is both unpredictable and non-dose dependent (Fisher et al., 2015).

Most drugs with a predictable adverse reaction on the liver are screened out before, or during, clinical trials (Jaeschke, 2015). An exception to this rule is acetaminophen. While acetaminophen is safe at therapeutic levels, it actually represents the most common cause of acute liver failure in the United States (Chen et al., 2015; Fisher et al., 2015; Jaeschke, 2015; Reuben et al., 2010). This is due in no small part to its availability as an over-the-counter medication, as well as its presence in many prescription opioid formulations such as Vicodin® and Percocet® (Bunchorntavakul and Reddy, 2013; Herndon and Dankenbring, 2014; Yoon et al., 2016). Many patients on medication to manage long-term pain take a combination of these opioid formulations as well as acetaminophen, thereby unwittingly overdosing on the drug. Intentional overdose with acetaminophen also accounts for a significant number of acetaminophen toxicities.

1.3.2 DILI Secondary to Acetaminophen

Acetaminophen is a commonly used analgesic and antipyretic (Bunchorntavakul and Reddy, 2013; Yoon et al., 2016). It is well tolerated at therapeutic doses (<4g/day) but leads to toxicity at higher doses. APAP is metabolized through a combination of Phase I and Phase II detoxifying enzymes. In the case of acetaminophen, Phase II metabolism occurs first with the majority of the parent compound being conjugated to glucuronide or sulfate, and being excreted as inactive conjugates (Larson, 2007). Even at therapeutic doses, a small amount of the parent compound is metabolized by cytochrome P-450 enzymes 2E1, 1A2, and 3A4 (Lee et al., 1996; Snawder et al., 1994; Thummel et al., 1993). into the toxic and electrophilic intermediate N-acetyl-p-benzoquinone imine, or NAPQI (Dahlin et al., 1984). NAPQI is subsequently detoxified by the tripeptide

glutathione (Larson, 2007). At higher doses of acetaminophen, the conjugation systems become overwhelmed and a much higher percent of the initial dose is shunted through the P-450 system, leading to increased NAPQI formation (Du et al., 2013). Although glutathione exists in very high concentrations within the cytoplasm, increased NAPQI formation rapidly depletes glutathione stores (Lee et al., 1996; Mitchell et al., 1973; Xie et al., 2015a). As an electrophile, NAPQI can covalently bind to proteins free-floating within the cytoplasm, or proteins contained on organelle membranes forming protein adducts. It has been extensively shown that mitochondrial proteins are affected by NAPQI (Cohen et al., 1997; McGill et al., 2012; Qiu et al., 1998; Tirmenstein and Nelson, 1989; Xie et al., 2015b). This leads to mitochondrial oxidative stress and JNK activation (Du et al., 2015; Gunawan et al., 2006; Henderson et al., 2007; Meyers et al., 1988; Saito et al., 2010; Xie et al., 2014a). Activated JNK (pJNK) then translocates into the mitochondria and amplifies the oxidative stress (Hanawa et al., 2008; Saito et al., 2010). Eventually, the mitochondrial membrane permeability transition (MPT) occurs leading to matrix swelling and lysis of the outer mitochondrial membrane (Hanawa et al., 2008; Jaeschke et al., 2012a; Kon et al., 2004; Saito et al., 2010). Mitochondrial lysis leads to the release of apoptosis-inducing factor (AIF) and endonuclease G (EndoG) from the intermembrane space (Bajt et al., 2004, 2006; Cover et al., 2005). These two endonucleases then translocate to the nucleus leading to fragmentation of nuclear DNA and, ultimately, hepatocyte cell death by oncotic necrosis (Bajt et al., 2011; McGill et al., 2012).

1.3.3 Importance of hepatocyte models

While the mechanisms of liver injury following acetaminophen toxicity are well described in mice and man, knowledge into the mechanisms leading to liver injury following idiosyncratic drug

induced liver injury is lacking. Furthermore, hepatotoxicity is the most common cause of drug failure during development or clinical trials. Thus, to prevent a significant expenditure of financial and other resources, drug development companies must be able to determine early on in the development process which drugs will cause hepatotoxicity, and which drugs will not. Therefore, convenient, reliable, and inexpensive models, such as cell culture models, must be developed to accurately identify hepatotoxic drugs before the clinical phase.

One of the more commonly used hepatocyte cell line is the HepG2 cell line. Since the discovery of this cell line in 1979 (Aden et al., 1979), it has been used extensively in research, including drug metabolism studies. One major drawback, however, is that the HepG2 cell line, while beneficial for many aspects of liver study, do not possess a full complement of the drug metabolizing enzymes, cytochrome-P450s (Wilkening et al., 2003), limiting their usefulness for these studies. Another human hepatoma cell line, HepaRG, was identified and shown to express a level of cytochrome P450s more consistent with primary human hepatocytes (Aninat et al., 2006; Gripon et al., 2002), making them a superior choice for studies of drug metabolism, relative to HepG2. While primary human hepatocytes remain the gold standard for hepatocyte cell culture studies, they are only sporadically available, require specialized isolation techniques and do not tolerate the freeze/thaw cycle well (Rijntjes et al., 1986). Thus, HepaRG cells provide an attractive alternative to primary cells. Even still, lengthy growth and differentiation process limits their usefulness for quick studies. To overcome this, a pre-differentiated cryo-preserved HepaRG cell line was developed, dramatically decreasing the growth period, and increasing their usefulness.

The final chapter of this dissertation will be dedicated to the exploration of the use of the pre-differentiated HepaRG cell line for the studies of drug metabolism, specifically for studies of acetaminophen toxicity.

2. PLASMA BIOMARKERS OF ISCHEMIA-REPERFUSION INJURY IN HUMAN LIVER TRANSPLANTATION

2.1 INTRODUCTION:

Liver transplantation (LT) remains the only therapeutic option for patients with end-stage liver disease (ESLD). During LT, the donor liver undergoes a period of ischemia during harvest and cold storage up until the time of reperfusion in the recipient. This ischemic period consists of both warm and cold ischemia. Paradoxically, the return of blood flow to the ischemic organ predisposes it to injury.

Ischemia-reperfusion injury (IRI) has been described in multiple organs. In the mouse liver, the reperfusion period itself is relatively well tolerated as demonstrated by low levels of ALT for several hours following reperfusion (Yang et al., 2014). However, this low level of injury ultimately initiates an inflammatory cascade through the release of cellular debris, activation of Kupffer cells, and finally, the recruitment of neutrophils, which are responsible for necrotic cell death (Ellett et al., 2009; Jaeschke and Farhood, 1991b). In human patients undergoing liver transplantation, relatively little is known about IRI. This is due, in part, to the fact that invasive biopsies at extended time-points following transplantation are not possible in these patients. Therefore, a non-invasive method for describing IRI in human transplant patients is required.

Recently, circulating biomarkers have been used to describe molecular mechanisms and events following several types of liver injury, such as cholestasis and drug induced liver injury (DILI) (Antoine et al., 2012; McGill and Jaeschke, 2014; Woolbright et al., 2013). These biomarkers accurately describe both mode and mechanisms of cell death during these conditions, and also show promise in predicting outcome (McGill et al., 2012). Previous results from our laboratory has demonstrated that these same biomarkers can be used to accurately describe the events occurring following IRI in rodents (Yang et al., 2014). Because these biomarkers are non-invasive,

and can be easily measured in plasma, they represent an ideal technique to describe the events contributing to liver injury following LT in humans.

While other groups have also used this approach, their studies have been limited to earlier reperfusion times (Ilmakunnas et al., 2008, 2009; Pesonen et al., 2000; Ulukaya et al., 2010), following transplantation. However, in the rodent model of IRI, a model often used to recapitulate human transplantation, peak neutrophil infiltration and extravasation doesn't occur until 6 hours post reperfusion, and peak injury doesn't occur until 24 hours post reperfusion. Therefore, a more comprehensive time course is necessary to fully understand the events which occur following LT in humans. In addition, there are currently no studies which evaluate multiple biomarkers for a prolonged time course. Thus, an accurate clinical picture of the cellular events that occur several days following transplantation is lacking. Therefore, we sought to obtain a comprehensive characterization of the cellular events which occur following liver transplantation by evaluating biomarkers known to accurately describe extent of injury (ALT, miRNA-122), mode of cell death (cytokeratin-18), and mitochondrial involvement (GDH, mtDNA), in patients undergoing liver transplantation before, during, and up to 72 hours following the procedure. Furthermore, we evaluated the role of neutrophils in the post-reperfusion injury process by evaluating CD11b expression, ROS production, and phagocytic capability, all parameters of neutrophil activation.

We found that in contrast to the mouse model of IRI, most of the injury occurs within several hours of reperfusion. Importantly, we found no evidence for the involvement of neutrophils in this process, but rather a trend toward the decrease of neutrophil involvement. Thus, we conclude that the mouse model of IRI is not a good surrogate for the study of liver transplantation.

2.2 PATIENTS AND METHODS:

Study Design: All consenting patients undergoing liver transplantation for any reason at the University of Kansas Hospital were included in this study. Blood from patients enrolled in this study was collected at the following times: Pre-OLT (≤6hrs before procedure), anhepatic period, 0.25, 0.5, 1, 6, 12, 24, 48, and 72 hours post-reperfusion. At each time point, blood was collected in a red top tube (no additives) for serum, and a green top tube (heparin) or pink top tube (EDTA) for plasma. Upon collection, blood was stored at 4^{o}C until procurement by study personnel, at which point blood tubes were centrifuged and plasma/serum was aliquoted and stored at -80^{o}C until use. All procedures conducted in this study were done with approval by, and in accordance with, the Institutional Review Board at the University of Kansas.

Biochemistry: ALT was measured using a commercially available kit (Pointe Scientific, Roche, IL) according to the manufacturer's instructions. GDH was measured using the modified method of Passonneau and Lowry as previously described (McGill et al., 2012).

Mitochondrial DNA: DNA from serum was isolated using the QiaAMP Mini Blood Kit (Qiagen, USA) according to the manufacturer's instructions. Isolated DNA was then subjected to qPCR using primers for the mitochondrial-DNA specific gene cytochrome C oxidase subunit III (CytC; Fwd-ATGACCCACCAATCACATGC, Rev-ATCACATGGCTAGGCCGGAG). Quantification of mtDNA was compared to a standard curve consisting of known amounts of DNA isolated from primary human hepatocytes as previously described (Xie et al., 2014a).

Nuclear DNA fragments: Nuclear DNA fragments were measured using a commercially available cell death detection kit (Roche, Indianapolis, IN) according to the manufacturer's instructions. This ELISA kit uses a primary anti-histone antibody and a secondary anti-DNA antibody. Upon addition of substrate, the absorbance at 405nm over 1 hour was measured and compared to control (pre-OLT sample for each patient).

miRNA-122: qPCR was used to measure miRNA levels as described previously (Starkey Lewis et al., 2011).

HMGB1 and cytokeratin: Total HMGB1 and cytokeratin-18 (cleaved and full-length) were measured by LC-MS/MS as described previously (Antoine et al., 2009).

Neutrophil assays: All neutrophil assays were performed within 6 hours of the blood draw. Neutrophil activation was measured using flow cytometry to identify neutrophils expressing the CD11b surface marker. Whole blood was incubated on ice with saturating concentrations of PE-labeled anti-CD11b antibody. Red blood cells were subsequently lysed and neutrophils expressing CD11b were identified via flow cytometry. The oxidative burst assay was used to measure production of ROS from activated neutrophils. Briefly, whole blood was incubated with PBS, PMA, or *E. coli* at 37^{o}C for 10 min. DHR-123 was then added, followed by a second ten minute incubation period. The samples were washed, red cells lysed, centrifuged, and the pellet reconstituted. In the presence of ROS, DHR-123 is converted to its fluorescent metabolite rhodamine-123. Flow cytometry was then used to quantitate production of ROS as a function of increased fluorescence. The neutrophil phagocytosis assay was used to assess neutrophil

function. Whole blood was incubated with FITC-labelled *E-coli* for 15 minutes. Flow cytometry was used to identify neutrophils that have phagocytosed FITC-labelled *E. coli.*

2.3 RESULTS:

Patient Data. Consenting patients undergoing transplant for any etiology of ESLD were included in this study. The age of patients in this study ranged from 19 to 69 (mean = 57) and consisted of 47 males and 25 females. The most common diagnosis was viral hepatitis (HCV) with or without the presence of other confounding factors. Patient data is summarized in Table 2.3.1. Data from one consenting patient was excluded due to immediate post-operative complications (thrombosis). Every attempt was made to collect each time point for every patient but some time points were missed in order to maintain standard of care. The most commonly missed time point was +72 hours.

Time course of injury following LT. We first set out to describe the time course of injury following OLT in humans. In contrast to the rodent model of IRI, in which ALT remains low during the early time point and peaks at later time points, we found a sharp rise in ALT at 1hr (446±49 U/L) followed by a gradual decline over the next 72 hours (Figure 2.3.1A). Previous studies have demonstrated that miRNA-122 is a more sensitive indicator of liver injury than ALT (Laterza et al., 2009), so we measured this biomarker to confirm this pattern of injury. Again, we found, a similar pattern: a sharp rise to maximum injury (11.7±1.7 U/L) at 1 hour followed by a gradual but steady decline over the next 72 hours (Figure 2.3.1B). The similarity between ALT and miRNA-122 is best observed when compared directly (Figure 2.3.1C).

Mitochondrial injury during OLT. Previous studies have implicated mitochondrial injury as an initiator of cell death following periods of ischemia (Lemasters et al., 1997; Theruvath et al., 2008). To test the possibility that injury following OLT is a result of mitochondrial injury, we measured

Table 2.3.1 Patient Data

Median Age (Range)	57 (19-69)
M/F	47/25
Diagnosis:	
AIH	2 (2.8%)
Alcoholic Cirrhosis	8 (11.1%)
Cryptogenic Cirrhosis	1 (1.4%)
Hepatitis C	37 (51.4%)
Alone	8 (11.1%)
+ HCC	16 (22.2%)
+ Alcoholism	9 (12.5%)
+ Both	3 (4.2%)
+ NASH	1 (1.4%)
HCC	2 (2.8%)
Alone	1 (1.4%)
+ NASH	1 (1.4%)
NASH	8 (11.1%)
PBC	2 (2.8%)
PSC	8 (11.1%)
PLD	1 (1.4%)
Wilson's Disease	1 (1.4%)
A1AT Deficiency	1 (1.4%)
Biliary Atresia	1 (1.4%)
Warm Ischemia Time (min)	28.5±0.8
Cold Ischemia Time (min)	325.4±10.6

Table 2.3.1. Patient Data. Representative data for patients undergoing orthotopic liver transplantation including diagnosis as well as cold and warm ischemic times

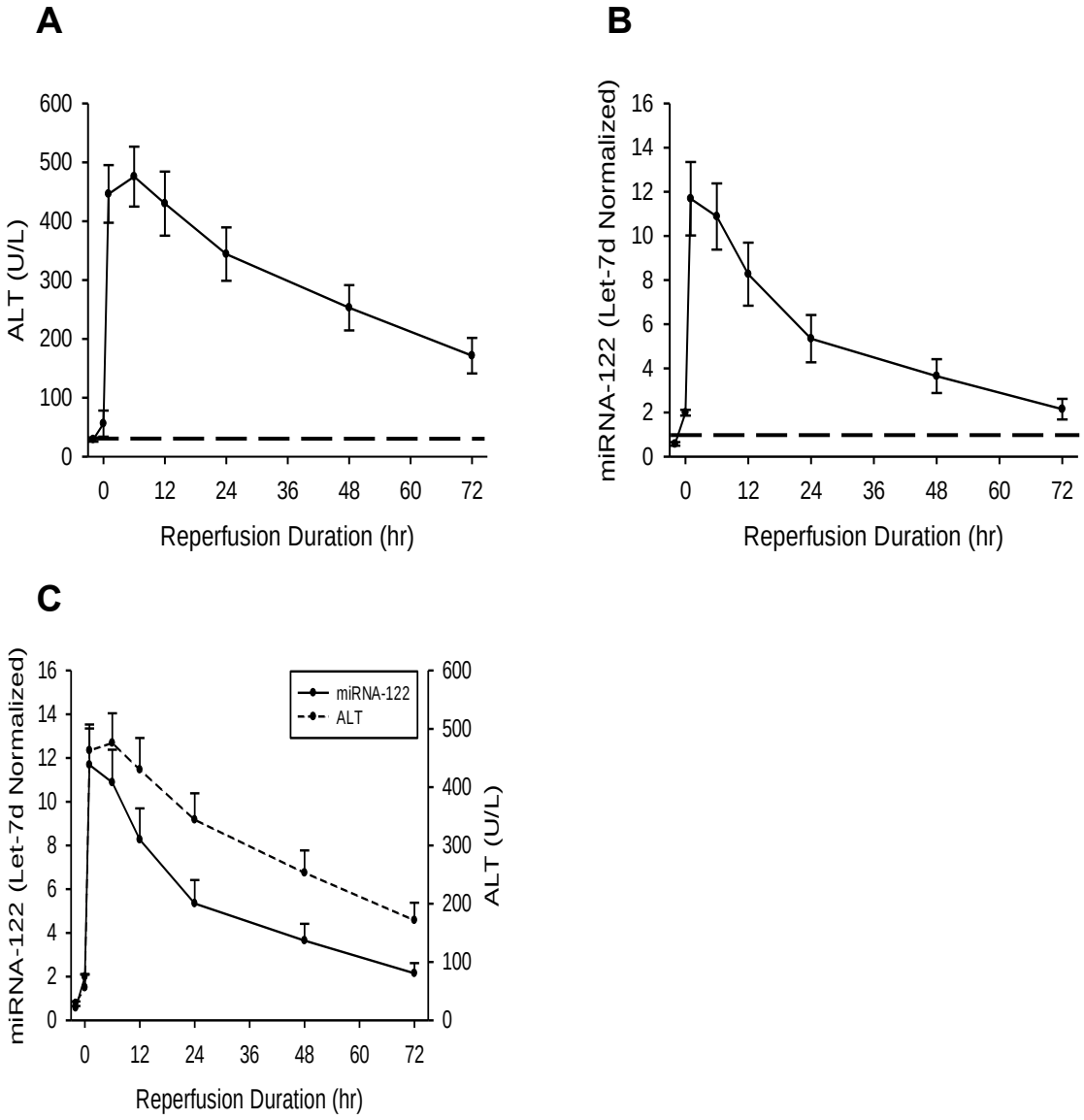

Figure 2.3.1. Time course of reperfusion injury following transplantation. Plasma levels of (A) ALT and (B) miRNA-122 from ≤6hrs before to 72 hours after liver transplantation. (C) ALT and miRNA-122 for these same time points on the same graph to show correlation of miRNA-122 and ALT. Data from each time point is represented as average ± SEM for 43-74 patients (ALT) or 18-25 patients (miRNA-122). Horizontal dashed bar represents average of healthy volunteers.

the matrix macromolecules glutamate dehydrogenase and mitochondrial DNA. Upon mitochondrial injury and cell death, these macromolecules would be released into the sinusoids and systemic circulation. Interestingly, we found that peak mtDNA for CytC reached 7.5±1.3 ng/ml and occurred 1 hour following reperfusion (Figure 2.3.2A). In contrast, peak levels of GDH (153±27 U/L) didn't occur until 24 hours post-reperfusion (Figures 2.3.2B&C).

Nuclear DNA fragmentation. In addition to GDH and mtDNA, mitochondrial injury leads to the release of endonuclease G, causing nuclear fragmentation and cell death (McGill et al., 2012). To explore the relationship between mitochondrial injury and nuclear DNA fragmentation following transplantation, we measured the amount of DNA fragments in plasma from these patients. As with ALT, miRNA-122, and mtDNA, we found a sharp increase in DNA fragmentation at 1 hour post-reperfusion (~700 fold increase vs. control) which gradually reached baseline over the next 3 days (Figure 2.3.2D). Taken together, these data indicate that the majority of injury following OLT is due to ischemia, rather than reperfusion injury.

Necrosis, not apoptosis, is the predominant mode of cell death. Despite substantial evidence to the contrary, many studies still point to apoptosis as the primary mode of cell death following ischemic injury. To differentiate between these two forms of cell death in the current study, we used levels of cytokeratin-18, an intermediate filament protein. During apoptosis, CK-18 is cleaved by caspases into its shorter form, ccK-18. However, during necrosis, the full-length form (FK-18) is passively released. We found a dramatic increase in both the caspase-cleaved and full-length forms of cytokeratin (2950±580 vs 29,300±4200 U/L, respectively) 1 hour following reperfusion (Figures 2.3.3A&B). While the increase in ccK-18 was unexpected, when compared to the FK-18

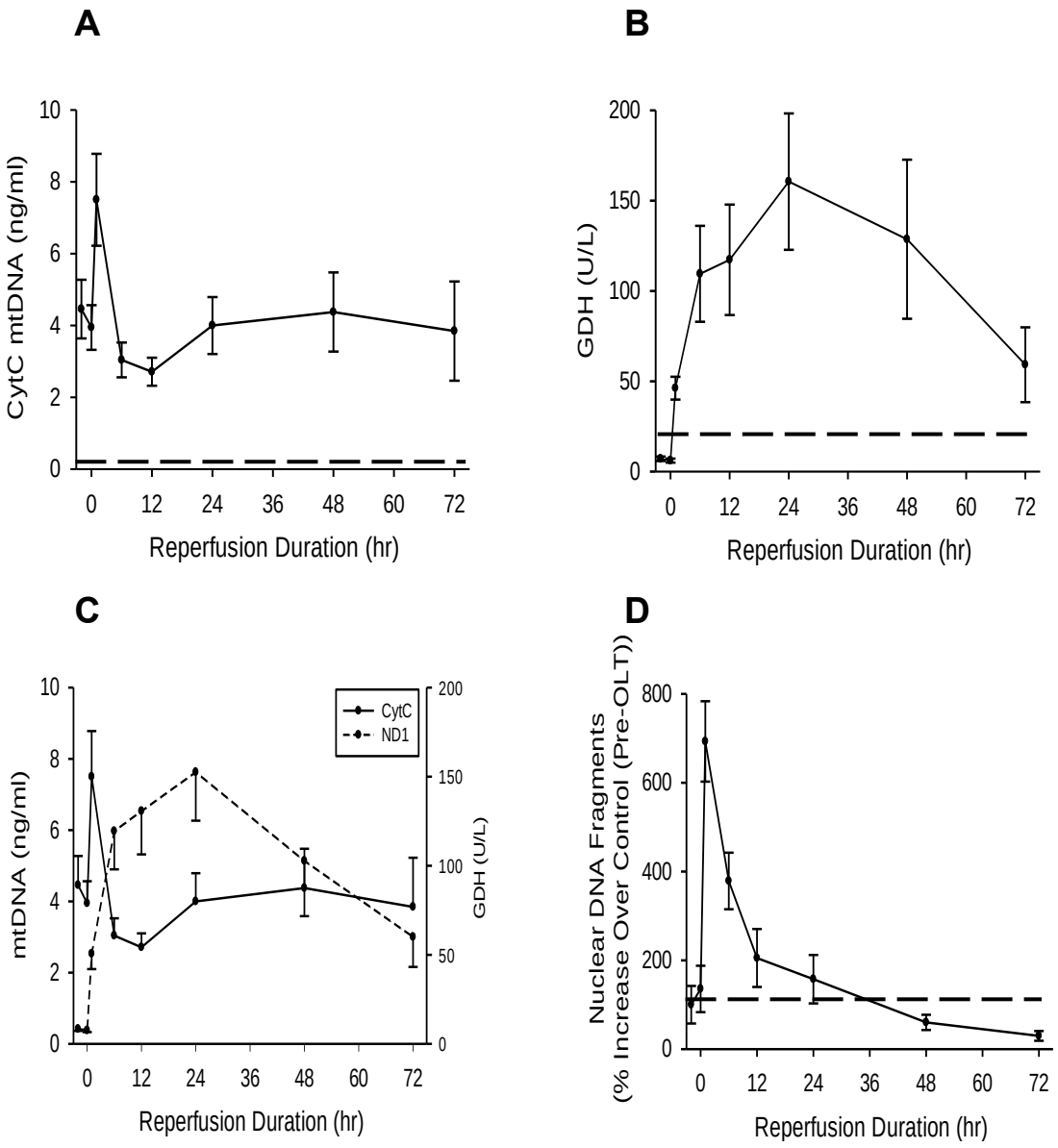

Figure 2.3.2. Mitochondrial biomarkers following transplantation. Plasma levels of (A) Cytochrome C oxidase subunit III mtDNA and (B) GDH from ≤6hrs before to 72 hours after liver transplantation. (C) mtDNA and GDH for these same time points on the same graph demonstrates lack of correlation between mtDNA and GDH. (D) Nuclear DNA fragments from ≤6hrs before to 72 hours after liver transplantation. Data from each time point is represented as average ± SEM for 44-59 (CytC) or 43-74 patients (GHD). Horizontal dashed line represents average of healthy volunteers.

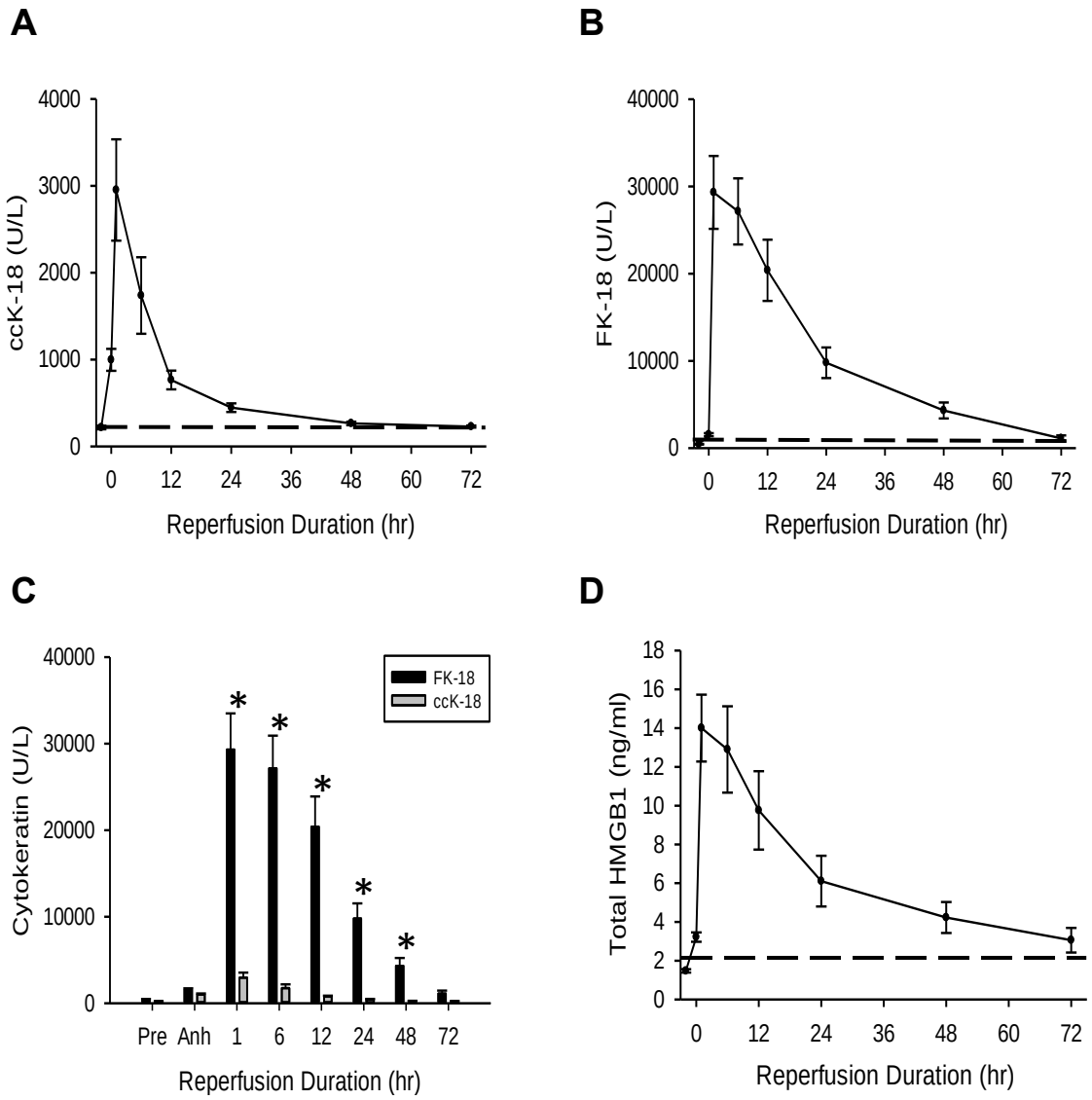

Figure 2.3.3. Necrosis predominates following reperfusion. Plasma levels of (A) caspase cleaved (ccK-18) and (B) full length (FK-19) cytokeratin-18 from ≤6 hours before to 72 hours after liver transplantation. (C) Comparison of ccK-18 and FK-19 showing significant elevation of FK-18 over ccK-18 at each post-reperfusion time point except 72hr. 'Pre' and 'Anh' represent the pre-OLT and anhepatic blood draws, respectively. (D) Plasma levels of nuclear DNA fragments for the same time points. Data from each time point is represented as average ± SEM for 18-25 patients. Horizontal dashed line represents average of healthy volunteers. * = $p<0.05$

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at each time point, the contribution of ccK-18 to overall cell death is only approximately 10%. (Figure 2.3.3C). Furthermore, the nuclear protein HMGB1 also peaks at this time point (15.7±2.4 ng/ml). Since HMGB1 is passively released during necrosis, this increase gives further evidence to the necrosis at this time point (Figure 2.3.3D). Taken together, this data indicates that necrosis, rather than apoptosis predominates during all time points.

No evidence for neutrophil involvement in OLT injury. In the rodent model of IRI, liver injury is largely dependent on Kupffer cell activation and neutrophil recruitment/activation (Ellett et al., 2009; Jaeschke and Farhood, 1991b; Nace et al., 2013). Therefore, we set out to determine the extent to which neutrophils are involved in injury following LT in humans. To do this, we measured neutrophil activation as determined by CD11b expression, (Figure 2.3.4A), ROS production (Figure 2.3.4B), and phagocytic capability (Figure 2.3.4C). In contrast to markers of injury and cell death, which peaked early and gradually subsided, we found no significant change over the 72 hours in these parameters following LT. In fact, all markers of neutrophil activity were observed to be elevated initially, and then trend downward during this period. When compared to ALT activity, there was no significant change in neutrophil activity at the same time as peak ALT concentration suggesting liver injury is not a result of neutrophil activation (Figure 2.3.4D). Taken together, these data suggest no relevant role for neutrophils in the injury process following OLT, particularly in the later time points.

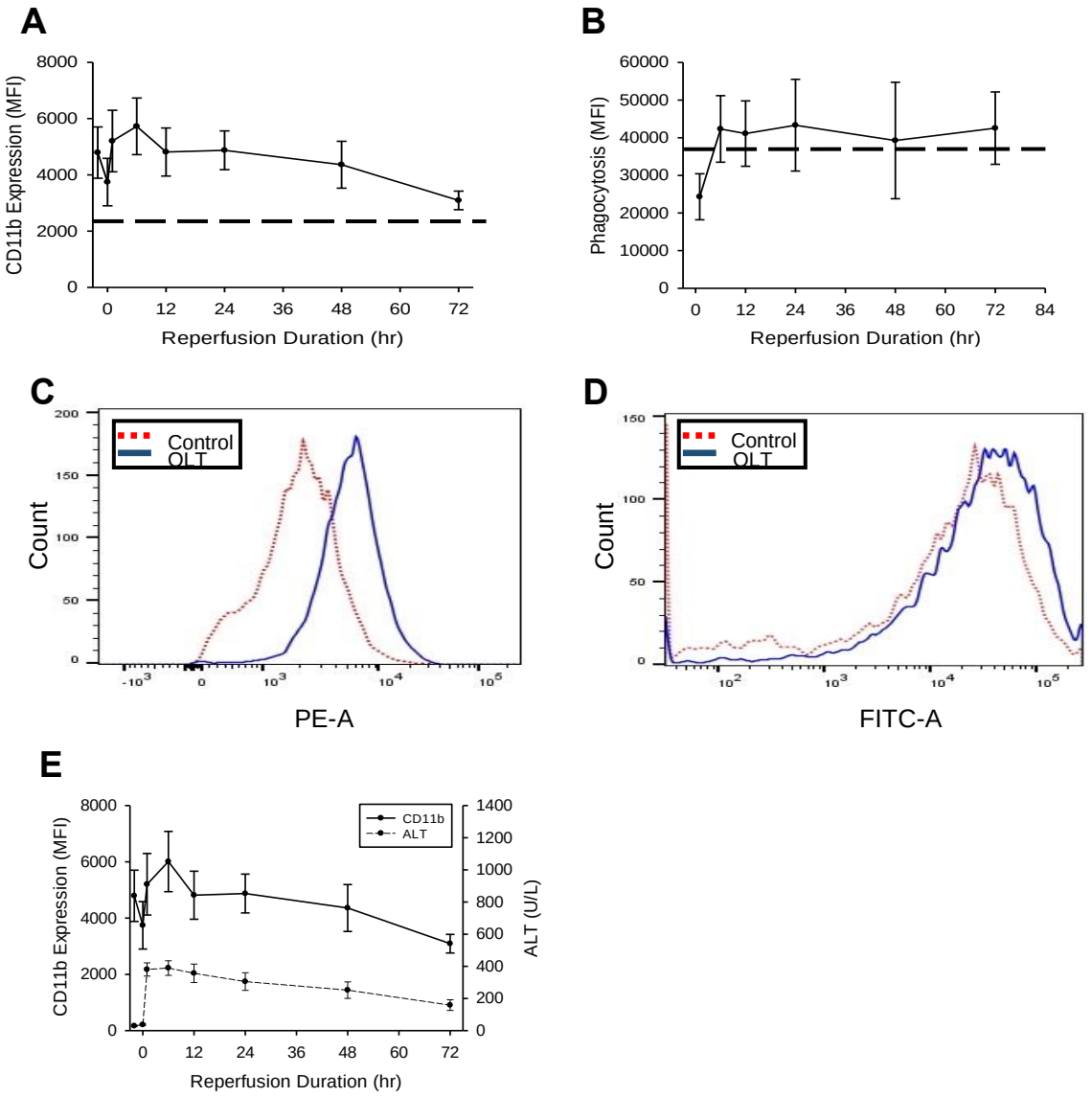

Figure 2.3.4. Assessment of CD11b expression and phagocytosis following transplantation. Measurement of (A) CD11b expression and (B) phagocytic activity in neutrophils following OLT in human patients at various time points before and after reperfusion. Representative histogram showing shifts in (C) CD11b expression or (D) phagocytosis at 6 hours post-reperfusion relative to healthy volunteers. (E) Comparison of neutrophil activation (as measured by CD11b expression) and ALT, demonstrating that peak ALT injury occurs in the absence of increased neutrophil activity. Data from each time point is represented as average ± SEM for 6-7 patients (CD11b and phagocytosis) or 43-74 patients (ALT). Horizontal dashed bar represents average of healthy volunteers.

2.4 DISCUSSION:

In the present study, we set out to examine the cellular events leading to reperfusion injury in humans following OLT. To do this, we used several circulating plasma biomarkers which are predictive of mode and mechanism of cell death in other disease models, as well as the mouse model of IRI (Antoine et al., 2012; McGill and Jaeschke, 2014; Woolbright et al., 2013; Yang et al., 2014). Because the role of neutrophils following OLT in humans is currently unknown, we also measured markers of neutrophil activation including CD11b expression, phagocytic capability, and ROS production.

Based on the results of this study, it appears that there is very little reperfusion injury in the average transplant patient; most of the observed increase in ALT is likely a result of the ischemic period. Previous studies have demonstrated that during ischemia, the lack of oxygen delivery causes the hepatocyte to switch from aerobic metabolism to anaerobic metabolism leading to an increase in lactic acid concentrations, a rapid decrease in ATP levels, decreased intracellular pH, and an inability of the cell to maintain homeostasis (Barbiro et al., 1998; Lemasters et al., 1987). As a result of these changes, a small percentage of cells die during ischemia. However, the majority of cell death is caused during the reperfusion period during which osmotic forces drive extracellular fluid into the cell in an attempt to normalize the metabolic perturbations which occurred as a result of ischemia. This results in cellular swelling and oncotic necrosis.

In our study, nearly all of the markers of injury peak very shortly after reperfusion, which is in direct contrast to the rodent model of IRI in which injury peaks 12-24 hours post-ischemia. This rapid peak likely represents a ‘wash-out’ period where reperfusion of the previously ischemic liver causes release of these markers from necrotic cells. However, the use of preservative solutions and cold storage minimize the degree of damage (Vine et al., 1989), thus, the overall degree of injury

is quite low relative to models of warm ischemia, such as the rodent IRI model, and hypoxic hepatitis (Weemhoff et al., 2017). This also explains the lack of a robust immune response in that neutrophils are recruited to eliminate cellular debris from necrotic cells and in the process, release ROS, such as hypochlorous acid (Hasegawa et al., 2005), which accidentally kill injured hepatocytes which may have otherwise recovered from the initial hypoxic insult. In the warm ischemia mouse model, there is extensive injury and thus a robust immune response. Furthermore, it could be assumed that the number of unhealthy, but not dead, hepatocytes are greater in models of warm ischemia than in patients following OLT as a result of the use of preservative solutions which reduce the ionic changes described above, decreased operative time, and decreased storage time.

Previous studies have suggested that the mitochondria play a key role in ischemic injury through opening of the MPTP (Kim et al., 2003; Lemasters et al., 1997; Theruvath et al., 2008). Our data supports this hypothesis in that there is an elevation of mitochondrial specific macromolecules DNA and GDH, which would only be expected to be present in serum if mitochondrial rupture has occurred. Interestingly, whereas mtDNA shows a rapid increase and decline, GDH actually shows a rapid, yet sustained increase (Figures 2.3.2A & 2.3.2B). However, since there is no significant difference between 6 and 24 hours, and a downward trend is noted after 6 hours, this discrepancy could be attributed to different half-lives of the macromolecules in plasma.

In contrast to the rodent model of IRI, in which significant injury occurs approximately 6 hours after reperfusion and is correlated with neutrophil infiltration (Jaeschke et al., 1990), human patients undergoing OLT experience peak injury much earlier (approximately 1 hour post-reperfusion) and does not correlated with an increase in neutrophil activity (Figures 2.3.1A and 2.3.4E). In fact, in these patients, markers of neutrophil activity actually tend to decrease following

reperfusion. This unexpected finding underscores the difference between the two models of IRI. In the rodent model, maximum damage is created by occlusion of the portal triad supplying the left lateral and medium lobe, and the liver undergoes warm ischemia, while during transplantation, every effort is made to minimize tissue injury though the use of preservatives such as University of Wisconsin solution, minimizing operative time, and optimizing donor liver for the recipient. Importantly, livers stored in UW solution prior to transplant are stored at sub-normothermic temperatures, which slows basal metabolic rate and delays the depletion of ATP (Jain et al., 2008; Reckendorfer et al., 1992). Thus, the two major inciting causes of reperfusion injury in the mouse model are ameliorated in human patients. Therefore, very little injury occurs, which would not be expected to generate a robust inflammatory response.

In addition to storage and operative protocols which minimize damage, other factors may play a role in the pattern of neutrophil activation observed in the current study. During experimental induction of IRI in the mouse model, the livers of the ischemic mice are healthy but then greatly injured, leading to neutrophil infiltration and exacerbation of injury (Jaeschke et al., 1990). On the other hand, human patients preparing to receive a liver transplant already have injured livers, which might explain why pre-operative neutrophil activity is already high (Figure 2.3.4A). Following transplantation, the diseased liver is replaced with a healthier liver, causing neutrophil activity to actually decrease over the first few days. In addition, many of these patients are on immunosuppressive medications which may contribute to the tapering of neutrophil activation observed following reperfusion.

A final point of interest in this study is the observation that necrosis, not apoptosis predominates during all time points following reperfusion in OLT. While many studies point to apoptosis as the primary mode of cell death following ischemia, many of these studies rely solely on TUNEL

staining, which is not specific for apoptotic cell death. Histopathology can be used to differentiate between the two forms, but this is not practical for human patients following OLT. Thus, the cytokeratin-18 assay can be used. During apoptosis, caspases cleave cytokeratin-18 into a fragmented form, but during necrosis, cytokeratin-18 is passively released in its full-length form. Thus, the degree of elevation of each form can indicate which mode of cell death is predominating. In the current study, it is clear that necrosis, not apoptosis, is the mode of injury following reperfusion. This is in direct contrast with previous studies showing protection against injury when an experimental caspase inhibitor is added to the preservation solution (Baskin-Bey et al., 2007). However, protection is only afforded if added to the preservative solution, and at a concentration high enough to affect other proteases which may be active during necrosis (Schotte et al., 1999). Thus, therapeutic efforts aimed at minimizing injury should be directed towards minimizing necrosis, rather than apoptosis.

In the current study, we have characterized the mechanisms and mode of cell death following liver transplantation in humans. Despite minimal injury, there is still evidence for mitochondrial involvement in cellular injury which is in agreement with other studies. Furthermore, we have shown that necrosis, rather than apoptosis predominates following OLT. Overall, the current study underscores the importance of choosing an animal model which accurately reflects the clinical experience.

3. PLASMA BIOMARKERS TO STUDY MECHANISMS OF LIVER INJURY IN PATIENTS WITH HYPOXIC HEPATITIS

3.1 INTRODUCTION

Hypoxic hepatitis (HH) is a condition resulting from prolonged periods of hypoxia to the liver. Hypoxic hepatitis is recognized clinically by a hypoxic insult accompanied by sharp increases in plasma transaminase activity to >20 times normal, and lack of other confounding etiologies of liver disease, such as viral or drug-induced hepatitis (Henrion et al., 2003; Horvatits et al., 2013). Typically, the inciting cause for HH involves an episode of cardiogenic, circulatory, or respiratory failure leading to decreased oxygen delivery to the liver (Henrion et al., 2003). Hypoxic hepatitis represents a serious source of morbidity and mortality, with a prevalence of approximately 10% in intensive care patients (Fuhrmann et al., 2010). HH resolves with treatment of underlying causes, but mortality can reache 50-60% within one month (Fuhrmann et al., 2010; Hawker, 1991; Horvatits et al., 2013).

Despite the prevalence and mortality of HH, very few studies have examined the cellular mechanisms underlying liver injury, in part due to the lack of an animal model. The most relevant model employed experimentally is the hemorrhagic shock/resuscitation model in which rodents are hemorrhaged to a hypotensive state (low flow ischemia) for a period of time, followed by a resuscitation period, after which cellular mechanisms of liver injury can be assessed. A major problem with this model is that liver injury does not always approach the degree of injury seen during clinical cases of HH, during which ALT levels easily exceed 20 times normal (Jaeschke and Farhood, 2002; Wetzel et al., 2014; Zuckerbraun et al., 2005), indicating that the model does not completely recapitulate what is occurring in a clinical setting. Additionally, HH can be precipitated by a number of causes other than hemorrhagic shock (Henrion et al., 2003). Furthermore, most of these studies are limited in that they evaluate a time point shortly after

(<6hrs) resuscitation, rather than a prolonged time course. Therefore, a different approach to study the mechanisms of injury during HH is needed.

One such approach is the use of mechanistic biomarkers of liver injury, such as mitochondrial DNA, microRNA-122 (miRNA-122), total and acetylated HMGB-1 (acHMGB1), and the ratio of caspase-cleaved to full-length cytokeratin-18. Our laboratory and others have previously used this approach to characterize both modes of cell death (apoptosis vs. necrosis) and cellular mechanisms of liver injury in ischemia/reperfusion injury in mice, as well as acetaminophen toxicity and cholestasis in both mice and humans (Antoine et al., 2012; McGill et al., 2012; Starkey Lewis et al., 2011; Woolbright et al., 2015; Yang et al., 2014). In addition, a previous study has demonstrated that miRNA-122 may be a more sensitive marker of liver injury during HH in a porcine model of cardiogenic shock (Andersson et al., 2012). However, to date, no studies have employed the use of these biomarkers to characterize the mechanisms of liver injury following HH in humans. Therefore, the aim of the current study was to assess the mechanisms of liver injury following HH using circulating plasma biomarkers in order to better understand cellular mechanisms leading to injury in these patients.

3.2 PATIENTS, MATERIALS AND METHODS

Patient Characteristics and Study Design: Subjects with HH were selected from 266 patients who presented to Banner-University Medical Center Phoenix with initially suspected acetaminophen (APAP) toxicity. However, only those patients with peak plasma ALT > 1,000 IU/L whose hepatic necrosis was not caused by APAP (as determined by plasma APAP and APAP-protein adduct levels), medical history demonstrating a definitive history or strong likelihood of hypotension/shock, and who were subsequently diagnosed with HH were selected (total of 14 patients). A total of 15 age- and gender-matched subjects with APAP toxicity were selected among inpatients at the University of Kansas Medical Center or Banner-University Medical Center Phoenix as comparison. Patients with comorbidities contributing to liver injury (such as alcoholism or viral hepatitis) were excluded. Blood was obtained upon admission after informed consent, and then approximately every 24 hours. Because duration of hospitalization differed for each patient, for the purposes of this study, the day of peak injury (as assessed by clinically measured ALT levels) was considered 'day 0'. Following blood collection, blood tubes were centrifuged, plasma collected, frozen, and sent to the University of Kansas Medical Center or the University of Liverpool for analysis. All biochemical parameters reported are averages of maximum values, not necessarily values at the time of peak ALT. All patient samples were procured with approval by, and in accordance with the Institutional Review Board at both the University of Kansas and Banner Health Center.

Animals. C57Bl/6J mice were purchased from Jackson Laboratories (Bar Harbor, Maine) and treated with 700 mg/kg galactosamine/100 μg/kg *Salmonella enteritidis* endotoxin (Gal/ET). After 6 h, blood was obtained for measurement of plasma caspase-3 activities as described (McGill et

al., 2012). All experimental protocols were approved by the Institutional Animal Care and Use Committee of the University of Kansas Medical Center.

Biochemistry. ALT values reported for all subjects from Banner-University Medical Center Phoenix were from the hospital laboratory. ALT values for APAP patients from University of Kansas were measured in our laboratory using a commercially available kit (Pointe Scientific; Canton, MI). GDH activity was measured in our laboratory as previously described (McGill et al., 2012).

Mitochondrial DNA. Mitochondrial DNA was measured as previously described (McGill et al., 2012). Briefly, total DNA was isolated from plasma using the QIAamp DNA Blood Mini Kit (QIAGEN) and subjected to RT-qPCR using primers for human mtDNA specific NADH dehydrogenase (ND1: Fwd: ATACCCATGGCCAACCTCCT Rev: GGGCCTTTGCGTAGTTGTAT). Absolute quantification was achieved with use of a standard curve using known amounts of DNA from mitochondrial pellets obtained from primary human hepatocytes, isolated and processed as described previously (Xie et al., 2014a).

Nuclear DNA Fragments. Nuclear DNA fragments from HH subjects and healthy volunteers were measured using the cell death detection ELISA kit (Roche, Indianapolis, IN) according to the manufacturer's instructions (McGill et al., 2012). Change in absorbance over time at 405 nm was measured and values are reported as percent of control (healthy volunteers).

HMGB1 and cytokeratin-18. HMGB1 (total and acetylated) and cytokeratin-18 (cleaved and full-length) were measured by LC-MS/MS as previously described (Antoine et al., 2012, 2013).

miRNA-122. miRNA levels were measured by qPCR as previously described (Antoine et al., 2013; Starkey Lewis et al., 2011) and normalized to let-7d, is stably expressed in patients with acute liver injury and healthy volunteers (Antoine et al., 2013; Qi et al., 2012).

Caspase Activity. Caspase activity was measured using a fluorometric assay as described (Jaeschke et al., 1998). Briefly, plasma was added to caspase substrate (DEVD; 50μM final concentration) with or without inhibitor (Z-VAD-fmk; 10μM final concentration). Fluorescence was measured (excitation 480nm and emission 560nm) over one hour. Caspase activity was measured by subtracting activity with inhibitor from activity without inhibitor.

Cytokine Measurements. Plasma cytokines were measured by a multi-plex ELISA (Millipore, Billerica, MA) according to the manufacturer's instructions. Quality controls were run in duplicate with the samples and found to be within the normal range.

APAP-CYS Adduct Measurements. Plasma levels of APAP-CYS were measured using HPLC-ECD as previously described (James et al., 2009; Xie et al., 2015a). Time points as close as possible to Day 0 (peak ALT) for both APAP and HH patients were used in this analysis.

Statistics. All data are expressed as average ± SEM. Statistics was performed using 1-way ANOVA with appropriate ad hoc test, or t-test where appropriate.

3.3 RESULTS

Hypoxic hepatitis causes profound liver injury. All patient data is represented in Table 3.3.1. We first measured the time course of injury in these patients to ensure they fit the clinical profile of HH. We found that plasma ALT activities rapidly rose to peak injury (4082±606 U/L; Figure 3.3.1A) and steadily decreased over the next 5 days. When compared to healthy volunteers (HV), the difference in ALT was significantly higher at peak injury (4082±606 vs. 23.8±3.1 U/L) but not significantly different from patients with liver injury from APAP overdose (4082±606 vs. 5744±588 U/L; Figures 3.3.1A&B). Because ALT is not specific to the liver, and HH affects all organs simultaneously, we measured levels of the liver-specific microRNA-122 to ensure the majority of the ALT was from liver injury. Again, in HH patients, we found a dramatic increase to peak injury followed by a gradual but steady decline (Figure 3.3.1C). When compared to HV, levels of miRNA-122 in HH patients were significantly higher than controls (13.2±3.1 vs. 0.52±0.26, respectively; Figure 3.3.1D). To confirm liver injury was not caused by APAP, plasma levels of APAP-CYS were measured in both APAP and HH patients (Figure 3.3.2).

Hypoxic hepatitis causes hepatocellular necrosis. To differentiate whether liver injury is caused by necrosis or apoptosis, we measured plasma levels of full-length and caspase-cleaved cytokeratin-18. We observed a rapid increase in both forms of cytokeratin-18 at the time of peak ALT, followed by a gradual and steady decline over 5 days (Figure 3.3.3A&B). However, the magnitude of increase in the full-length form was approximately 18-fold higher than the caspase-cleaved form (45837±12085 vs 2528±1074) at the time of peak ALT. Furthermore, levels of full-length cytokeratin-18 were significantly higher than caspase-cleaved cytokeratin-18 at each time point except for day 5, demonstrating that necrosis predominates during HH (Figure 3.3.3C).

Table 3.3.1 Patient Data

Parameter	Hypoxic Hepatitis	Acetaminophen
% M:F	56:44	33:67
Median Age (Range)	39 (18-62)	35 (19-65)
Survival (%)	92.9 (13/14)	93.3 (14/15)
Median Hospital Stay (Range)	10.8 (2-30.2)	8 (3-19)
AST (U/L)	8574 ± 1696	9324 ± 1251
ALT (U/L)	4082 ± 606	5744 ± 588
Creatinine (mg/dL)	5.3 ± 0.9	3.1 ± 0.7
Bilirubin (mg/dL)	1.8 ± 0.4	9.7 ± 2.4*

Table 3.3.1. Patient characteristics. Clinical parameters and other characteristics of patients diagnosed with HH and acetaminophen toxicity. Peak values for AST, ALT, creatinine and bilirubin are reported as average ± SEM of peak value, not necessarily at the time of peak ALT. NR = Not Reported. * = $p<0.05$.

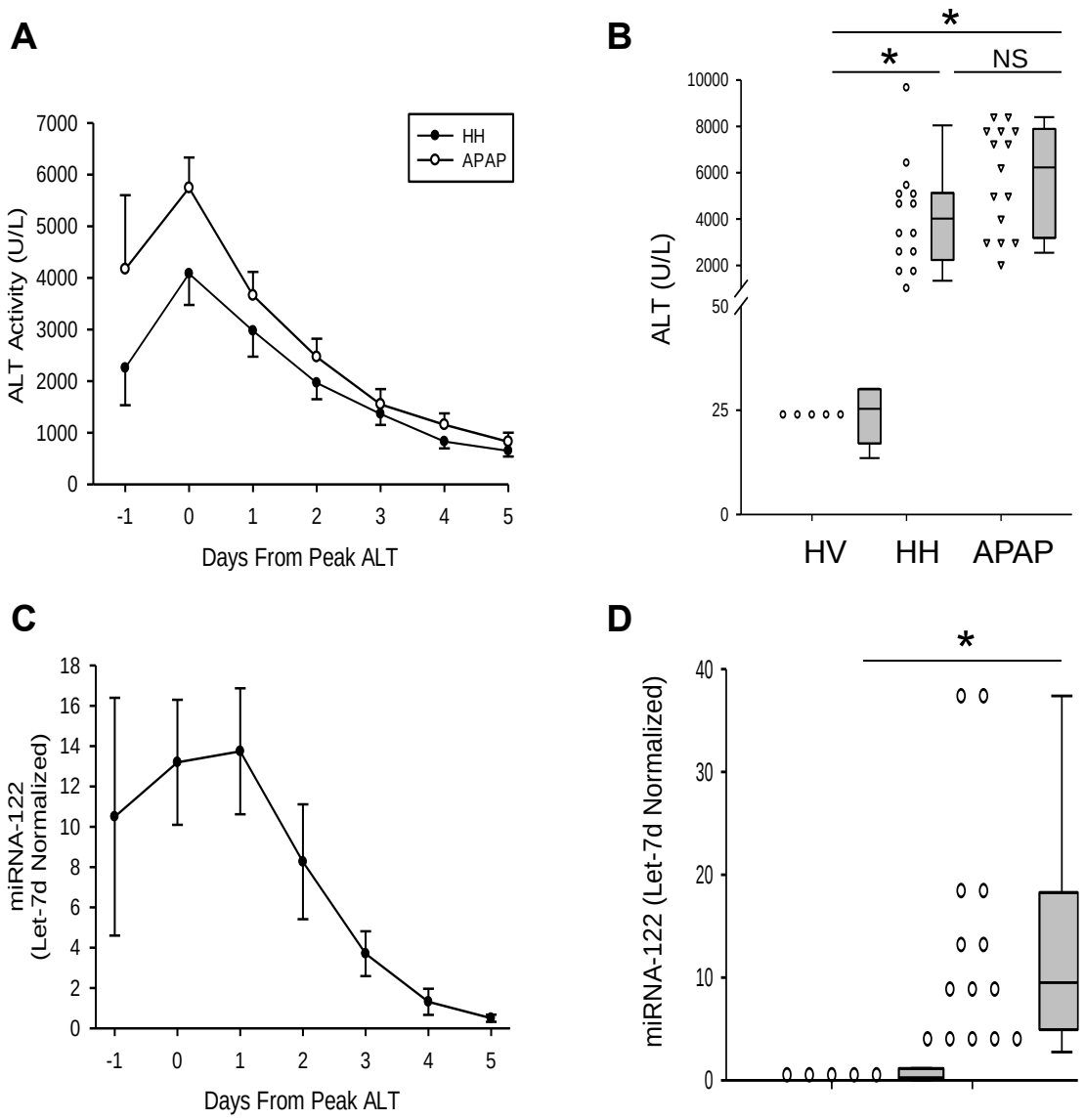

Figure 3.3.1. Time course of injury following hypoxic hepatitis. (A) Plasma levels of ALT in both HH and APAP patients from -1 to +5 days after peak injury. (B) Dot histogram comparing plasma levels of ALT between HH patients and HV or patients with APAP toxicity at the time of peak ALT. (C) miRNA-122 for patients with HH are shown from -1 to +5 days after peak injury. (D) Dot histogram comparing plasma levels of miRNA between HH patients and HV at the time of peak ALT. Line graph data are represented as average ± SE. Box plots show the 25th and 75th percentiles. Whiskers show 5th and 95th percentiles. * = $p<0.05$.

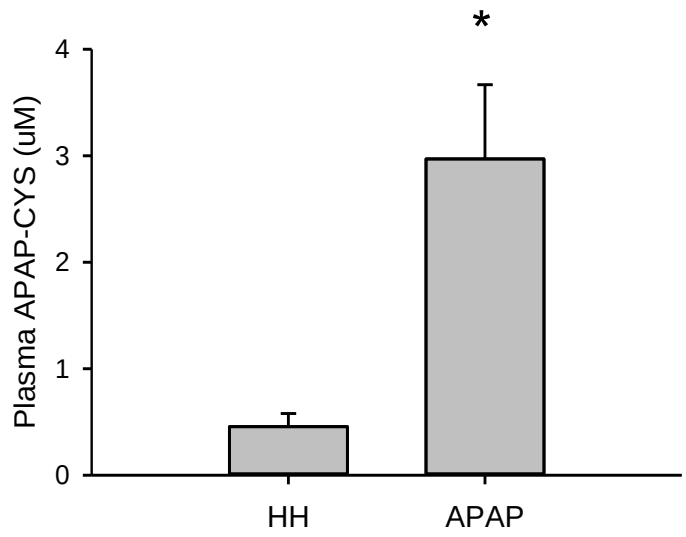

Figure 3.3.2 Plasma APAP-CYS adducts. Comparison of plasma APAP-CYS protein adducts in HH or APAP overdose patients at the time of peak injury. Data are expressed as average ± SE. * = $p<0.05$

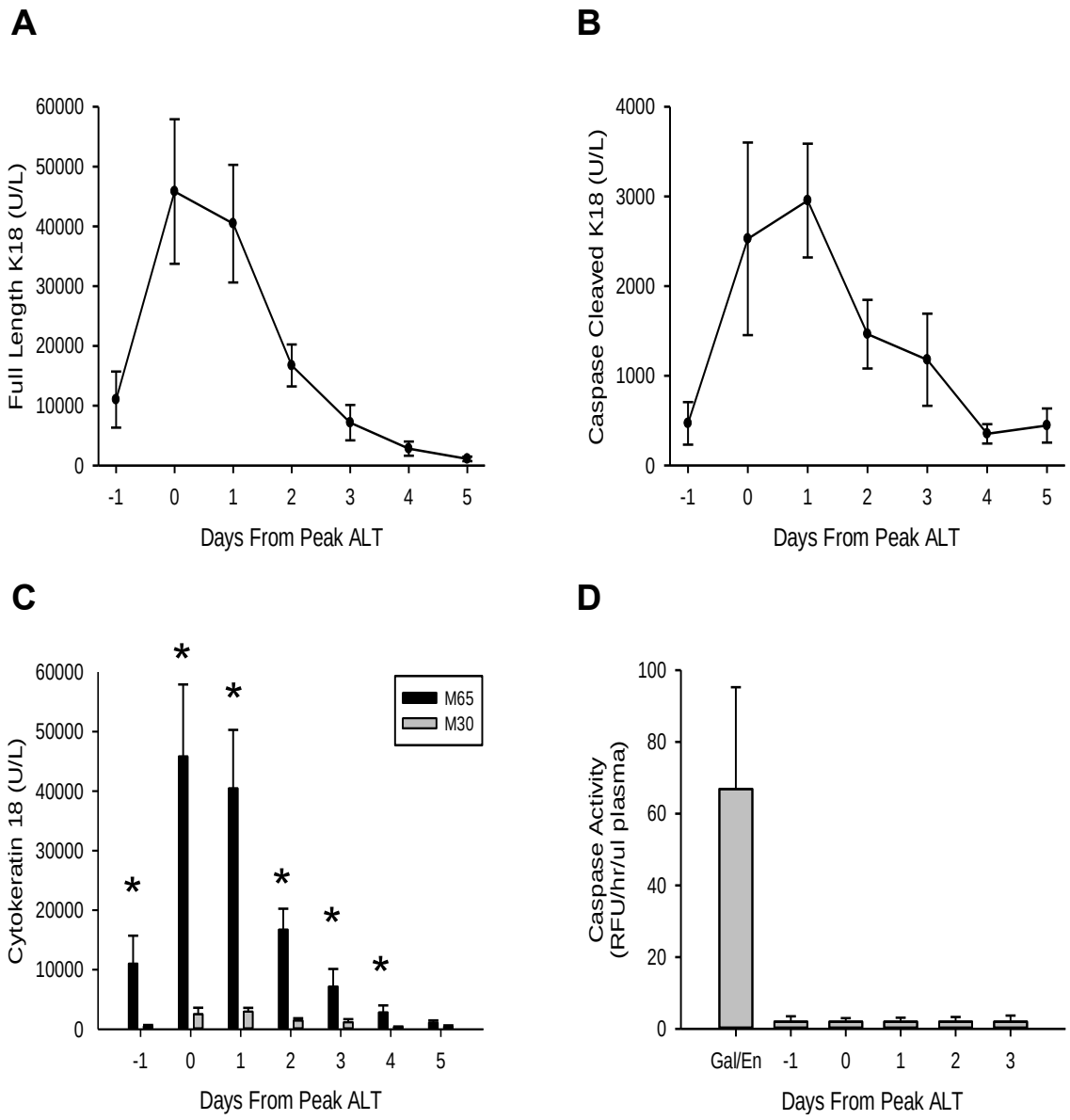

Figure 3.3.3. Necrosis predominates during hypoxic hepatitis. Plasma levels of (A) full-length cytokeratin-18 and (B) caspase-cleaved cytokeratin-18 from -1 to +5 days after peak injury. (C) Total cytokeratin-18 levels during the same time course demonstrating the relative contributions of each form of cytokeratin. (D) Caspase activity in plasma at various time points following peak injury. As positive control for apoptosis, plasma samples were obtained from mice treated with galactosamine/endotoxin for 6 h. Data are represented as average ± SE. * = $p<0.05$.

However, because there was an increase in caspase cleaved cytokeratin-18, we measured caspase activity in the plasma but found no measureable caspase activity in these samples (Figure 3.3.3D). Plasma from galactosamine//endotoxin (Gal/ET)-treated animals served as controls for parenchymal cell apoptosis (Jaeschke et al., 1998). The readily detectable caspase-3 activity in these animals suggested that if significant caspase-dependent apoptotic cell death occurs, plasma caspase-3 activity can be measured.

Mitochondrial injury occurs in HH. Numerous *in vitro* studies suggest mitochondria are targets of ischemia-reperfusion injury (Lemasters et al., 1997). To test this hypothesis in these patients, we measured levels of the mitochondria-specific biomarkers GDH and mitochondrial DNA (mtDNA). Because these macromolecules are located within the mitochondrial matrix, only mitochondrial damage would be expected to lead to their release in plasma (McGill et al., 2012). Accordingly, we found an increase in GDH which closely mimicked the increase in ALT – that is, a rapid increase at the time of peak ALT (1381±229) followed by a gradual but steady decline (Figures 3.3.4A, 3.3.5, 3.3.6, & 3.3.7). Similarly, plasma levels of mtDNA as measured by RT-PCR of the cytochrome c oxidase (CytC) gene showed a similar downward trend following peak injury (Figure 2.3.4C). Interestingly, neither levels of GDH nor CytC mtDNA in plasma of HH patients were elevated compared to APAP overdose patients (Figures 3.3.4B & D).

Nuclear DNA fragmentation in HH. Because mitochondrial injury during necrosis releases endonucleases leading to nuclear DNA fragmentation and cell death (Bajt et al., 2006), we measured plasma levels of nuclear DNA fragments. Our results show an increasing concentration of nuclear DNA fragments in the plasma of HH patients which peaks at day zero

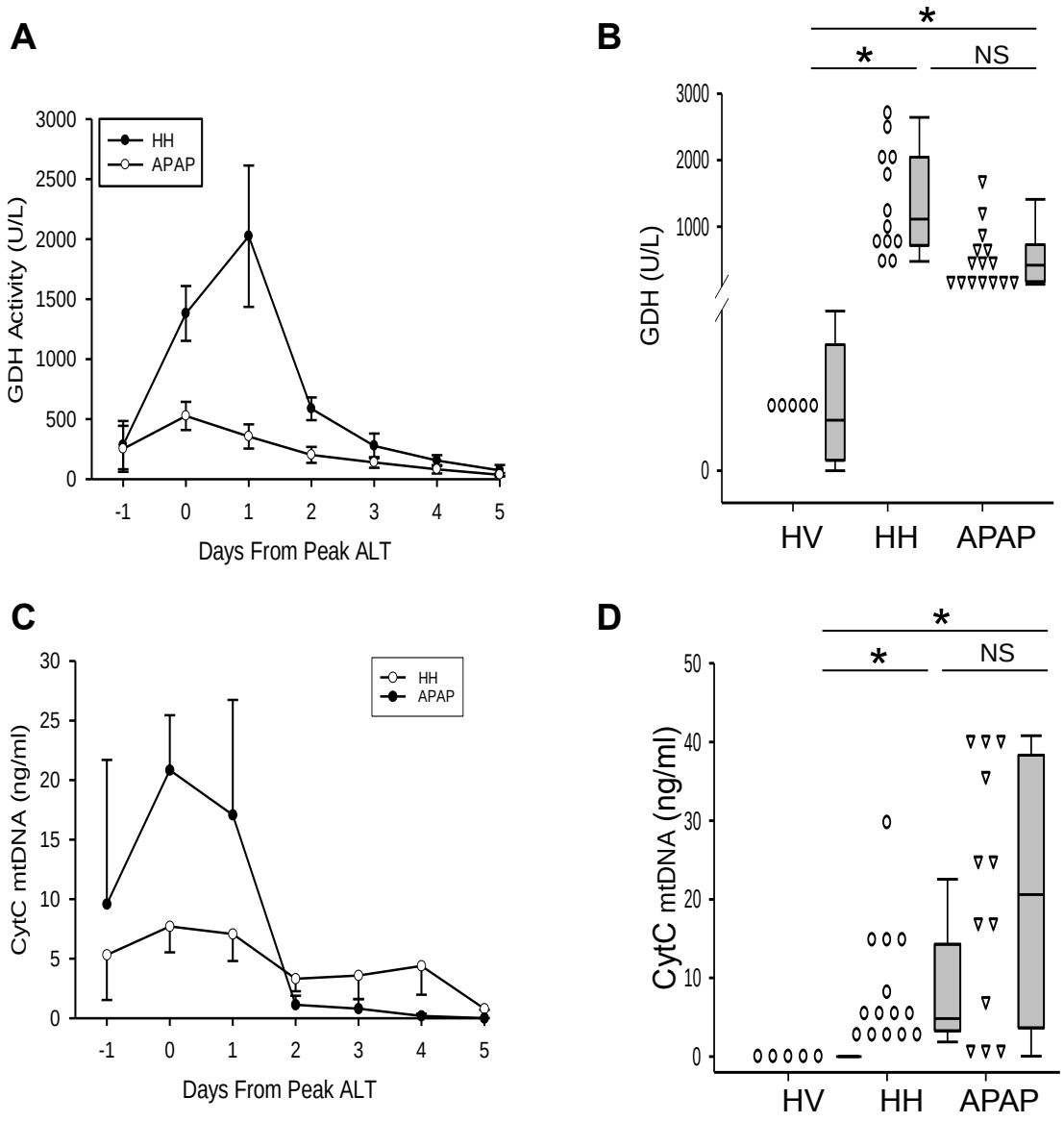

Figure 3.3.4. Mitochondrial injury occurs in hypoxic hepatitis. Plasma levels of (A) GDH and (C) cytochrome c oxidase (CytC) mtDNA for patients with HH or APAP overdose shown from -1 to +5 days after peak injury. Data are presented as average ± SE. Dot histograms comparing plasma levels of (B) GDH and (D) CytC mtDNA between HH patients and HV or patients with APAP toxicity at the time of peak ALT. Box plots show the 25th and 75th percentiles. Whiskers show 5th and 95th percentiles. * = $p<0.05$.

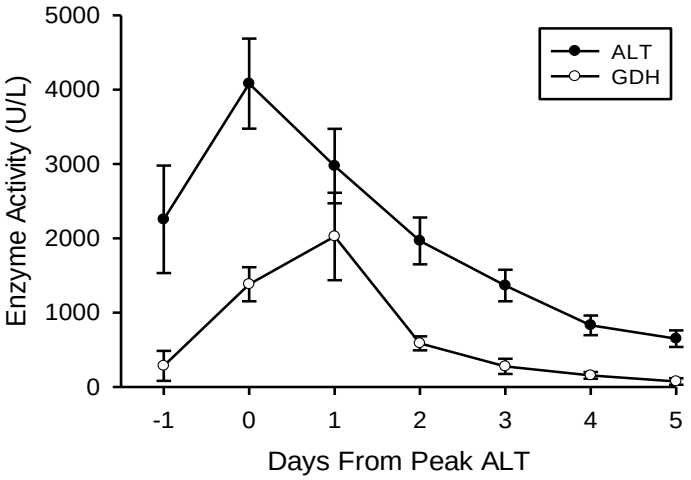

Figure 3.3.5 Time course of hepatocellular and mitochondrial injury. Comparison of plasma levels of ALT (hepatocellular injury) and GDH (mitochondrial injury) in hypoxic hepatitis patients from one day before to 5 days after peak ALT. Data are expressed as average ± SE.

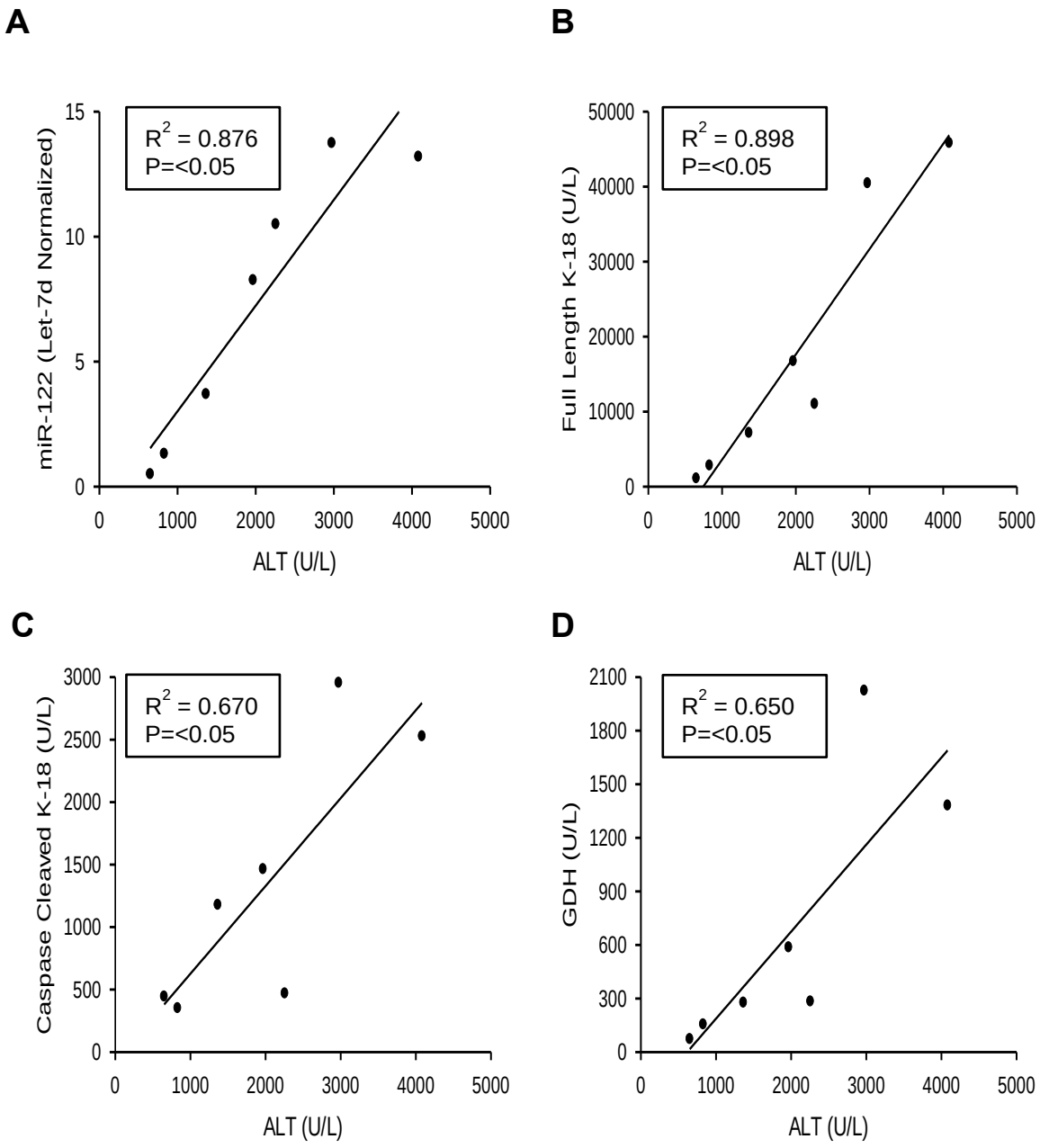

Figure 3.3.6. Regression analysis of biomarkers. Linear regression analysis of (A) miR-122, (B) FK-18, (C) ccK-18, and (D) GDH with ALT. Data is presented from the day before to 5 days after peak injury as measured by ALT. * = $p<0.05$ vs. control.

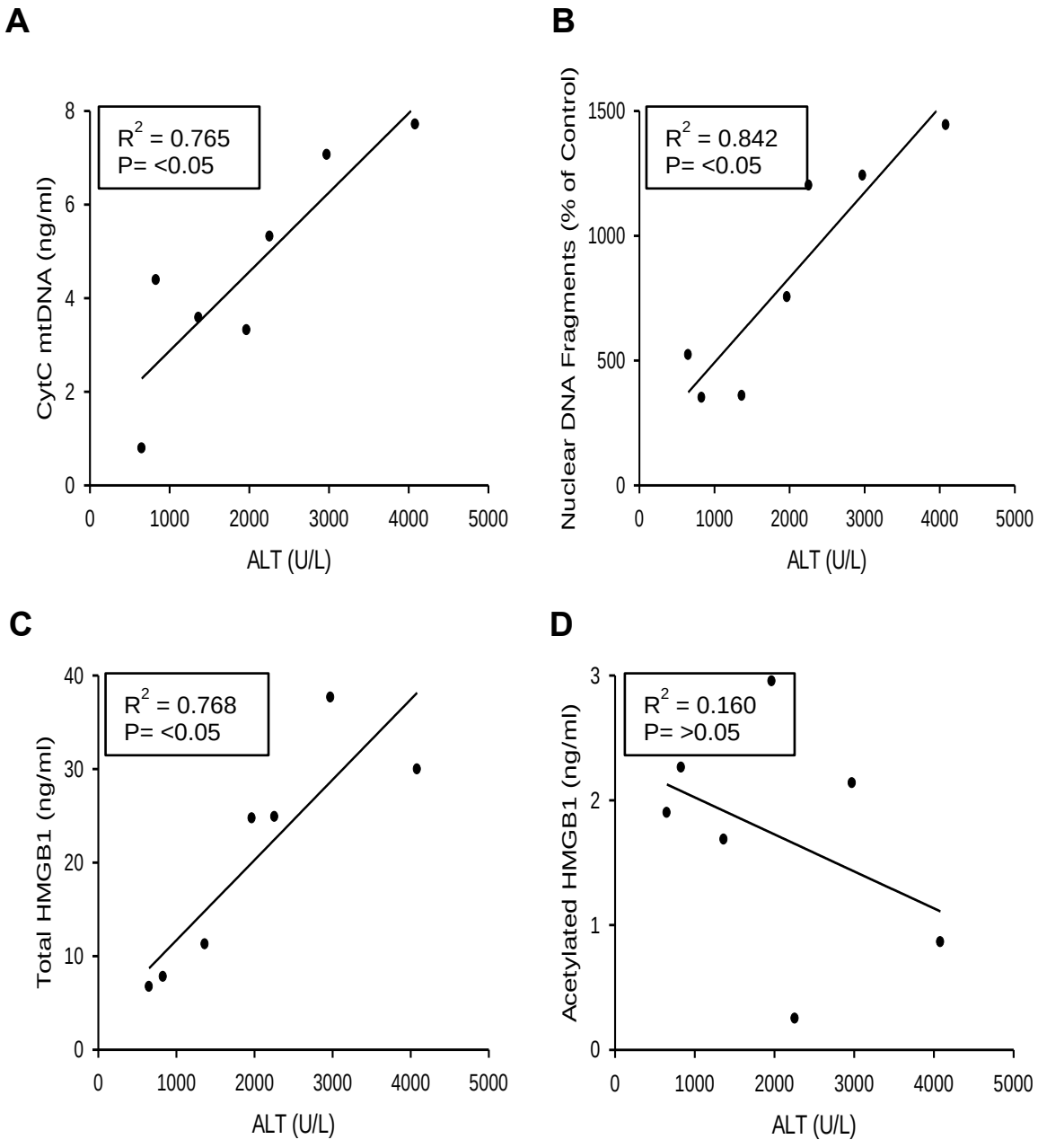

Figure 3.3.7. Regression analysis of biomarkers. Linear regression analysis of (A) CytC, (B) Nuclear DNA Fragments, (C) Total HMGB1, and (D) Acetylated HMGB1 with ALT. Data is presented from the day before to 5 days after peak injury as measured by ALT. * = $p<0.05$ vs. control.

(1444±182%; Figure 3.3.8A). As with GDH, this time course of injury closely mimics that of ALT. We then compared the levels of nuclear DNA fragments to those observed in APAP patients. As with GDH and mtDNA, there was no significant difference between HH and APAP patients (1444±182 vs. 2531±552%, respectively; Figures 3.3.8A & B).

The inflammatory response does not exacerbate HH in later stages. HMGB1 is a nuclear protein that sits in the minor grove of DNA and modulates transcription of numerous genes. HMGB1 can be either passively released during necrosis or actively secreted in its acetylated form by inflammatory cells. Therefore, the two forms of HMGB1 can provide information on the mode of cell death as well as possible inflammatory cell activation during injury. To confirm necrosis as the mode of cell death, we measured total levels of HMGB1 and found a similar pattern of change over the course of injury as all other parameters measured thus far (Figure 3.3.8C). These data are consistent with our cytokeratin-18 measurements and provide further evidence for necrosis as the primary mode of cell death. To assess whether or not there is an immune component, we also measured levels of acetylated HMGB1 and found a reverse pattern – an increase in acHMGB1 at the later time points. When comparing acHMGB1 to total HMGB1, there is a time-dependent increase in the percent of acHMGB1 from less than 5% at the peak of injury to 20-30% during the recovery phase (Figure 3.3.8D). Several studies point to elevated levels of pro-inflammatory cytokines such as IL-6 and IL-10 during the early phase of HH (Bajt et al., 2006; Wetzel et al., 2014; Zuckerbraun et al., 2005)**.** To determine whether the elevated acHMGB1 ratio we observed was an indication of a robust inflammatory response during the later stages of HH, we measured multiple cytokines. For most cytokines and chemokines, the highest levels were observed at the early injury with a downward trend at the later stages (Figures 3.3.9A – D).

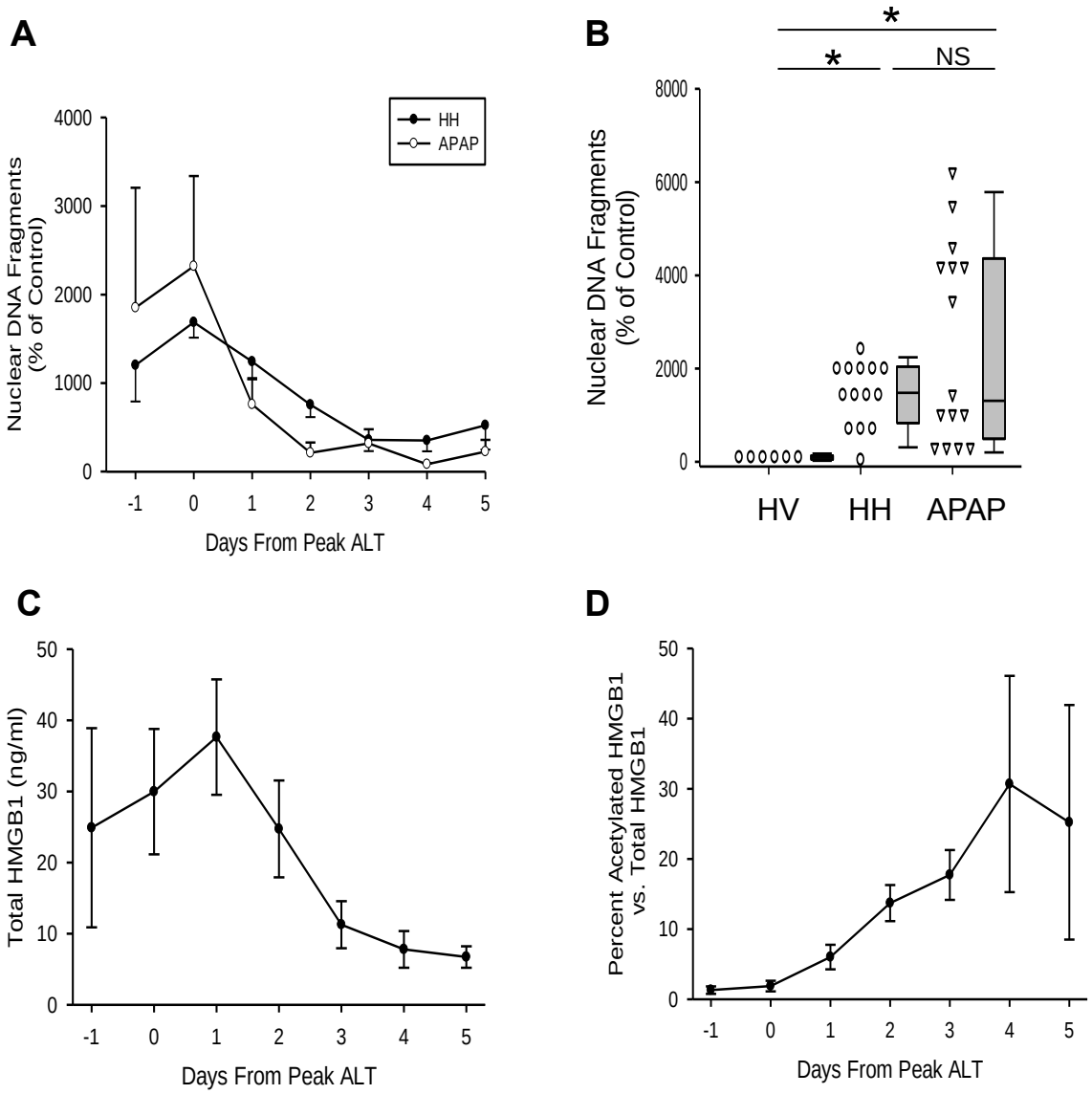

Figure 3.3.8. Cell death involves DNA fragmentation and release of HMGB1. (A) Plasma levels of DNA fragments in HH and APAP patients from -1 to +5 days after peak injury. (B) Dot histogram comparing plasma levels of DNA fragments between HH patients and HV or patients with APAP toxicity at the time of peak injury. (C) Total HMGB1 in HH patients measured from -1 day to +5 days after peak injury. (D) Percent of hyperacetylated HMGB1 of total HMGB1 in the plasma of HH patients from -1 to +5 days after peak injury. Line graph data are represented as average ± SE. Box plots show the 25th and 75th percentiles. Whiskers show 5th and 95th percentiles. * = $p<0.05$.

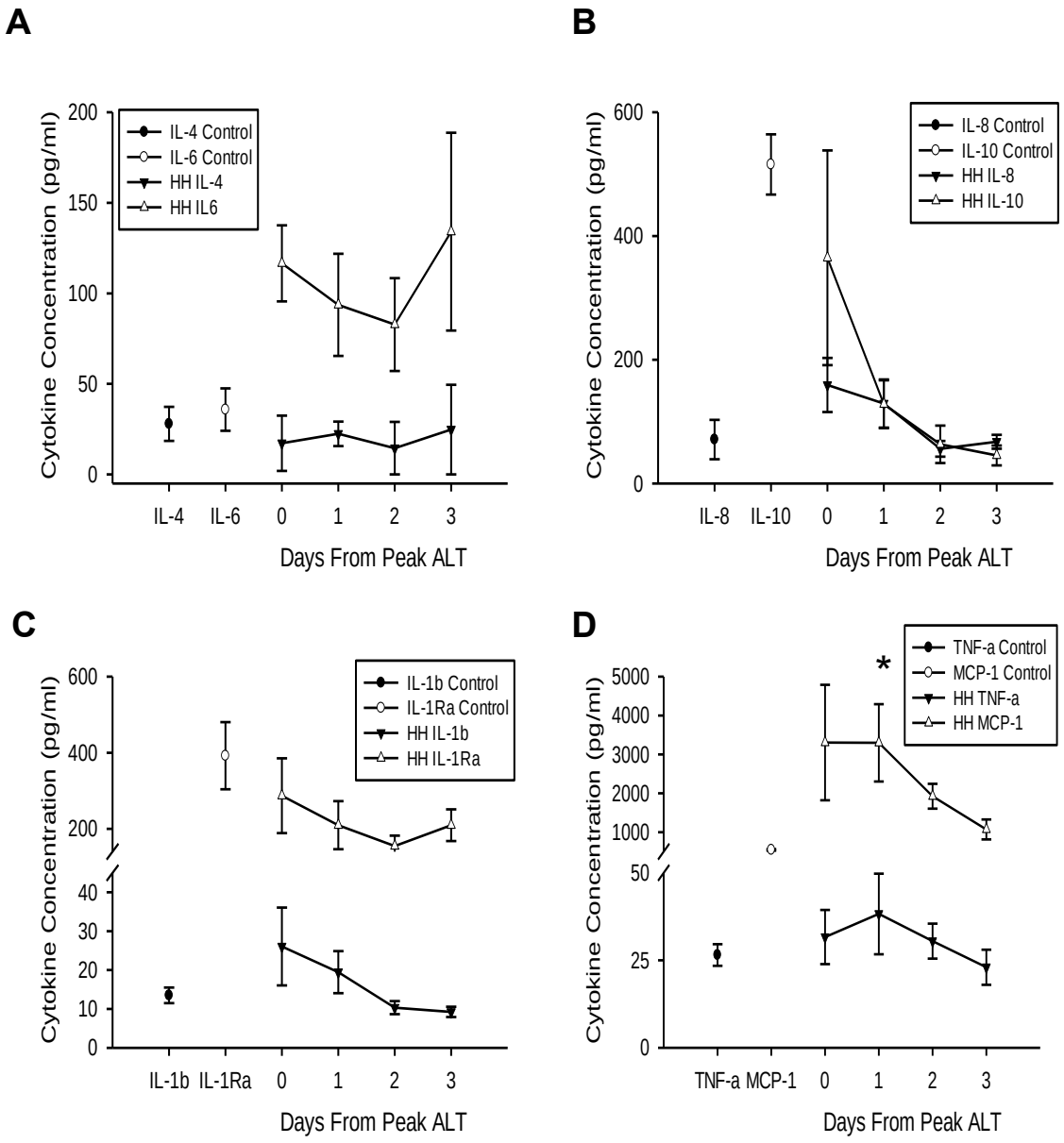

Figure 3.3.9 Plasma levels of inflammatory mediators during hypoxic hepatitis. Plasma levels of (A) IL-4 and IL-6, (B) IL-8 and IL-10, (C) IL-1β and IL-1Ra, and (D) TNF-α and MCP-1 in hypoxic hepatitis patients from day 0 (peak of ALT) to 3 days after peak of injury (line graphs) are compared to control levels of the same cytokines (healthy volunteers, single plots). Data represent average ± SE (n = 5-11 patients per time point). * = $p<0.05$ vs. control.

In order to highlight the strong correlation between each of these plasma parameters with ALT, the time course of ALT vs miR-122, full-length cytokeratin-18, caspase-cleaved cytokeratin-18, mtDNA, nuclear DNA fragments, and total HMGB1 is shown in the Figure 3.3.10 and 3.3.11. All parameters show a similar time-dependent increase and subsequent decrease in plasma as ALT (Figure 3.3.10 & 3.3.11). This is further supported by linear regression analysis, which demonstrated a significant correlation of each parameter with ALT on each day (Figures 3.3.6 & 3.3.7). The only exception is plasma levels of acetylated HMGB1, an indicator of inflammatory cell activation, which shows neither a temporary correlation (Figure 3.3.11) nor a significance with linear regression (Figures 3.3.7).

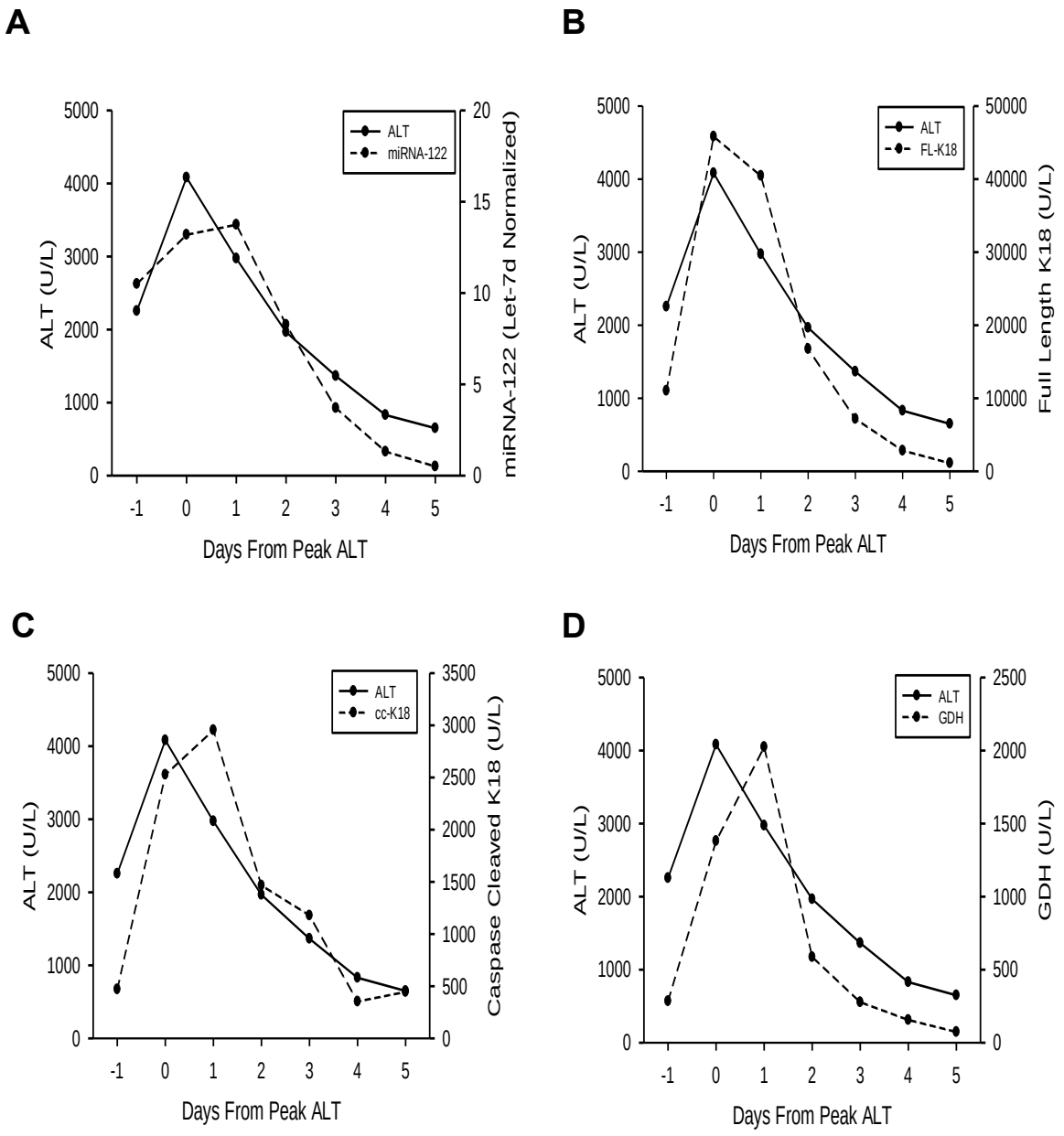

Figure 3.3.10 Relationship between ALT and plasma biomarkers. Side by side comparison demonstrating relationship between ALT and (A) miR-122, (B) FL-K18, (C) cc-K18, and (D) GDH Data is presented as the average value from the day before, to 5 days after, peak injury as measured by ALT. Error bars have been omitted for clarity.

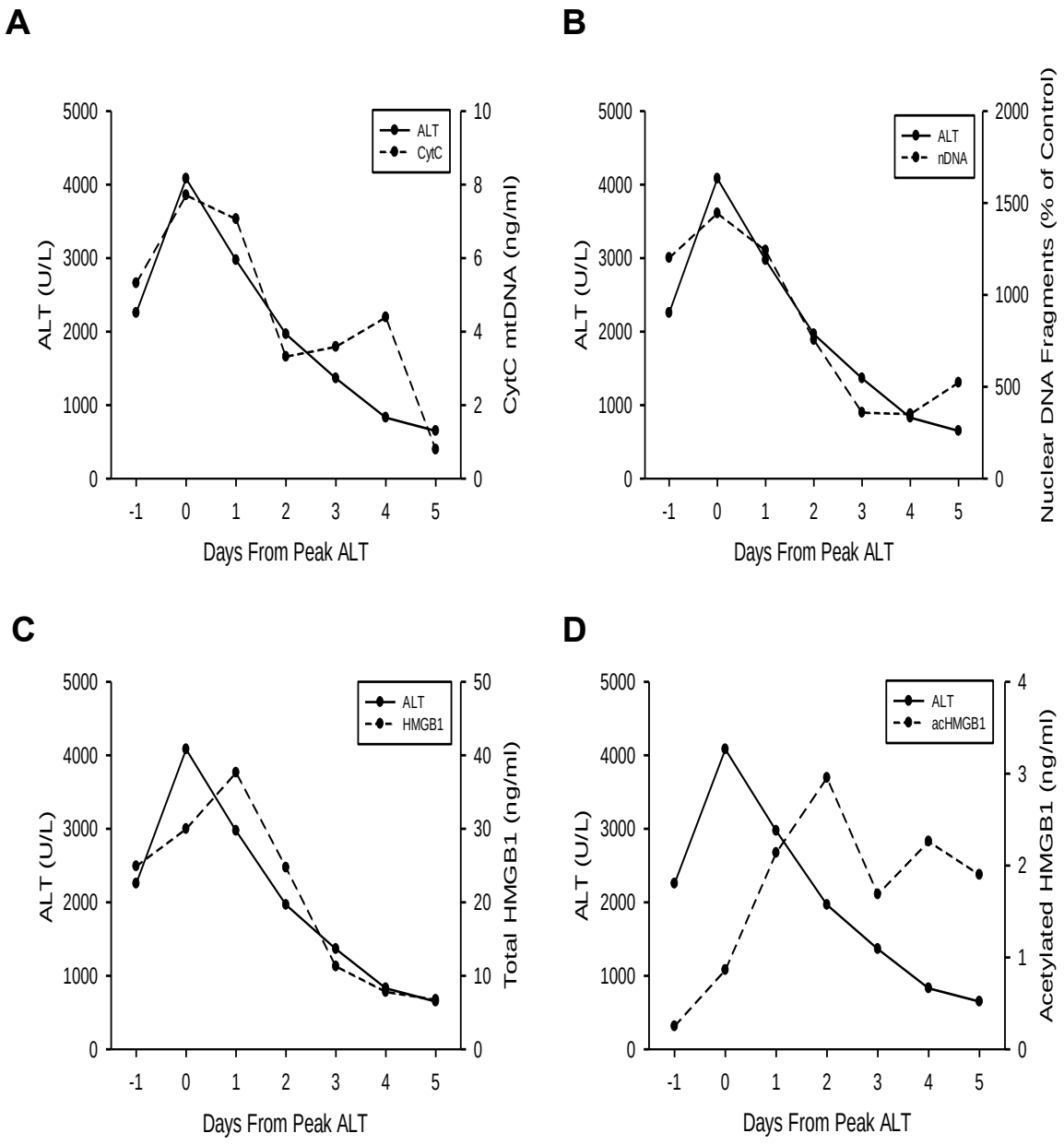

Figure 3.3.11 Relationship between ALT and plasma biomarkers. Side by side comparison demonstrating relationship between ALT and (A) CytC, (B) Nuclear DNA fragments, (C) Total HMGB1, and (D) Acetylated HMGB1. Data is presented as the average value from the day before, to 5 days after, peak injury as measured by ALT. Error bars have been omitted for clarity.

3.4 DISCUSSION

In the present study, we set out to investigate the mechanisms of injury during HH in patients. To accomplish this, we investigated a series of circulating plasma biomarkers which characterize mode and mechanisms of cell death in experimental models and other human liver diseases (Antoine et al., 2012; McGill et al., 2012; Starkey Lewis et al., 2011; Woolbright et al., 2015; Yang et al., 2014). Because APAP hepatotoxicity is well-characterized in both animal models and humans (Jaeschke, 2015), we compared the findings in patients with HH to those in patients with APAP toxicity.

Liver injury during HH. We found liver injury in our patients fits the clinical profile of HH, mostly characterized by a sharp rise in ALT greater than 20 times normal. Although not liver specific, ALT is clinically used for assessment of liver injury. Multiple studies show that miRNA-122 is a more sensitive and specific indicator of ongoing liver injury, rising earlier and to a much greater degree than ALT (Roderburg et al., 2015; Starkey Lewis et al., 2011; Wang et al., 2009b). Because we detected a dramatic increase of the liver-specific miRNA-122 in HH patients similar to that previously observed in APAP hepatotoxicity, we conclude that the majority of ALT release in these patients was derived from hepatocytes – an important distinction in the setting of HH, since hypoxia is not limited to the liver. Our results are consistent with previously reported values of miRNA-122 in a porcine model of cardiogenic shock (Andersson et al., 2012). Interestingly, our cohort of HH patients had low plasma levels of adducts (Figure 3.3.2), however such low levels of adducts are well under the level which would be expected if APAP caused a significant amount of liver injury (James et al., 2009). More likely, these individuals consumed a therapeutic dose of

APAP and adducts were passively released as a result of the hypoxic event leading to HH (McGill et al., 2013).

Mode of cell death during HH. To differentiate whether or not hepatocellular injury was occurring as a result of apoptosis or necrosis, we measured plasma levels of cytokeratin-18, an intermediate filament protein which composes part of the cytoskeleton. During apoptosis, active caspases cleave cytokeratin creating a neo-epitope which can be recognized by the M30 antibody. In contrast, during necrosis, caspases are not active and mainly full-length cytokeratin-18 is released. Thus, the ratio of cleaved/full-length cytokeratin-18 can be used to determine whether cells undergo apoptosis or necrosis. Given that at any time during the observation period >95% of the detectable cytokeratin-18 levels in blood of these patients was the full-length form, our data strongly suggest that necrosis was the dominant form of liver cell death during HH. Even still, we saw significant elevations in the caspase-cleaved form of cytokeratin-18 at various time points. However, no detectable caspase activity could be found in plasma of HH patients despite the fact that plasma caspase-3 activities are readily measurable in experimental models of apoptosis (McGill et al., 2012). In light of elevated levels of caspase-cleaved cytokeratin-18, however, these results should be interpreted with caution, since little is known about the stability or half-life of active caspases in plasma. It is possible that caspases are rapidly degraded or eliminated from plasma before blood samples are obtained. Therefore, caspase-3 activities, especially at later time points during the recovery phase could be lower than in the liver. Regardless, when compared quantitatively with the full-length form of cytokeratin-18 (Figure 3.3.3C), it becomes clear that the vast majority of injury is caused by necrosis, and that apoptosis, as indicated by cleaved cytokeratin-18, contributes relatively little to liver injury in this setting.

Mitochondrial damage as a hallmark of cell death in HH. In other causes of liver injury, such as APAP toxicity, in which necrosis is the predominant form of cell death, mitochondria play a major role in the pathophysiology of injury (Jaeschke et al., 2012a). In the experimental model and in APAP overdose patients, mitochondrial rupture leads to release of matrix macromolecules which can be measured in plasma (McGill et al., 2012). Furthermore, endonuclease-G released from damaged mitochondria translocates to the nucleus where it causes DNA fragmentation and cell death (Bajt et al., 2006; Jaeschke et al., 2012a). Our results show that the increase in mitochondrial injury as measured by GDH and mitochondrial DNA mirror the pattern of injury as measured by ALT. Similarly, levels of nuclear DNA fragments follow this trend. These results provide strong evidence for mitochondrial damage as a key event in the mechanism of liver cell death in HH patients. Interestingly, our *in vivo* results in humans correlate with many experimental studies which demonstrate a critical role of mitochondria in reperfusion injury (Lemasters et al., 1997; Powell et al., 2014; Vairetti et al., 2006). It has been shown that during hypoxia, intracellular pH decreases which actually exerts a protective effect against hypoxic injury (Qian et al., 1997). However, upon re-oxygenation, intracellular pH returns to normal, precipitating formation of the MPTP and mitochondrial rupture (Qian et al., 1997; Schwartz et al., 2013). Mechanisms behind pH-induced mitochondrial injury are thought to involve mitochondrial calcium uptake (Schwartz et al., 2013). Collectively, a clinical picture begins to emerge: during a period of hypoxia, ATP levels fall leading to a decreased ability to sequester calcium within mitochondria (Gasbarrini et al., 1992; Lemasters et al., 1987). Return of oxygen allows for oxidative phosphorylation and elevated cytosolic calcium levels, leading to mitochondrial calcium sequestration. This incites formation of the MPTP, which triggers matrix swelling, rupture and necrotic cell death. Based on

our previous data, it is possible that endonuclease-G released from ruptured mitochondrial produces DNA fragmentation, but further studies are needed for verification.

Role of inflammation in HH. Interestingly, we found an increase in acetylated HMGB1 (acHMGB1) protein in plasma of HH patients at later time points. Since macrophages actively secrete acHMGB1 upon activation (Bonaldi et al., 2003), our data suggest activation of inflammatory cells during the recovery phase. These findings are consistent with activation of macrophages and other phagocytes for removal of cell debris and regeneration of the damaged tissue. However, the majority of cytokines were slightly elevated earlier. Since the gastrointestinal tract is very susceptible to microcirculatory changes during ischemic injury (Ceppa et al., 2003), it is possible that intestinal injury allows for bacterial translocation and macrophage activation before liver injury occurs. Thus, the initial inflammatory response is on the decline at the point when these blood samples were drawn and the increased acHMGB1 levels, reflecting a sterile inflammatory response, could be the result of macrophage activation in an attempt to clear debris rather than exacerbate injury (Jaeschke et al., 2012b). This has also been shown in APAP overdose patients, where neutrophil and monocyte activation and hepatic infiltration are crucial for liver repair and regeneration (Antoniades et al., 2012; Williams et al., 2014). This seems very likely given that injury is decreased at later time points despite an apparent activation of inflammatory cells. This is in stark contrast to models where inflammation is involved in propagation of injury, such as no-flow ischemia, in which sterile inflammation leads to exacerbation of injury well past the point of reperfusion (Jaeschke, 2003).

Overall, our data demonstrated that HH, similar to APAP hepatotoxicity, is characterized by extensive mitochondrial damage and necrotic cell death. Although these mechanistic biomarkers gave novel insight into the mechanism of these disease states in humans, there was no relevant difference between the parameters in APAP and HH that would allow a more accurate diagnosis. However, our previous studies looking at the miRNA profile between these patient groups indicated that similar miRNAs with different levels and unique miRNAs can be detected in plasma of these patients suggesting that the miRNA profile may be used to differentiate between APAP hepatotoxicity and HH (Ward et al., 2014). Thus, plasma biomarkers are useful in both better understanding the mechanisms of the disease in humans and can be used to aid in the differential diagnosis.

4. COMPARISON OF FRESHLY DIFFERENTIATED AND CRYOPRESERVED PRE-DIFFERENTIATED HEPARG CELLS FOR STUDIES OF ACETAMINOPHEN TOXICITY

4.1 INTRODUCTION

Drug safety, specifically hepatotoxicity, is a common cause for drug failure during clinical trials (Arrowsmith and Miller, 2013). The development of a reliable and convenient *in vitro* model to assess drug toxicity would help identify hepatotoxic compounds earlier during the drug discovery process, saving time and minimizing animal experiments. Currently, primary human hepatocytes (PHH) remain the gold standard for *in vitro* studies of hepatotoxicity; however, they are not widely available and are known to lose cytochrome-P450 activity following cryopreservation, limiting their usefulness in drug metabolism studies (Hengstler et al., 2000). A suitable alternative is the human cell line HepaRG (Guillouzo et al., 2007; Hewitt et al., 2007). These cells are favorable for drug toxicity studies because they contain a full complement of drug metabolizing enzymes, including cytochromes P450 (Aninat et al., 2006; Hart et al., 2010). Although HepaRG cells can withstand the cryopreservation process, the subsequent growth and differentiation period is lengthy and inconvenient for high throughput studies. Recently, pre-differentiated cryopreserved HepaRG (cHepaRG) cells have been developed, but no studies have directly compared these two preparations in a controlled drug toxicity study.

Acetaminophen (APAP) overdose continues to be a major problem in the United States and accounts for the majority of acute liver failure cases on a yearly basis (Lee, 2013). At therapeutic doses, the bulk of APAP is glucuronidated or sulfated and excreted in the urine as inactive conjugates (Larson, 2007). However, a small percent of the dose is metabolically activated by P450-mediated conversion to the electrophile NAPQI by cytochrome-P450 mediated metabolism (Dahlin et al., 1984). This metabolic activation is driven predominantly by CYP2E1 (Lee et al., 1996), although CYP3A4 (Thummel et al., 1993) and CYP1A2 (Snawder et al., 1994) have also been shown to contribute to NAPQI formation. Despite the formation of the highly reactive

compound NAPQI, APAP is safe at therapeutic doses because the metabolite is detoxified by the tripeptide glutathione (GSH), which exists at high concentrations within the hepatocyte cytoplasm (Larson, 2007). However, in cases of APAP overdose, sulfation is overwhelmed and a much higher percent of the dose is shunted through the P450 system, leading to depletion of GSH (Mitchell et al., 1973; Xie et al., 2015a) and increased levels of NAPQI (Lee et al., 1996). NAPQI covalently bind to proteins, particularly mitochondrial proteins (Cohen et al., 1997; McGill et al., 2012; Qiu et al., 1998; Tirmenstein and Nelson, 1989), leading to mitochondrial stress, membrane depolarization, and release of endonucleases which translocate to the nucleus leading to cell death (Jaeschke et al., 2003; McGill et al., 2012). Thus, APAP hepatotoxicity is dependent upon 3 major processes: metabolic activation, glutathione depletion, and mitochondrial injury, ultimately culminating in oncotic necrosis. Inhibition of any one or more of these processes prevents the liver injury.

The acetaminophen model of hepatotoxicity is well characterized, relatively simple, and as previously stated, requires multiple intracellular events to occur before toxicity is observed. Therefore, APAP provides an ideal model for identifying any potential differences between freshly differentiated HepaRG cells and cHepaRG cells.

4.2 MATERIALS AND METHODS

Cell culture. All HepaRG cells were obtained from Biopredic International (Rennes, FR). All cells were stored in LN_2 until use. HepaRG cells were grown and differentiated as previously described (McGill et al., 2011). cHepaRG cells were seeded according to the manufacturer's instructions and were acclimated for 1 week prior to initiation of experiments. Frozen PHH were obtained from Biopredic International (Rennes, FR), CellzDirect (now Life Technologies) and ZenBio (Research Triangle Park, NC). The frozen PHH were thawed and prepared according to the manufacturer's instructions. Before seeding, frozen PHH were centrifuged with 90% Percoll to purify live cells. Fresh PHH were acquired through the University of Kansas Liver Center from consenting donor patients presenting to the University of Kansas Hospital. All samples were obtained with approval from the Institutional Review Board. Once approved, liver tissue was processed as described in detail (Xie et al., 2014b). All cell lines were maintained at 37^{o}C and 5% CO_2 before and during experiments.

Acetaminophen treatment. At the time of treatment, growth medium was removed and cells were washed once with 1x PBS and treated with the indicated concentrations of APAP dissolved in DMSO-free William's E medium or with Williams' E medium alone. The cells were harvested at the indicated time points.

Biochemistry. After acetaminophen treatment, 1 ml cell medium was collected for measurement of enzyme (LDH or ALT) release. Cells were then washed once with PBS, scraped in cell lysis buffer, and frozen at -80^{o}C until use. Once thawed, cells were sonicated and LDH concentration in both medium and cell lysate was measured as previously described (McGill et al., 2011). ALT

levels were similarly measured using a commercially available kit according to the manufacturer's instructions (Pointe Scientific, Canton, MI).

APAP-protein Adducts. Following acetaminophen treatment, cells were washed once with PBS, scraped in 10 mM sodium-acetate buffer (pH 6.5), and frozen at -80^{o}C until use. APAP-protein binding was measured using high-pressure liquid chromatography with electrochemical detection (HPLC-ECD) as previously described (McGill et al., 2013). The results were normalized to total protein concentration in cell lysates as determined by the BCA assay.

GSH Depletion. GSH was measured as previously described (Jaeschke and Mitchell, 1990). Results were normalized to total protein concentration of cell lysate as determined by the BCA assay.

JC-1 Assay. Mitochondrial membrane permeability was measured with the use of a commercially available JC-1 Mitochondrial Membrane Permeability Kit (Cell Technology, Fremont, CA) as described (Bajt et al., 2004).

DNA isolation and qPCR. Cells subjected to P450 mRNA analysis were scraped and stored in Tri® Reagent (Sigma Chemical, St. Louis, MO) at -80^{o}C until use. After thawing, mRNA was isolated using a standard protocol and converted to cDNA. cDNA was subject to qPCR reaction using the following primers: CYP2E1 (fwd: TTGAAGCCTCTCGTTGACCC, rev: CGTGGTGGGATACAGCCAA), CYP3A4 (fwd: CTTCATCCAATGGACTGCATAAAT, rev: TCCCAAGTATAACACTCTACACAGACAA), and 1A2 (fwd:

TGGAGACCTTCCGACACTCCT, rev: CGTTGTGTCCCTTGTTGTGC) and normalized to β-actin (fwd: CATGTACGTTGCTATCCAGGC rev: CTCCTTAATGTCACGCACGAT).

Statistics. All data are expressed as average ± SEM. Statistical significance was assessed using Student's t-test or one-way analysis of variance (ANOVA) with Tukey's post-hoc test where appropriate. All graphs were made using SigmaPlot® software (vers. 12.5). $p < 0.05$ was considered significant.

4.3 RESULTS

We first compared cell death in four different cell preparations: fresh PHH, frozen PHH, HepaRG cells, and cHepaRG cells. ALT release was measured in PHH cultures, while LDH was chosen for HepaRG cultures due to lower expression of ALT in this cell line. Following 24 hour treatment with 20 mM APAP, cell death was observed in fresh PHH, HepaRG, and cHepaRG cells (enzyme release: 64±6%, 23±2% and 50±10%, respectively) (Figures 4.3.1A, C, D). Notably, viability in cryopreserved PHH cultures was poor as indicated by excessive cell death in control samples (36±10%), which actually decreased after 24 hrs of APAP treatment (27±5%) (Figures 4.3.1B and 4.3.2). Importantly, cell death between cHepaRG and fresh PHH was similar (64±6% vs. 50±10%). These data suggest that frozen PHH cultures are generally a poor model for drug toxicity studies, while fresh PHH, HepaRG and cHepaRG cells are useful.

Because no direct comparison of HepaRG and cHepaRG cells has been performed, we wanted to determine whether or not these cells respond similarly to APAP. We began by measuring full time courses of enzyme release after APAP treatment. We found that APAP caused a time dependent increase in cell death in both HepaRG and cHepaRG cells, however, injury occurred faster, and was more severe, in cHepaRG cells compared to HepaRG cells (16hr vs. 24hr and 50±10% vs. 23±2%, respectively) (Figures 4.3.3A, B). We then performed dose-response studies in both cell types. Interestingly, in HepaRG cells, there was a significant increase in cell death at 10 mM APAP but no additional cell death was observed with 20mM APAP (Figure 4.3.3C), despite a clear dose-response in cHepaRG cells (Figure 4.3.3D). In addition, the degree of injury in cHepaRG cells at 10mM was similar to that in HepaRG cells (28±15% vs. 32±4%). These results are similar to those observed in Figure 4.3.1C (28±1% vs. 23±2%), but differ from what we have previously reported (McGill et al., 2011)

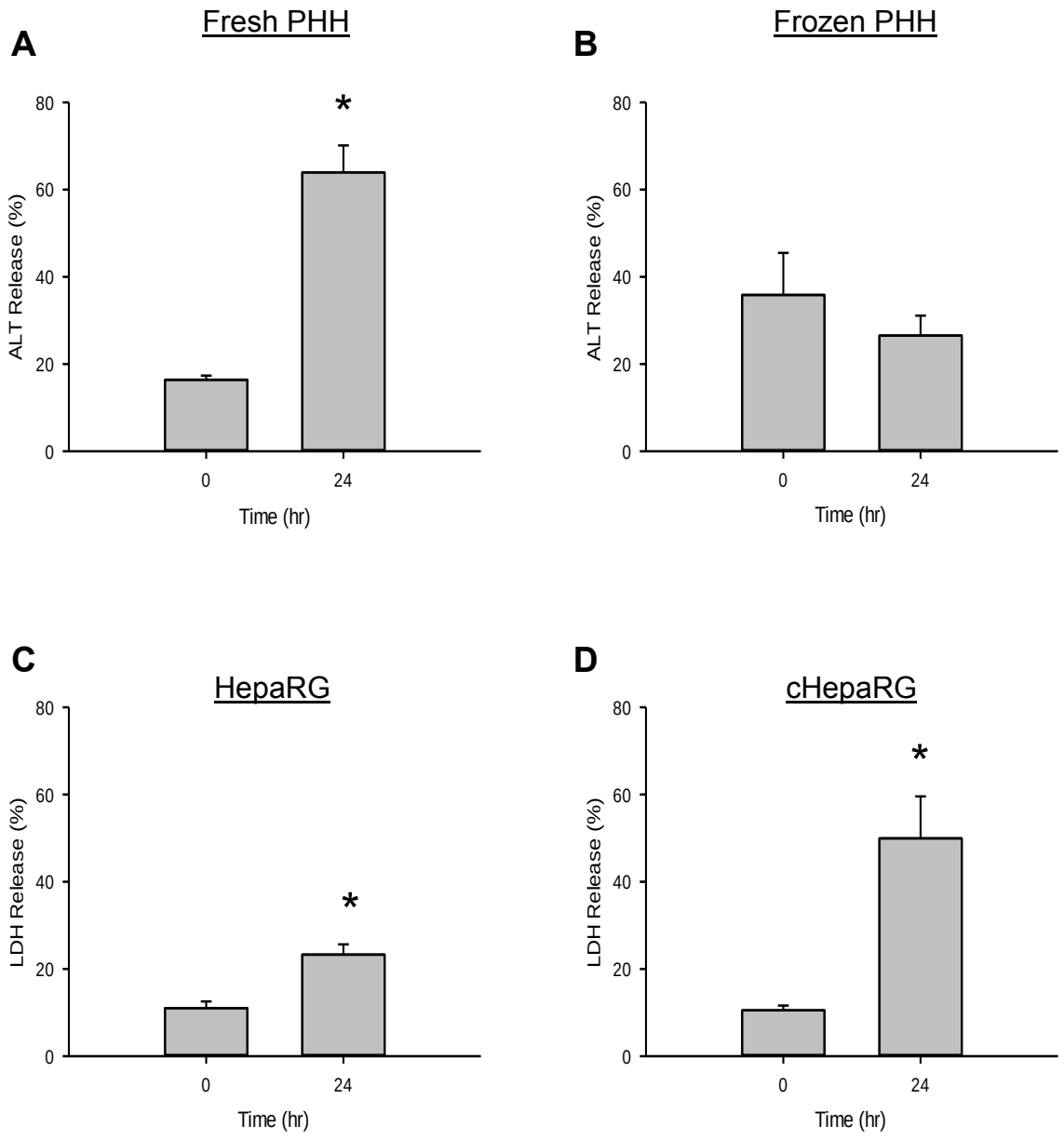

Figure 4.3.1. Comparison of cell death between primary human hepatocytes and HepaRG cells. Release of ALT or LDH was measured in (A) fresh and (B) frozen primary human hepatocytes (PHH) as well as in (C) freshly differentiated (HepaRG) or (D) pre-differentiated cryopreserved (cHepaRG) HepaRG cells following treatment with vehicle or 20mM acetaminophen. Data are expressed as average ± SE for 3 independent experiments. * = $p<0.05$ vs. control.

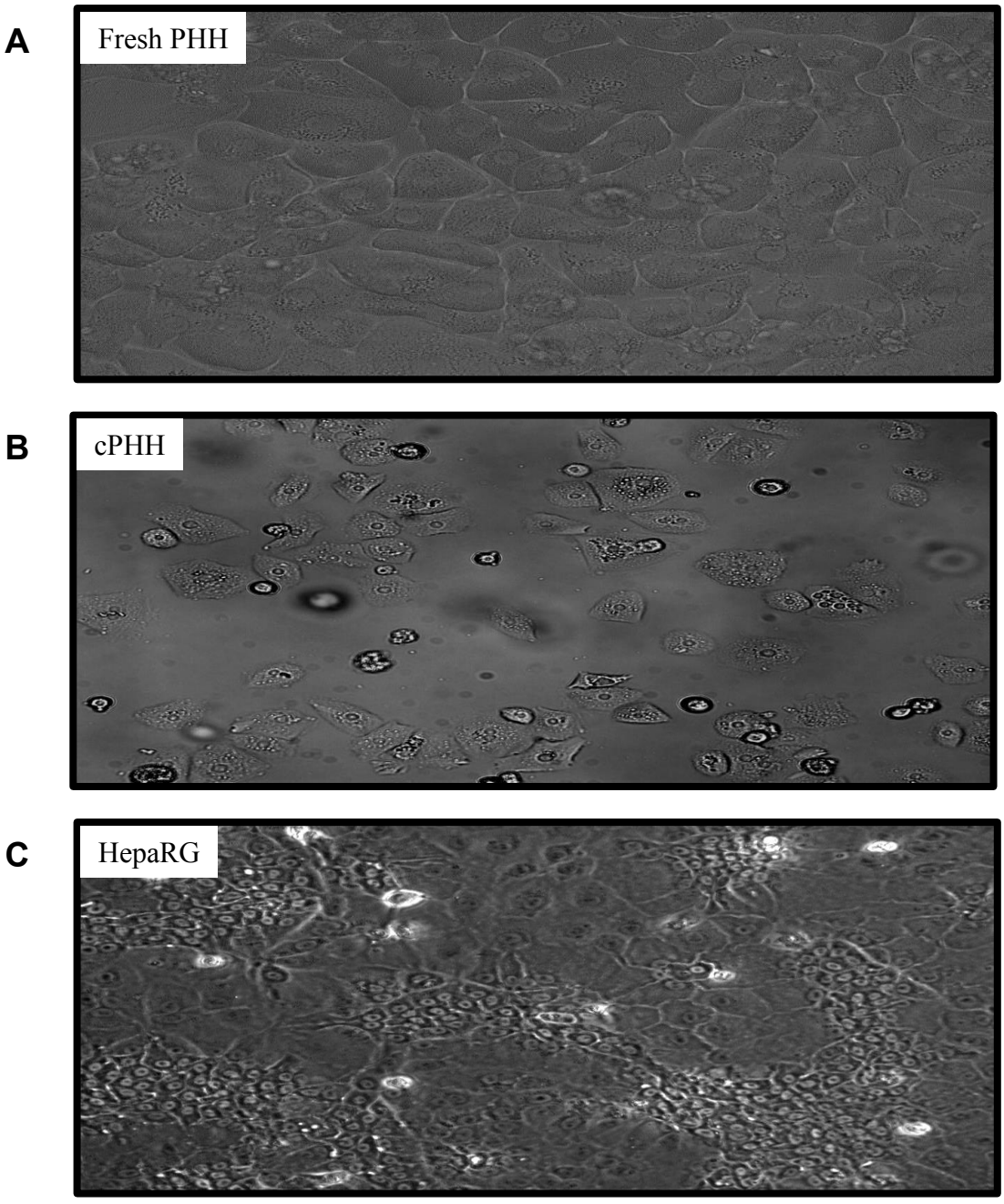

Figure 4.3.2. Microscopic comparison of primary human hepatocytes and HepaRG cells. Phase contrast images (400X) of (A) fresh primary human hepatocytes; (B) cryopreserved primary human hepatocytes; and (C) Undifferentiated cryopreserved HepaRG cells 24 hours after seeding.

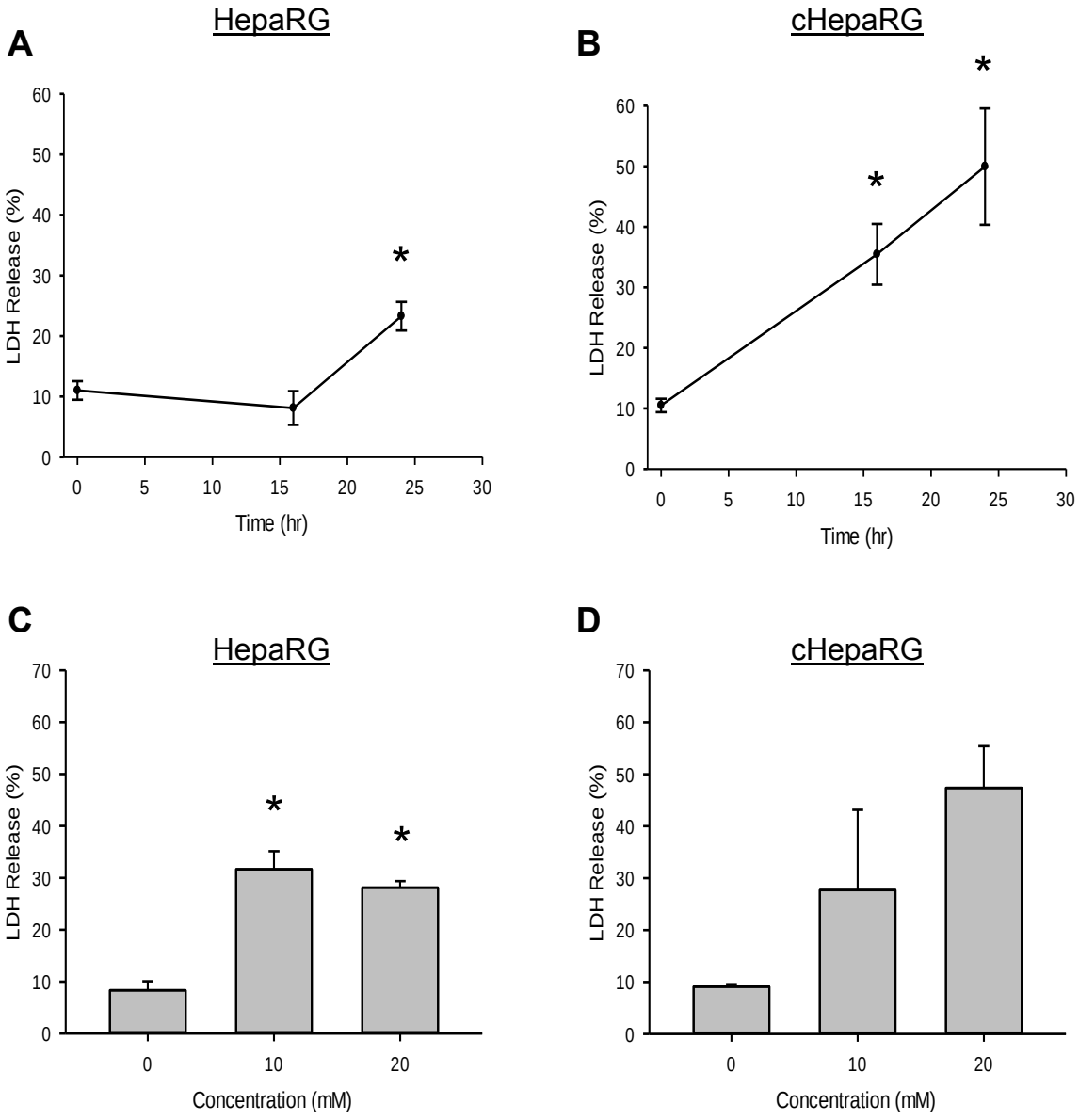

Figure 4.3.3. Comparison of time-course and dose-response of cell death between HepaRG and cHepaRG cells. Release of LDH was measured in (A) freshly differentiated (HepaRG) or (B) pre-differentiated cryopreserved (cHepaRG) HepaRG cells following treatment with vehicle or 20mM acetaminophen. Dose response curves following 24hr treatment with vehicle or various concentrations of acetaminophen in (C) freshly differentiated (HepaRG) or (D) pre-differentiated cryopreserved (cHepaRG) HepaRG cells. Data are expressed as average ± SE for 3 independent experiments. * = $p<0.05$ vs. control.

Metabolic activation of APAP leads to NAPQI formation which is detoxified by GSH, leading to decreased GSH levels. Therefore, depletion of GSH can be used as a measurement of metabolism of APAP into its toxic metabolite. To compare the ability of HepaRG and cHepaRG to form this reactive intermediate, we treated cells with 20 mM APAP for multiple time points up to 24 hours and measured GSH. Although HepaRG cells were observed to have higher basal levels of GSH (98±11 vs. 62±5 nmol/mg protein), we found a similar pattern of GSH depletion with an initial rapid decrease between 0 and 3 hr which then plateaued (Figures 4.3.4A, B). The dose-response of GSH depletion was also similar, resulting in approximately 34% and 37% depletion of GSH in HepaRG and cHepaRG, respectively, after 10 mM APAP, and 68% and 70% depletion of GSH in HepaRG and cHepaRG, respectively, following 20mM APAP (Figures 4.3.4C, D).

If NAPQI is not detoxified by GSH, it can covalently bind to sulfhydryl groups on cysteine residues to form APAP-protein adducts. To further test formation of NAPQI in the cells, we measured the levels of protein-derived APAP-cys and found that although maximum adduct formation was higher in cHepaRG cells compared to HepaRG cells (0.44±0.05 vs. 0.28±0.04 nmol/mg protein, respectively) (Figures 4.3.5A, B), the time course was similar between the two preparations. Both HepaRG and cHepaRG cells demonstrated a sharp rise in adduct formation between 0 and 6 hrs post-APAP treatment, followed by a gradual decline to 24 hrs.

We also compared the levels of P450 mRNA between HepaRG and cHepaRG for those P450s known to play a role in APAP metabolism. Interestingly, we observed a trend toward higher CYP2E1 and CYP3A4 mRNA levels in cHepaRG cells which may explain the higher APAP-protein adduct concentrations in these cells. We also found a slight decrease in CYP1A2 mRNA levels relative to HepaRG cells. However, these differences were not found to be statistically

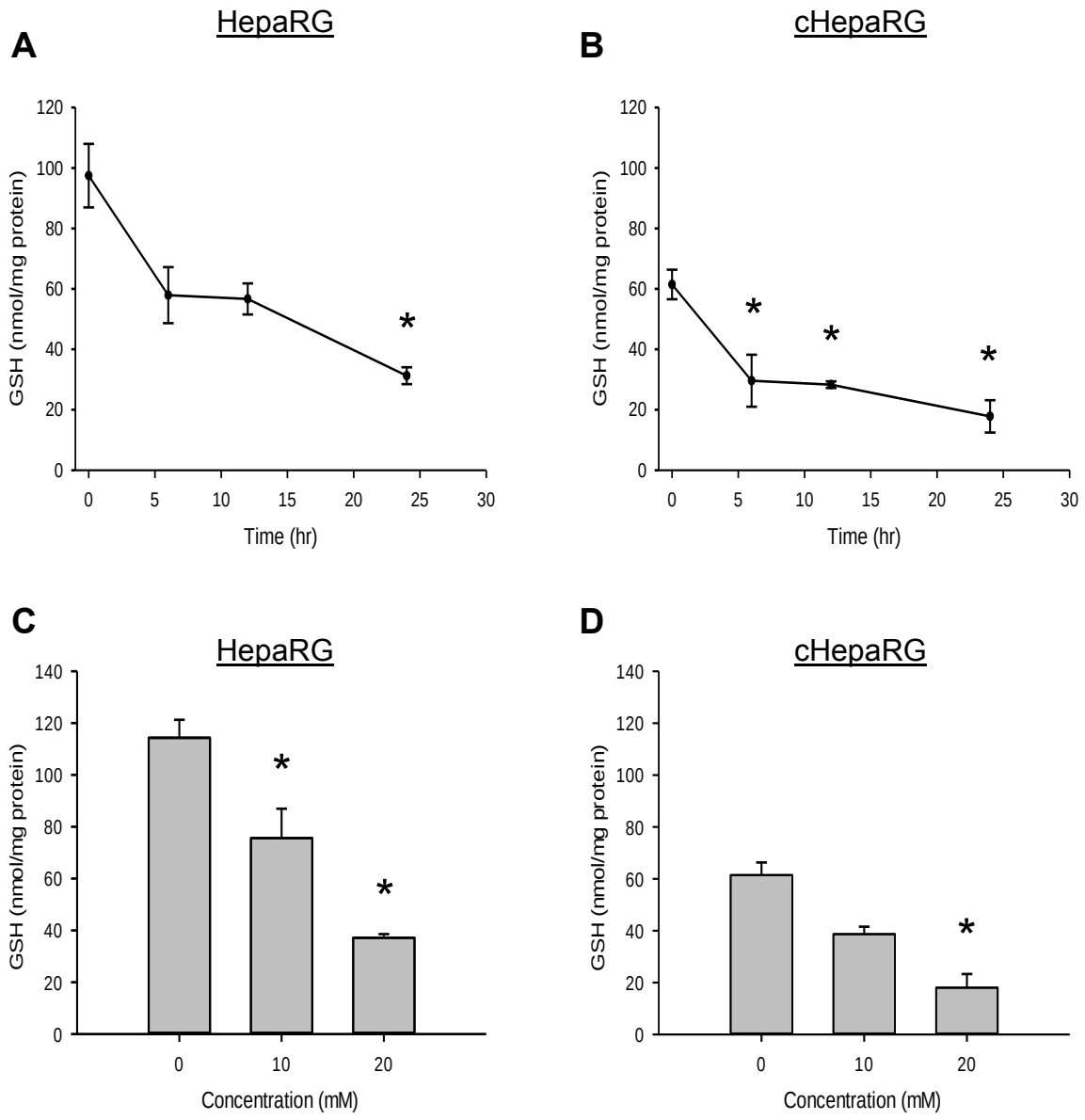

Figure 4.3.4. GSH Depletion is similar between 'Regular' and cryopreserved HepaRG cells. Time course of GSH depletion following acetaminophen treatment (20mM) in (A) freshly differentiated (HepaRG) and (B) pre-differentiated cryopreserved (cHepaRG) HepaRG cells. Dose-response of GSH depletion following acetaminophen treatment in (C) freshly differentiated (HepaRG) and (D) pre-differentiated cryopreserved (cHepaRG) HepaRG cells at 24 h. Data are expressed as average ± SE for 3 independent experiments. * = $p<0.05$ vs. control.

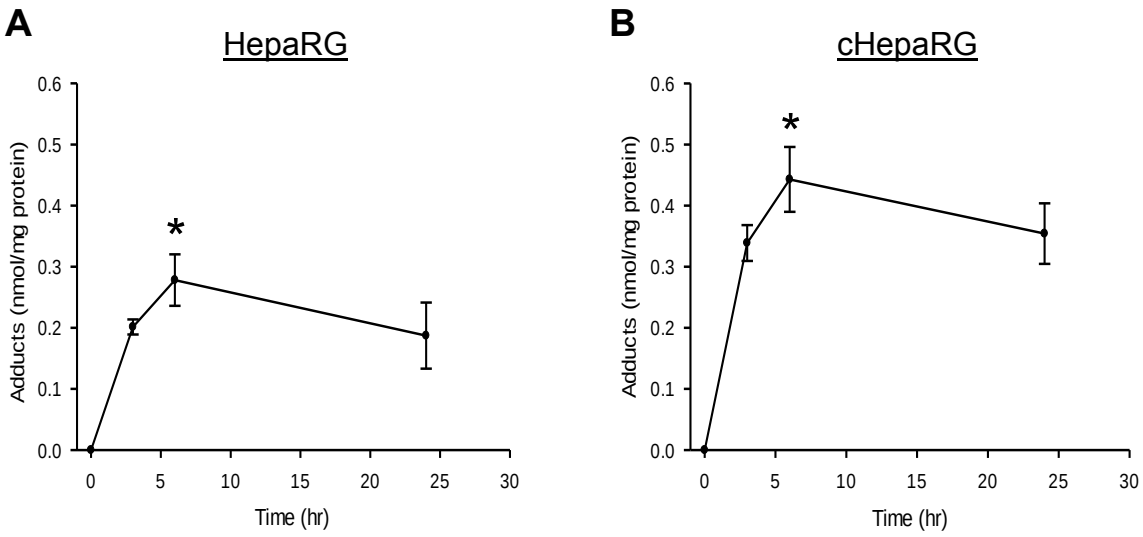

Figure 4.3.5. Time course of APAP-protein adducts in HepaRG cells. Acetaminophen-cysteine protein adducts were measured in (A) freshly differentiated (HepaRG) and (B) pre-differentiated cryopreserved (cHepaRG) HepaRG cells following treatment with 20mM acetaminophen for various times. Data are expressed as mean±SE for 3 independent experiments. * = $p<0.05$ vs. control.

significant (Table 4.3.1). Taken together, our results suggest similar phase I drug metabolism capabilities in HepaRG and cHepaRG cells.

Mitochondrial damage and membrane permeability plays a key role in APAP toxicity. With this in mind, we set out to determine the extent to which mitochondrial membrane permeability plays a role in the cell death measured in Figure 4.3.1. We performed the JC1 assay to assess mitochondrial membrane potential after APAP treatment in both HepaRG and cHepaRG cells. Again, we found that the two cell preparations showed a similar response to APAP treatment. After 3 hrs, mitochondrial membrane potential decreased 32±1% in HepaRG cells compared to 32±5% in cHepaRG cells (Figures 4.3.6A, B). Furthermore, mitochondrial membrane potential remained low relative to control in both HepaRG and cHepaRG cells over 24 hrs. These data suggest that downstream events occurring during APAP toxicity are similar between the two cell preparations. Taken together, these data indicate similar mechanisms of toxicity between HepaRG and cHepaRG cells.

Table 4.3.1 CYP Activity Level

		$2^{\Delta Ct}$	Average Fold Increase	SEM	*p*-value
CYP 2E1	Cryopreserved	13.36	12.38	6.11	0.057
	Regular	1.08	1.00	0.22	
CYP 3A4	Cryopreserved	4.57	4.49	2.92	0.057
	Regular	1.02	1.00	0.11	
CYP 1A2	Cryopreserved	0.35	0.27	0.14	0.101
	Regular	1.28	1.00	0.40	

Table 4.3.1. Comparison of mRNA levels of major cytochrome P450 enzymes involved in APAP metabolism between cryopreserved predifferentiated HepaRG cells and undifferentiated crypreserved HepaRG cells.

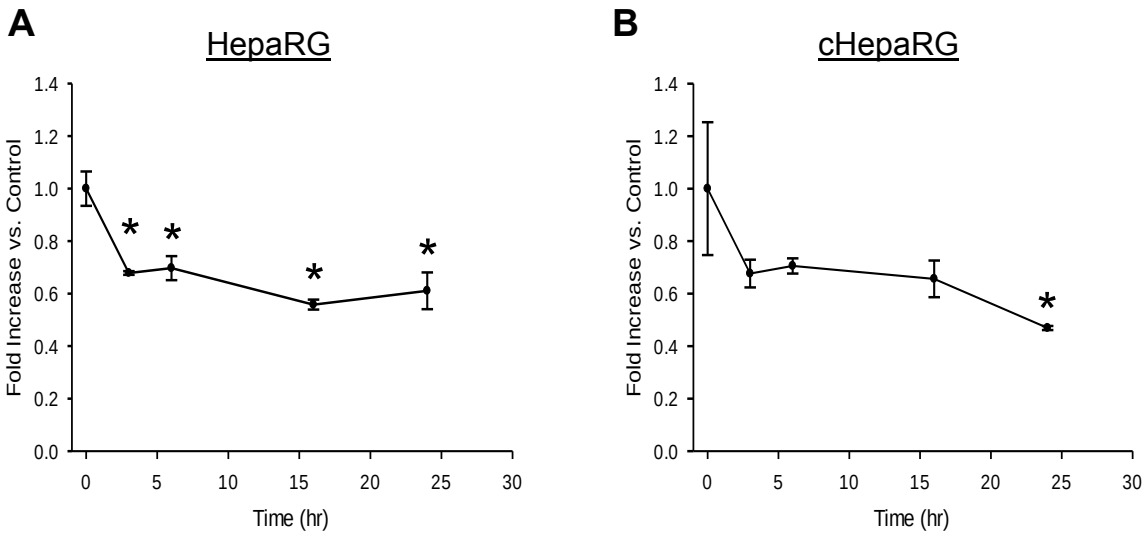

Figure 4.3.6. Time course of mitochondrial permeability in HepaRG cells. Red/green ratio from JC-1 assay (normalized to control) in (A) freshly differentiated (HepaRG) and (B) pre-differentiated cryopreserved (cHepaRG) HepaRG cells following treatment with 20mM acetaminophen. Each time point represents average data from at least three groups. * = $p<0.05$ vs. control.

4.4 DISCUSSION

In the present study, HepaRG and cHepaRG cells were compared for their ability to metabolize APAP and develop subsequent toxicity. To this end, we focused on several aspects of APAP metabolism and toxicity: GSH depletion, adduct formation, and mitochondrial membrane permeability, all events shown to be critical for APAP toxicity (Jaeschke et al., 2012a; McGill et al., 2011). We found that although there were quantitative differences between the two cell preparations, the overall pattern of injury in cHepaRG cells were similar to HepaRG in all measured parameters.

Due to inter-species differences, primary human hepatocytes are the gold standard for in vitro drug toxicity studies. However, limited availability of these cells hinder their usefulness for most labs. The development of a long-term maintenance medium for PHH (Runge et al., 2000) has helped with viability of PHH in culture for longer periods of time, however CYP mRNA decreases within 24hrs (Rodríguez-Antona et al., 2002), preventing the use of PHH in prolonged studies of drug metabolism. Additionally, as we have demonstrated, the viability of frozen PHH following cryopreservation is poor (Figure 4.3.2E**)**, necessitating their immediate use following procurement. Thus, the ability to perform rapid high-throughput drug screening with PHH remains difficult.

The human hepatoma cell line HepaRG has been shown to be a comparable alternative to primary human hepatocytes for studies of drug metabolism (McGill et al., 2011). This cell line is superior to frozen human hepatocytes for several reasons. First, unlike primary human hepatocytes which are only sporadically available, HepaRG cells are commercially available. Second, HepaRG cells have the same level of drug metabolizing enzymes and transporters as primary human hepatocytes (Anthérieu et al., 2010) making them far more useful for drug metabolism studies than other liver cell lines such as HepG2, which do not retain CYP levels (Aninat et al., 2006). Third, functional

CYP levels in HepaRG cells remain stable over prolonged periods allowing for the possibility of long term drug toxicity studies (Jossé et al., 2008). Finally, HepaRG have little phenotypic variation (Lambert et al., 2009) minimizing variation between experiments. Despite these advantages, the major disadvantage to the use of these cells is a lengthy and inconvenient growth and differentiation period required prior to the initiation of experiments. Thus, pre-differentiated cryopreserved (cHepaRG) HepaRG cells represent a convenient alternative.

Interestingly, we found that cHepaRG cells exhibited both decreased basal levels of GSH and increased CYP2E1 and CYP1A2 mRNA (Figures 4.3.5A, B and Table 4.3.1) when compared to HepaRG cells. These differences could explain the variation in the magnitude of cell death between HepaRG and cHepaRG cells observed in the current study (Figure 4.3.1). Although the differences in CYPmRNA were not statistically significant, the 12-fold increase in CYP2E1, the major CYP responsible for APAP activation (Manyike et al., 2000), could explain the difference in adduct formation between the two preparations. With less GSH to detoxify NAPQI, levels of the reactive intermediate would be expected to increase, leading to greater adduct formation and increased cell death. This seems likely given the fact that fewer adducts were measured during all time points in HepaRG cells compared to cHepaRG cells. Nevertheless, the basis for decreased basal levels of GSH and increased CYP mRNA levels in cHepaRG cells requires further investigation.

Other *in vitro* models for the study of drug toxicity are lacking in either convenience, availability, or functional stability. First among these are the use of various immortalized cell lines, such as HepG2. While the HepG2 line may be useful for certain in vitro studies of liver function, their lack of CYP enzymes makes them a poor choice for drug metabolism studies (Hewitt and Hewitt, 2004). Other cell lines maintain high CYP levels, such as Huh7, however poor stability at high confluence in culture prevents their use for long term drug metabolism studies (Guguen-Guillouzo

and Guillouzo, 2010). Fresh precision cut liver slices have also been used. While the major advantage is the retention of the 3D liver architecture, both viability and enzyme level rapidly decrease following acquisition, as with primary human hepatocytes (Guguen-Guillouzo and Guillouzo, 2010). Finally, 3D models (spheroids) have been developed which preserve structure and function of hepatocytes as well as CYP levels (Xia et al., 2012). However, during prolonged culture of spheroids, extreme care must be taken to maintain spheroid size to optimize results. Failure to do so would likely lead to differential oxygen or drug delivery to spheroids of various sizes (Xia et al., 2012) which would be expected to influence the results. Despite multiple in vitro methods for study of the liver, HepaRG remains the only model which both recapitulates PHH drug metabolism and possesses the necessary stability in culture for long term studies. The development of pre-differentiated cryopreserved HepaRG (cHepaRG) cells which are functionally similar to undifferentiated cryopreserved (HepaRG) cells eliminates the need for lengthy growth and differentiation required of HepaRG cells.

In summary, we have compared, for the first time, APAP metabolism between freshly differentiated HepaRG cells (HepaRG) and pre-differentiated cryopreserved HepaRG cells (cHepaRG). We found that although there were slight differences in the overall magnitude of all measured parameters between HepaRG and cHepaRG cells, the pattern of toxicity between the two preparations is remarkably similar following APAP administration. Therefore, we conclude that pre-differentiated cryopreserved HepaRG cells represent a suitable and convenient alternative to freshly differentiated HepaRG cells for studies of acetaminophen toxicity.

5. DISCUSSION AND FUTURE DIRECTIONS

5.1 SUMMARY

In a series of studies, we employed non-invasive methods for the description of the molecular events leading up to cell death following liver injury from ischemic injury. We explored both cold and warm ischemic injury in the context of OLT and hypoxic hepatitis, respectively. To do this, we used a previously established set of biomarkers which are useful in differentiating between apoptosis and necrosis, determining the role of mitochondria in injury, and identifying a role of the immune system in injury. Additionally, we demonstrated that there was no significant difference in acetaminophen metabolism between two preparations of HepaRG cells, paving the way for the widespread use of the pre-differentiated cryopreserved HepaRG cells in studies of drug toxicity. This finding will allow for the rapid screening for the potential of hepatotoxicity of numerous drugs and aid in identification of drugs which may cause idiosyncratic drug induced liver injury.

5.2 NOVELTY OF THE USE OF BIOMARKERS TO STUDY ISCHEMIC LIVER INJURY

Our findings demonstrate that the use of biomarkers of injury, mode and mechanism of cell death, and inflammation, are not only novel for the study of ischemic injury in humans, but also underscore the discrepancy between certain animal models and the ability of these models to recapitulate the human condition. For instance, following warm ischemia in the rodent, there is a marked immune response which leads to exacerbation of injury. In contrast, during warm ischemia in humans, such a response does not occur. One detail of these studies which sets it apart from other studies using biomarkers is that we collected samples for an extended time frame, rather than studying a single time point, or a short time course shortly after the ischemic event. The importance

of this can be underscored by comparing our findings to previously published data from the mouse model of ischemic injury. In this model, there are two phases of injury – the early stage, in which ischemic injury leads to cell death, and the late stage, in which cellular debris (DAMPs) released from necrotic cells in the early stage leads to activation of macrophages, recruitment of neutrophils, and subsequent injury. In studies which only examine a short time course following ischemia, the late phase, during which inflammation plays a major role may be overlooked. Thus, these biomarkers represent a convenient way to obtain detailed information into the events leading to liver injury well after the initial injurious event. The fact that the injury pattern in the later stages of ischemia in humans did not have a similar pattern seen in the mouse model of ischemia, demonstrates the critical importance of prolonged monitoring and highlights the importance of selecting appropriate animal models when studying human conditions. In the case of the rodent model of ischemia, the ischemic time is titrated in order to study mechanisms of injury. A major drawback to using the rodent model of ischemia is that due to surgical advances, the level of injury observed is not well represented by a mouse model based on prolonged ischemic times. Future studies using the rodent model of ischemic injury as a surrogate for human liver transplantation should be designed and interpreted with this in mind.

5.3 APOPTOSIS VS. NECROSIS IN ISCHEMIC LIVER INJURY

Quite possibly one of the biggest ongoing disputes within the study of ischemic liver injury is whether cells die via apoptosis or necrosis. While this may seem like an academic argument, there exists a great deal of clinical significance; targeting the appropriate mode of cell death could be expected to minimize the progression of injury following ischemic insult in man. While some literature suggests apoptosis is the primary form of injury following ischemia (Compagnon et al.,

2017; Freitas et al., 2017; Ko et al., 2017), we have demonstrated that following warm ischemia, necrosis appears to be the predominant form of cell death (Weemhoff et al., 2017; Yang et al., 2014). Likewise, unpublished results from our laboratory demonstrate the same is true with cold ischemia following liver transplantation. While biopsy with histopathologic analysis would be needed to confirm necrosis following OLT and HH, our laboratory has shown previously, that following an episode of warm ischemia, >99% of cells die by necrosis. (Yang et al., 2014). One problem with many studies claiming that apoptosis is the primary mode of cell death is the use of only a single time point or inappropriate conclusions drawn from the use of the TUNEL assay. Even cytokeratin biomarker data can be misinterpreted if not presented or interpreted properly. For instance, one study demonstrates that following ischemic injury there is an increase in caspase-cleaved keratin-18 at 6hrs post reperfusion. However, data for full length keratin-18, or additional time points, are not shown. Indeed, even in our studies, we have shown that in both types of ischemic injury, caspase cleaved keratin can be elevated during reperfusion relative to healthy volunteers, and can even be elevated relative to other time points. However, when taken in context of the entire time course of injury, and in relationship to full length keratin, it becomes obvious that necrosis is the predominant mode of cell death. Similarly, many studies rely only on TUNEL staining to conclude that apoptosis is the primary form of cell death. However, it is well known that the TUNEL assay is not specific for apoptotic cell death (Grasl-Kraupp et al., 1995). Thus, data obtained from the TUNEL assay should be interpreted with caution.

Although both necrotic and apoptotic cells are identified using the TUNEL assay, we have previously shown that the pattern of TUNEL staining can be used to help differentiate between the two forms of cell death (Yang et al., 2014). As discussed previously, apoptotic cells will typically appear as small, condensed cells, either individually or in clusters, due to the apoptotic process. In

contrast, necrotic cells typically appear as large diffuse areas of staining due to membrane rupture and release of stain into the surrounding area. Because of the propensity for misinterpretation of the TUNEL assay, researchers intending to differentiate between the two forms of cell death in the absence of histopathology should use a secondary method in conjunction with the TUNEL assay. Such methods include, but are not limited to, caspase activity assays, Western blotting for cleaved caspase-3, and interventional studies using caspase inhibitors and/or necrostatins when possible. Even still, studies can be misinterpreted; a study claiming that apoptosis predominates after OLT was based on the fact that caspase inhibitors added to the preservative offered some protection against cell death. However, this study did not take into the account that the caspase inhibitors were added to the preservative at a concentration high enough to non-specifically inhibit proteases which may be responsible for necrotic cell death (Schotte et al., 1999).

5.4 MITOCHONDRIAL INVOLVEMENT IN ISCHEMIC LIVER INJURY

In addition to the differentiation between apoptosis and necrosis, we also demonstrated that mitochondria play a role cell death following ischemic injury. This finding confirms earlier cell culture data in which treatment of hepatocytes with mitochondrial protectants minimized injury following ischemia. In our studies, we measured GDH and mitochondrial DNA to identify mitochondrial injury and found a rise in GDH which mimicked that of ALT. The rise in GDH is significant because it implicates the mitochondria as an important component of cell death. An argument against this could be that the assay is measuring GDH from intact mitochondria released into circulation following cell death, implying that cell death occurs independently of mitochondrial injury. However, in our studies, plasma samples were centrifuged to remove intact mitochondria prior to the measurement of GDH. In addition, it has been shown that cell death can

occur in the absence of mitochondrial injury (McGill et al., 2012). Based on these findings, future research should explore the exact role mitochondrial injury plays in the pathophysiology of ischemic injury in patients. Currently, mitochondrial targeted therapies (such as Mito-tempo) are being developed to protect against drug induced liver injury (Du et al., 2017). The use of these agents could also be explored in the context of ischemic liver injury for potential use in the clinic in these patients.

5.5 INFLAMMATION FOLLOWING ISCHEMIC LIVER INJURY IN HUMANS

Based on our data, we conclude that inflammation does not play a major role in liver injury following transplantation or hypoxic hepatitis. This is in stark contrast to conclusions made about liver transplantation based on the mouse model. The most likely explanation for the discrepancy between the injury pattern between the two models is the preservation period during the ischemic period in OLT. Factors such as preservation techniques, optimal surgical strategy, and appropriate donor-recipient matching all help minimize the degree of injury which occurs during the ischemic period. As we know from the rodent model, it is the initial injury and release of DAMPs which leads to macrophage activation, the recruitment of neutrophils, and additional injury (van Golen et al., 2012; Jaeschke et al., 1990, 1992). Therefore, it stands to reason that with very little injury to begin with, there would be little to no response by the immune system. Indeed, data from both studies suggest that inflammation plays little to no role in the development of injury in our patients. For liver transplantation, the combination of preservatives and limited cold ischemia time could explain the lack of injury (and subsequent inflammation). However, during hypoxic hepatitis (HH), the lack of inflammation can less easily be explained, as this condition experiences warm ischemia for prolonged periods of time, similar to the rodent model. Nevertheless, based on the pattern of

liver injury observed in these patients, inflammation does not appear to exacerbate liver injury. Likewise, with liver transplantation, we would expect an increase in ALT at times well after reperfusion if inflammation played a key role in the late stages of injury. Furthermore, this rise in ALT would likely correlate with activation of the immune systems, as in the rodent model. However, following liver transplantation, we did not observe a change in neutrophil activation suggestive of an immune component of injury. Instead, we observed an increase in ALT only shortly after ischemia with a gradual decline to baseline levels over time. Because neutrophil-depleting antibodies are protective against ischemic injury in mice (Jaeschke et al., 1990), the lack of neutrophil activation in our patients strongly suggests the lack of an inflammatory component. However, the direct measurement of pro- and anti-inflammatory cytokines would be necessary for a more direct and conclusive assessment of the immune response following liver transplantation. Even still, neutrophil and cytokine data would need to be interpreted in conjunction with liver injury, as neutrophil infiltration occurs in other models of liver injury, namely acetaminophen toxicity, without causing additional injury (Williams et al., 2014). In this model, neutrophil infiltration is thought to be beneficial for initiation of the regenerative response (Williams et al., 2014).

Not surprisingly, the degree of injury in HH patients was significantly higher than in OLT patients. In our study however, survival following HH was higher than reported in the literature (93% vs. 50%) (Fuhrmann et al., 2010; Hawker, 1991). While there exist several possible explanations for this discrepancy, the most likely is that in our study, patients were mostly healthy individuals with a single documented episode of hypotension, often induced by an opioid overdose. In contrast, numbers reported in literature often reflect elderly patients with significant health issues including

cardiac or respiratory disease (Fuhrmann et al., 2009). Other reasons for the discrepancy could involve other co-morbidities not identified or accounted for in our study. Another likely cause for the discrepancy in survival rate amongst hypoxic hepatitis patients is simply the sample size of our study, which included only 13 patients. The incorporation of more patients into our hypoxic hepatitis study would be beneficial for the advancement of our knowledge of this field and allow us to subdivide patients based on co-morbidities and underlying cause of the initial ischemic insult. Furthermore, a larger patient population would possibly allow us to use this biomarker data to aide in prognosis of these patients. One major obstacle for the addition of additional patients to this study is that a diagnosis of HH is made only after ruling out other causes of liver injury (namely DILI). Therefore, any patient whose clinical symptoms match those of hypoxic hepatitis would need to be enrolled in a study prior to a diagnosis of HH. This alone does not present much of a challenge, but multiple blood collections would need to be obtained while a diagnosis is pending. Furthermore, markers of neutrophil activation, such as CD11b expression, ROS production, and phagocytic activity need to be measured within hours of blood collection. While none of these are insurmountable obstacles, an ongoing study of HH would need to account and plan for the consumption of added time and expenses for patients who, in the end, may not even be diagnosed with HH.

Due to the discrepancies between the rodent model of ischemia and the pattern of injury following cold and warm ischemia studied in this dissertation, it is at least clear that the rodent IR models as they are currently used are not appropriate for translation to OLT patients. Furthermore, based on the degree of injury in our HH patients, it is equally clear that the HH model in rodents, mainly

hemorrhagic shock/resuscitation, needs to be refined to be more consistent and representative of the degree of injury in humans.

5.6 COMPLICATIONS FOLLOWING ORTHOTOPIC LIVER TRANSPLANTATION

A major conceptual difference between the mouse model of ischemia and extrapolation to human transplantation is that the mouse model focuses on degree of injury as an end-point. In human liver transplantation, every effort is made to minimize injury, but the degree of injury as measured by ALT is not the end-point. In fact, the degree of injury following surgery provides the physician very little information as to the success or likely outcome of the procedure. This is exemplified by the fact that graft rejection following transplantation is seldom due to acute hepatocellular injury, but rather due to complications arising from the biliary system (Seehofer et al., 2013; Song et al., 2014; Wojcicki et al., 2008). The most common cause of complications from OLT today are, in fact, biliary strictures (Karimian et al., 2014). Unpublished data from our study show that about 20% percent of patients developed biliary stricture and this is in line with previously published reports (Giacomoni et al., 2006; Kochhar et al., 2013; Soejima et al., 2006)**.** However, we did not find any correlation between the development of stricture and graft failure, or survival. Furthermore, of major importance is that the majority of these strictures were extrahepatic, rather than intrahepatic biliary stricture. One common cause for these types of strictures are typically due to surgical technique or donor matching (biliary ductal size mismatch) (Karimian et al., 2014; Verdonk et al., 2006). In our study, most patients received biliary stents at the time of transplantation, minimizing or eliminating the risk of stricture formation. Of more concern, are intrahepatic biliary strictures, which are thought to be caused due to ischemic injury of the biliary epithelial cells (Buis et al., 2006; Sanchez-Urdazpal et al., 1992). Indeed, it has been shown that

biliary epithelial cells are more susceptible to ischemia than hepatocytes (Imamura et al., 1997; McKeown et al., 1988; Noack et al., 1993) and this leads to areas of stenosis within the biliary tree, leading to chronic injury and complications. Intrahepatic biliary strictures seem to be more a result of warm ischemic-time than cold ischemic time because when they occur, they generally occur in patients who received a liver from a donor after cardiac death (Pine et al., 2009). During organ harvest, the liver can be harvested following declaration of brain death or cardiac death. During brain death, the liver may still be receiving adequate blood flow from the heart up until the time of removal. Thus, there is minimal warm ischemia. Conversely, the organ may be harvested following cardiac death, which can be a prolonged process involving hypotension, hypoperfusion, and prolonged warm ischemic injury well before the organ is harvested. In our study, it is unknown as to which patients received a liver from a donor following brain or cardiac death, but the low incidence of non-anastomotic biliary strictures suggests that most xenografts were obtained following brain death. Though it is generally accepted that non-anastomotic biliary strictures are primarily a function of warm ischemia, (Abt et al., 2003; Cursio and Gugenheim, 2012; Pine et al., 2009; Taner et al., 2012; de Vera et al., 2009) they are not a commonly reported complication of hypoxic hepatitis. This is an interesting observation because during hypoxic hepatitis, the liver undergoes prolonged warm ischemic times and thus these strictures would be expected to occur in high frequency in these patients. One explanation for this discrepancy could simply be that due to the high mortality rate with hypoxic hepatitis, many patients do not survive long enough to develop biliary stricture. Alternatively, the pathophysiology of biliary stricture could be multifactorial and involve more than ischemic injury to biliary epithelial cells. Thus, long term monitoring of patients following an episode of hypoxic hepatitis might provide valuable information into the role of warm ischemia on the pathophysiology of non-anastomotic strictures.

The fact that there were very few intrahepatic biliary stricture patients in our study is consistent with the overall degree of injury following liver transplantation and can also be attributed to both cold ischemia (vs. warm ischemia) and the use of preservatives. There have been studies suggesting that certain miRNAs released from biliary epithelial cells upon their death may be predictive for the formation of intrahepatic biliary stricture (Verhoeven et al., 2013). However, due to deviation from standard of patient care combined with the invasive nature of the procedure, the collection of bile over an extended period of time for the measurement of miRNA was not possible for our study and we were unable to explore this hypothesis. Thus, follow up studies for this purpose would provide significant information as to the pathophysiology of the development of biliary strictures. Regardless, our studies show that biomarkers represent a convenient and easy way to study both these conditions when invasive methods are unavailable or contraindicated.

5.7 UNDIFFERENTIATED VS. PRE-DIFFERENTIATED CRYOPRESERVED HEPARG CELLS

HepaRG cells have been used in the laboratory since 2002 (Gripon et al., 2002) and are useful compared to other cell lines for a variety of reasons. Importantly for studies of drug metabolism, HepaRG cells carry a full complement of active CYP enzymes while other cell lines, such as HepG2, do not (Aninat et al., 2006; Kanebratt and Andersson, 2008; Sassa et al., 1987). Because of the time-consuming nature of their growth and differentiation process, HepaRG cells are not always practical for short experiments in the lab. To overcome this obstacle, and promote the widespread use of this cell line, methods to cryopreserve differentiated HepaRG cells have been developed. While the growth and differentiation process remain the same, the cells are preserved in a differentiated state. Upon initiation of an experiment, the cells are thawed, plated, and allowed to grow for a short period (approximately 1 week) prior to their use in experiments. In contrast,

HepaRG cells purchased at passage 17 would require a minimum of 8 weeks for growth and differentiation. However, prior to the onset of our study, no group has directly compared the pre-differentiated HepaRG cells to the undifferentiated cells. Our findings detailed in Chapter 4 demonstrate that the two different preparations respond biologically similarly and thus can be used interchangeably. Perhaps of equal importance, we demonstrated that these cells are also comparable to primary human hepatocytes, the gold standard for *in vitro* hepatotoxicity research. Since primary human hepatocytes are only sporadically available, require special and lengthy isolation techniques, and do not tolerate the freeze/thaw cycle well, their use is often limited to laboratories equipped to handle their immediate isolation and use. These findings set the stage for the widespread use of HepaRG cells in drug toxicity studies.

Acetaminophen toxicity is the most common cause of liver failure in the United States (Budnitz et al., 2011; Lee, 2013). This is due, in part to the fact that acetaminophen is readily available as an over-the-counter analgesic and antipyretic. In fact, according to the FDA, more than 24 billion doses of acetaminophen were sold in 2008 and more than 48 million people use acetaminophen on a regular basis. In addition to this, acetaminophen is commonly added to opioid analgesics such as Vicodin (hydrocodone and acetaminophen) and Percocet (oxycodone and acetaminophen). This strategy allows for the reduction in opioid, and its potential for habit forming behavior, without compromising pain relief for the patient. However, if patients are unaware that these medications contain acetaminophen, they are likely to self-administer additional acetaminophen for the relief of break-through pain. This can lead to accidental overdose. Indeed, in 2011 there were over 80,000 cases of acetaminophen, 50% of which led to acute liver failure (Budnitz et al., 2011). While an antidote exists (N-acetylcysteine), this is only effective if given shortly after a toxic dose. Since many overdoses are either unknown or intentional, this becomes impractical in the clinical

setting. Thus, the study of the mechanisms of acetaminophen toxicity and interventions to minimize injury after the window during which NAC would be effective has tremendous application in the clinical setting.

APAP hepatotoxicity is well characterized in humans and rodents; it is known to involve CYP-mediated metabolic activation, glutathione depletion, and mitochondrial injury. Because of the frequency with which acetaminophen hepatotoxicity occurs, we used the acetaminophen model of drug induced liver injury to identify any potential differences between freshly differentiated HepaRG cells and cHepaRG cells. As discussed in the previous chapter, we found that the response to acetaminophen toxicity between both cell preparations were remarkably similar with respect to all facets of acetaminophen toxicity.

However, despite the similarities, we did see a difference in CYP mRNA levels. While this difference was not statistically significant and did not appear to affect the overall behavior of the cells, cryopreserved pre-differentiated HepaRG cells had a higher expression of CYP2E1, 3A4, and 1A2 than their counterpart. One possible explanation for this could lie in the growth and differentiation process, during which approximately 50% of the total cell population become hepatocytes and the rest develop into biliary epithelial cells (Gripon et al., 2002; Parent et al., 2004). This, however, is a rough estimate and each individual culture could have more of one type than the other. Thus, when working with HepaRG cells, CYP mRNA levels should be normalized to hepatocyte specific proteins, such as albumin. Nevertheless, despite the absolute expression of CYP enzymes, CYP activity between the two were not different. Thus, we conclude that the pattern of toxicity and injury is similar between the two preparations suggesting they can be used in other models of DILI. Since hepatotoxicity is the most common cause of drug failure and post-market withdrawal, the widespread use of HepaRG cells in the drug development process would likely

help identify drugs with the potential for hepatotoxicity before they make it to clinical trials or to market. By identifying these compounds earlier in the process, there exists not only a financial benefit to the pharmaceutical company, but more importantly, a significant decrease of risk to the general public.

The overall convenience of these cells can be extrapolated to other areas of hepatology. In fact, our laboratory has already demonstrated that in addition to similarities in the metabolism of acetaminophen, HepaRG cells respond similarly to primary human hepatocytes in studies of bile acid toxicity, eliminating the need for the primary cell line for these studies.(Woolbright et al., 2016) HepaRG cells could theoretically replace PHH for ischemic hepatitis studies. Furthermore, the use of HepaRG cells could expedite the field of biomarker research by serving as a platform for the identification of additional hepatocyte or biliary biomarkers of injury, or even regeneration, under a variety of conditions. Indeed, studies are already using these cells in biomarker research following drug toxicity.(Marrone et al., 2016) Finally, because HepaRG cells are a mixed population of hepatocytes and biliary epithelial cells, this cell line could potentially be used in studies of biliary epithelial cells.

5.8 CONCLUDING REMARKS

In summary, we have demonstrated that the use of previously identified biomarkers can be useful in describing the events following both warm and cold ischemic injury in humans. While our findings and conclusions provide significant advances to our understanding of the pathophysiology of ischemic injury in patients, more research is necessary to refine this knowledge. Future studies should be directed at determining the role of mitochondria in cell death following ischemia, identifying biomarkers of biliary injury and stricture, and identifying biomarkers of prognosis and

outcome. Based on our findings with the HepaRG cell line, the widespread use of these cells may help to accelerate research and discovery within the field of drug hepatoxicity and safety studies, particularly acetaminophen.

REFERENCES:

Abt, P., Crawford, M., Desai, N., Markmann, J., Olthoff, K., and Shaked, A. (2003). Liver transplantation from controlled non-heart-beating donors: an increased incidence of biliary complications. Transplantation *75*, 1659–1663.

Aden, D.P., Fogel, A., Plotkin, S., Damjanov, I., and Knowles, B.B. (1979). Controlled synthesis of HBsAg in a differentiated human liver carcinoma-derived cell line. Nature *282*, 615–616.

Alcorn, J.M., and Miyai, K. (1992). Aortic dissection and hepatic ischemia. J. Clin. Gastroenterol. *14*, 180–182.

Aldridge, D.R., Tranah, E.J., and Shawcross, D.L. (2015). Pathogenesis of hepatic encephalopathy: role of ammonia and systemic inflammation. J. Clin. Exp. Hepatol. *5*, S7–S20.

Alkhouri, N., Carter-Kent, C., and Feldstein, A.E. (2011). Apoptosis in nonalcoholic fatty liver disease: diagnostic and therapeutic implications. Expert Rev. Gastroenterol. Hepatol. *5*, 201–212.

Amacher, D.E. (2002). A toxicologist's guide to biomarkers of hepatic response. Hum. Exp. Toxicol. *21*, 253–262.

Andersson, P., Gidlöf, O., Braun, O.O., Götberg, M., van der Pals, J., Olde, B., and Erlinge, D. (2012). Plasma levels of liver-specific miR-122 is massively increased in a porcine cardiogenic shock model and attenuated by hypothermia. Shock *37*, 234–238.

Aninat, C., Piton, A., Glaise, D., Charpentier, T.L., Langouët, S., Morel, F., Guguen-Guillouzo, C., and Guillouzo, A. (2006). Expression of cytochromes P450, conjugating enzymes and nuclear receptors in human hepatoma HepaRG cells. Drug Metab. Dispos. *34*, 75–83.

Anthérieu, S., Chesné, C., Li, R., Camus, S., Lahoz, A., Picazo, L., Turpeinen, M., Tolonen, A., Uusitalo, J., Guguen-Guillouzo, C., et al. (2010). Stable expression, activity, and inducibility of cytochromes P450 in differentiated HepaRG cells. Drug Metab. *38*, 516–525.

Antoine, D.J., Williams, D.P., Kipar, A., Jenkins, R.E., Regan, S.L., Sathish, J.G., Kitteringham, N.R., and Park, B.K. (2009). High-mobility group box-1 protein and keratin-18, circulating serum proteins informative of acetaminophen-induced necrosis and apoptosis in vivo. Toxicol. Sci. *112*, 521–531.

Antoine, D.J., Jenkins, R.E., Dear, J.W., Williams, D.P., McGill, M.R., Sharpe, M.R., Craig, D.G., Simpson, K.J., Jaeschke, H., and Park, B.K. (2012). Molecular forms of HMGB1 and keratin-18 as mechanistic biomarkers for mode of cell death and prognosis during clinical acetaminophen hepatotoxicity. J. Hepatol. *56*, 1070–1079.

Antoine, D.J., Dear, J.W., Lewis, P.S., Platt, V., Coyle, J., Masson, M., Thanacoody, R.H., Gray, A.J., Webb, D.J., Moggs, J.G., et al. (2013). Mechanistic biomarkers provide early and sensitive detection of acetaminophen-induced acute liver injury at first presentation to hospital. Hepatology *58*, 777–787.

Antoine, D.J., Sabbisetti, V.S., Francis, B., Jorgensen, A.L., Craig, D.G.N., Simpson, K.J., Bonventre, J.V., Park, B.K., and Dear, J.W. (2015). Circulating Kidney Injury Molecule 1 Predicts Prognosis and Poor Outcome in Patients With Acetaminophen-Induced Liver Injury. Hepatol. *62*, 591–599.

Antoniades, C.G., Quaglia, A., Taams, L.S., Mitry, R.R., Hussain, M., Abeles, R., Possamai, L.A., Bruce, M., McPhail, M., Starling, C., et al. (2012). Source and characterization of hepatic macrophages in acetaminophen-induced acute liver failure in humans. Hepatol. *56*, 735–746.

Arrowsmith, J., and Miller, P. (2013). Trial watch: phase II and phase III attrition rates 2011-2012. Nat. Rev. Drug Discov. *12*, 569.

Bajt, M.L., Knight, T.R., Lemasters, J.J., and Jaeschke, H. (2004). Acetaminophen-induced oxidant stress and cell injury in cultured mouse hepatocytes: protection by N-acetyl cysteine. Toxicol. Sci. *80*, 343–349.

Bajt, M.L., Cover, C., Lemasters, J.J., and Jaeschke, H. (2006). Nuclear translocation of endonuclease G and apoptosis-inducing factor during acetaminophen-induced liver cell injury. Toxicol. Sci. *94*, 217–225.

Bajt, M.L., Farhood, A., Lemasters, J.J., and Jaeschke, H. (2008). Mitochondrial bax translocation accelerates DNA fragmentation and cell necrosis in a murine model of acetaminophen hepatotoxicity. J. Pharmacol. Exp. Ther. *324*, 8–14.

Bajt, M.L., Ramachandran, A., Yan, H.-M., Lebofsky, M., Farhood, A., Lemasters, J.J., and Jaeschke, H. (2011). Apoptosis-inducing factor modulates mitochondrial oxidant stress in acetaminophen hepatotoxicity. Toxicol. Sci. 1*22*, 598–605.

Bala, S., Marcos, M., and Szabo, G. (2009). Emerging role of microRNAs in liver diseases. World J. Gastroenterol. *15*, 5633–5640.

Bantel, H., Lügering, A., Heidemann, J., Volkmann, X., Poremba, C., Strassburg, C.P., Manns, M.P., and Schulze-Osthoff, K. (2004). Detection of apoptotic caspase activation in sera from patients with chronic HCV infection is associated with fibrotic liver injury. Hepatol. *40*, 1078–1087.

Barbiro, E., Zurovsky, Y., and Mayevsky, A. (1998). Real time monitoring of rat liver energy state during ischemia. Microvasc. Res. *56*, 253–260.

Baskin-Bey, E.S., Washburn, K., Feng, S., Oltersdorf, T., Shapiro, D., Huyghe, M., Burgart, L., Garrity-Park, M., van Vilsteren, F.G.I., Oliver, L.K., et al. (2007). Clinical Trial of the Pan-Caspase Inhibitor, IDN-6556, in Human Liver Preservation Injury. Am. J. Transplant. *7*, 218–225.

Bechmann, L.P., Jochum, C., Kocabayoglu, P., Sowa, J.-P., Kassalik, M., Gieseler, R.K., Saner, F., Paul, A., Trautwein, C., Gerken, G., et al. (2010). Cytokeratin 18-based modification of the MELD score improves prediction of spontaneous survival after acute liver injury. J. Hepatol. *53*, 639–647.

Beckwith-Hall, B.M., Nicholson, J.K., Nicholls, A.W., Foxall, P.J., Lindon, J.C., Connor, S.C., Abdi, M., Connelly, J., and Holmes, E. (1998). Nuclear magnetic resonance spectroscopic and principal components analysis investigations into biochemical effects of three model hepatotoxins. Chem. Res. Toxicol. *11*, 260–272.

Bonaldi, T., Talamo, F., Scaffidi, P., Ferrera, D., Porto, A., Bachi, A., Rubartelli, A., Agresti, A., and Bianchi, M.E. (2003). Monocytic cells hyperacetylate chromatin protein HMGB1 to redirect it towards secretion. EMBO J. *22*, 5551–5560.

Budnitz, D.S., Lovegrove, M.C., and Crosby, A.E. (2011). Emergency department visits for overdoses of acetaminophen-containing products. Am. J. Prev. Med. *40*, 585–592.

Buis, C.I., Hoekstra, H., Verdonk, R.C., and Porte, R.J. (2006). Causes and consequences of ischemic-type biliary lesions after liver transplantation. J. Hepatobiliary. Pancreat. Surg. *13*, 517–524.

Bunchorntavakul, C., and Reddy, K.R. (2013). Acetaminophen-related hepatotoxicity. Clin. Liver Dis. *17*, 587–607, viii.

Butterworth, R.F. (2015). Pathogenesis of hepatic encephalopathy and brain edema in acute liver failure. J. Clin. Exp. Hepatol. *5*, S96–S103.

Camargo, C.A., Madden, J.F., Gao, W., Selvan, R.S., and Clavien, P.A. (1997). Interleukin-6 protects liver against warm ischemia/reperfusion injury and promotes hepatocyte proliferation in the rodent. Hepatol. *26*, 1513–1520.

Ceppa, E.P., Fuh, K.C., and Bulkley, G.B. (2003). Mesenteric hemodynamic response to circulatory shock. Curr. Opin. Crit. Care *9*, 127–132.

Chatauret, N., Thuillier, R., and Hauet, T. (2011). Preservation strategies to reduce ischemic injury in kidney transplantation: pharmacological and genetic approaches. Curr. Opin. Organ Transplant. *16*, 180–187.

Chen, M., Suzuki, A., Borlak, J., Andrade, R.J., and Lucena, M.I. (2015). Drug-induced liver injury: Interactions between drug properties and host factors. J. Hepatol. *63*, 503–514.

Cohen, S.D., Pumford, N.R., Khairallah, E.A., Boekelheide, K., Pohl, L.R., Amouzadeh, H.R., and Hinson, J.A. (1997). Selective protein covalent binding and target organ toxicity. Toxicol. Appl. Pharmacol. *143*, 1–12.

Compagnon, P., Levesque, E., Hentati, H., Disabato, M., Calderaro, J., Feray, C., Corlu, A., Cohen, J.L., Mosbah, I.B., and Azoulay, D. (2017). An Oxygenated and Transportable Machine Perfusion System Fully Rescues Liver Grafts Exposed to Lethal Ischemic Damage in a Pig Model of DCD Liver Transplantation. Transplantation.

Cortez, M.A., and Calin, G.A. (2009). MicroRNA identification in plasma and serum: a new tool to diagnose and monitor diseases. Expert Opin. Biol. Ther. *9*, 703–711.

Cover, C., Mansouri, A., Knight, T.R., Bajt, M.L., Lemasters, J.J., Pessayre, D., and Jaeschke, H. (2005). Peroxynitrite-induced mitochondrial and endonuclease-mediated nuclear DNA damage in acetaminophen hepatotoxicity. J. Pharmacol. Exp. Ther. *315*, 879–887.

Craig, D.G.N., Lee, P., Pryde, E.A., Masterton, G.S., Hayes, P.C., and Simpson, K.J. (2011). Circulating apoptotic and necrotic cell death markers in patients with acute liver injury. Liver Int. *31*, 1127–1136.

Cursio, R., and Gugenheim, J. (2012). Ischemia-Reperfusion Injury and Ischemic-Type Biliary Lesions following Liver Transplantation. J. Transplant. *2012*, 164329.

Dahlin, D.C., Miwa, G.T., Lu, A.Y., and Nelson, S.D. (1984). N-acetyl-p-benzoquinone imine: a cytochrome P-450-mediated oxidation product of acetaminophen. Proc. Natl. Acad. Sci. *81*, 1327–1331.

Dear, J.W., Antoine, D.J., Starkey-Lewis, P., Goldring, C.E., and Park, B.K. (2014). Early detection of paracetamol toxicity using circulating liver microRNA and markers of cell necrosis. Br. J. Clin. Pharmacol. *77*, 904–905.

Dolganiuc, A., Petrasek, J., Kodys, K., Catalano, D., Mandrekar, P., Velayudham, A., and Szabo, G. (2009). MicroRNA expression profile in Lieber-DeCarli diet-induced alcoholic and methionine choline deficient diet-induced nonalcoholic steatohepatitis models in mice. Alcohol. Clin. Exp. Res. *33*, 1704–1710.

Du, K., Williams, C.D., McGill, M.R., Xie, Y., Farhood, A., Vinken, M., and Jaeschke, H. (2013). The gap junction inhibitor 2-aminoethoxy-diphenyl-borate protects against acetaminophen hepatotoxicity by inhibiting cytochrome P450 enzymes and c-jun N-terminal kinase activation. Toxicol. Appl. Pharmacol. *273*, 484–491.

Du, K., Xie, Y., McGill, M.R., and Jaeschke, H. (2015). Pathophysiological significance of c-jun N-terminal kinase in acetaminophen hepatotoxicity. Expert Opin. Drug Metab. Toxicol. *11*, 1769–1779.

Du, K., Farhood, A., and Jaeschke, H. (2017). Mitochondria-targeted antioxidant Mito-Tempo protects against acetaminophen hepatotoxicity. Arch. Toxicol. *91*, 761–773.

Duan, L., Davis, J.S., Woolbright, B.L., Du, K., Cahkraborty, M., Weemhoff, J., Jaeschke, H., and Bourdi, M. (2016). Differential susceptibility to acetaminophen-induced liver injury in sub-strains of C57BL/6 mice: 6N versus 6J. Food Chem. Toxicol. *98*, 107–118.

Ellett, J.D., Evans, Z.P., Atkinson, C., Schmidt, M.G., Schnellmann, R.G., and Chavin, K.D. (2009). Toll-like receptor 4 is a key mediator of murine steatotic liver warm ischemia/reperfusion injury. Liver Transpl. *15*, 1101–1109.

Eltzschig, H.K., and Eckle, T. (2011). Ischemia and reperfusion--from mechanism to translation. Nat. Med. *17*, 1391–1401.

El-Wahsh, M. (2007). Liver graft preservation: an overview. Hepatobiliary Pancreat. Dis. Int. *6*, 12–16.

Fisher, K., Vuppalanchi, R., and Saxena, R. (2015). Drug-Induced Liver Injury. Arch. Pathol. Lab. Med. *139*, 876–887.

Freitas, S.H., Dória, R.G.S., Bueno, R.S., Rocha, W.B., Filho, J.R.E., Moraes, J.R.E., Vidane, A.S., and Ambrósio, C.E. (2017). Evaluation of potential changes in liver and lung tissue of rats in an ischemia-reperfusion injury model (modified pringle maneuver). PloS One *12*, e0178665.

French, S.W., Benson, N.C., and Sun, P.S. (1984). Centrilobular liver necrosis induced by hypoxia in chronic ethanol-fed rats. Hepatol. *4*, 912–917.

Friedman, B.H., Wolf, J.H., Wang, L., Putt, M.E., Shaked, A., Christie, J.D., Hancock, W.W., and Olthoff, K.M. (2012). Serum cytokine profiles associated with early allograft dysfunction in patients undergoing liver transplantation. Liver Transplant. *18*, 166–176.

Fuhrmann, V., Kneidinger, N., Herkner, H., Heinz, G., Nikfardjam, M., Bojic, A., Schellongowski, P., Angermayr, B., Kitzberger, R., Warszawska, J., et al. (2009). Hypoxic hepatitis: underlying conditions and risk factors for mortality in critically ill patients. Intensive Care Med. *35*, 1397–1405.

Fuhrmann, V., Jäger, B., Zubkova, A., and Drolz, A. (2010). Hypoxic hepatitis - epidemiology, pathophysiology and clinical management. Wien. Klin. Wochenschr. *122*, 129–139.

Gasbarrini, A., Borle, A.B., Farghali, H., Bender, C., Francavilla, A., and Van Thiel, D. (1992). Effect of anoxia on intracellular ATP, Na+i, Ca2+i, Mg2+i, and cytotoxicity in rat hepatocytes. J. Biol. Chem. *267*, 6654–6663.

Giacomoni, A., Lauterio, A., Slim, A.O., Vanzulli, A., Calcagno, A., Mangoni, I., Belli, L.S., De Gasperi, A., and De Carlis, L. (2006). Biliary complications after living donor adult liver transplantation. Transpl. Int. *19*, 466–473.

van Golen, R.F., van Gulik, T.M., and Heger, M. (2012). The sterile immune response during hepatic ischemia/reperfusion. Cytokine Growth Factor Rev. *23*, 69–84.

Grasl-Kraupp, B., Ruttkay-Nedecky, B., Koudelka, H., Bukowska, K., Bursch, W., and Schulte-Hermann, R. (1995). In situ detection of fragmented DNA (TUNEL assay) fails to discriminate among apoptosis, necrosis, and autolytic cell death: a cautionary note. Hepatol. *21*, 1465–1468.

Gripon, P., Rumin, S., Urban, S., Le Seyec, J., Glaise, D., Cannie, I., Guyomard, C., Lucas, J., Trepo, C., and Guguen-Guillouzo, C. (2002). Infection of a human hepatoma cell line by hepatitis B virus. Proc. Natl. Acad. Sci.*99*, 15655–15660.

Guguen-Guillouzo, C., and Guillouzo, A. (2010). General review on in vitro hepatocyte models and their applications. Methods Mol. Biol. *640*, 1–40.

Guillouzo, A., Corlu, A., Aninat, C., Glaise, D., Morel, F., and Guguen-Guillouzo, C. (2007). The human hepatoma HepaRG cells: a highly differentiated model for studies of liver metabolism and toxicity of xenobiotics. Chem. Biol. Interact. *168*, 66–73.

Gujral, J.S., Bucci, T.J., Farhood, A., and Jaeschke, H. (2001). Mechanism of cell death during warm hepatic ischemia-reperfusion in rats: apoptosis or necrosis? Hepatol. *33*, 397–405.

Gunawan, B.K., Liu, Z.-X., Han, D., Hanawa, N., Gaarde, W.A., and Kaplowitz, N. (2006). c-Jun N-terminal kinase plays a major role in murine acetaminophen hepatotoxicity. Gastroenterology *131*, 165–178.

Guy, C.D., Suzuki, A., Burchette, J.L., Brunt, E.M., Abdelmalek, M.F., Cardona, D., McCall, S.J., Ünalp, A., Belt, P., Ferrell, L.D., et al. (2012). Costaining for keratins 8/18 plus ubiquitin improves detection of hepatocyte injury in nonalcoholic fatty liver disease. Hum. Pathol. *43*, 790–800.

Hanawa, N., Shinohara, M., Saberi, B., Gaarde, W.A., Han, D., and Kaplowitz, N. (2008). Role of JNK translocation to mitochondria leading to inhibition of mitochondria bioenergetics in acetaminophen-induced liver injury. J. Biol. Chem. *283*, 13565–13577.

Hart, S.N., Li, Y., Nakamoto, K., Subileau, E., Steen, D., and Zhong, X. (2010). A comparison of whole genome gene expression profiles of HepaRG cells and HepG2 cells to primary human hepatocytes and human liver tissues. Drug Metab. Dispos. *38*, 988–994.

Hasegawa, T., Malle, E., Farhood, A., and Jaeschke, H. (2005). Generation of hypochlorite-modified proteins by neutrophils during ischemia-reperfusion injury in rat liver: attenuation by ischemic preconditioning. Am. J. Physiol. Gastrointest. Liver Physiol. *289*, G760-767.

Hausenloy, D.J., and Yellon, D.M. (2013). Myocardial ischemia-reperfusion injury: a neglected therapeutic target. J. Clin. Invest. *123*, 92–100.

Hawker, F. (1991). Liver dysfunction in critical illness. Anaesth. Intensive Care *19*, 165–181.

Henderson, N.C., Pollock, K.J., Frew, J., Mackinnon, A.C., Flavell, R.A., Davis, R.J., Sethi, T., and Simpson, K.J. (2007). Critical role of c-jun (NH2) terminal kinase in paracetamol- induced acute liver failure. Gut *56*, 982–990.

Hengstler, J.G., Utesch, D., Steinberg, P., Platt, K.L., Diener, B., Ringel, M., Swales, N., Fischer, T., Biefang, K., Gerl, M., et al. (2000). Cryopreserved primary hepatocytes as a constantly available in vitro model for the evaluation of human and animal drug metabolism and enzyme induction. Drug Metab. Rev. *32*, 81–118.

Henrion, J., Colin, L., Schapira, M., and Heller, F.R. (1997). Hypoxic hepatitis caused by severe hypoxemia from obstructive sleep apnea. J. Clin. Gastroenterol. *24*, 245–249.

Henrion, J., Minette, P., Colin, L., Schapira, M., Delannoy, A., and Heller, F.R. (1999). Hypoxic hepatitis caused by acute exacerbation of chronic respiratory failure: a case-controlled, hemodynamic study of 17 consecutive cases. Hepatol. *29*, 427–433.

Henrion, J., Schapira, M., Luwaert, R., Colin, L., Delannoy, A., and Heller, F.R. (2003). Hypoxic hepatitis: clinical and hemodynamic study in 142 consecutive cases. Medicine *82*, 392–406.

Herndon, C.M., and Dankenbring, D.M. (2014). Patient perception and knowledge of acetaminophen in a large family medicine service. J. Pain Palliat. Care Pharmacother. *28*, 109–116.

Hewitt, N.J., and Hewitt, P. (2004). Phase I and II enzyme characterization of two sources of HepG2 cell lines. Xenobiotica *34*, 243–256.

Hewitt, N.J., Lechón, M.J.G., Houston, J.B., Hallifax, D., Brown, H.S., Maurel, P., Kenna, J.G., Gustavsson, L., Lohmann, C., Skonberg, C., et al. (2007). Primary hepatocytes: current understanding of the regulation of metabolic enzymes and transporter proteins, and pharmaceutical practice for the use of hepatocytes in metabolism, enzyme induction, transporter, clearance, and hepatotoxicity studies. Drug Metab. Rev. *39*, 159–234.

Horvatits, T., Trauner, M., and Fuhrmann, V. (2013). Hypoxic liver injury and cholestasis in critically ill patients. Curr. Opin. Crit. Care *19*, 128–132.

Ilmakunnas, M., Tukiainen, E.M., Rouhiainen, A., Rauvala, H., Arola, J., Nordin, A., Mäkisalo, H., Höckerstedt, K., and Isoniemi, H. (2008). High mobility group box 1 protein as a marker of hepatocellular injury in human liver transplantation. Liver Transpl. *14*, 1517–1525.

Ilmakunnas, M., Höckerstedt, K., Mäkisalo, H., Siitonen, S., Repo, H., and Pesonen, E.J. (2009). Hepatic neutrophil activation during reperfusion may not contribute to initial graft function after short cold ischemia in human liver transplantation. Transplant. Proc. *41*, 739–742.

Imamura, H., Brault, A., and Huet, P.M. (1997). Effects of extended cold preservation and transplantation on the rat liver microcirculation. Hepatol. *25*, 664–671.

Jaeschke, H. (1991). Vascular oxidant stress and hepatic ischemia/reperfusion injury. Free Radic. Res. Commun. *12–13 Pt 2*, 737–743.

Jaeschke, H. (2003). Molecular mechanisms of hepatic ischemia-reperfusion injury and preconditioning. Am. J. Physiol. Gastrointest. Liver Physiol. *284*, G15-26.

Jaeschke, H. (2015). Acetaminophen: Dose-Dependent Drug Hepatotoxicity and Acute Liver Failure in Patients. Dig. Dis. *33*, 464–471.

Jaeschke, H., and Farhood, A. (1991a). Neutrophil and Kupffer cell-induced oxidant stress and ischemia-reperfusion injury in rat liver. Am. J. Physiol. *260*, G355-362.

Jaeschke, H., and Farhood, A. (1991b). Neutrophil and Kupffer cell-induced oxidant stress and ischemia-reperfusion injury in rat liver. Am. J. Physiol. *260*, G355-362.

Jaeschke, H., and Farhood, A. (2002). Kupffer cell activation after no-flow ischemia versus hemorrhagic shock. Free Radic. Biol. Med. *33*, 210–219.

Jaeschke, H., and Lemasters, J.J. (2003). Apoptosis versus oncotic necrosis in hepatic ischemia/reperfusion injury. Gastroenterology *125*, 1246–1257.

Jaeschke, H., and Mitchell, J.R. (1990). Use of isolated perfused organs in hypoxia and ischemia/reperfusion oxidant stress. Methods Enzymol. *186*, 752–759.

Jaeschke, H., and Smith, C.W. (1997). Mechanisms of neutrophil-induced parenchymal cell injury. J. Leukoc. Biol. *61*, 647–653.

Jaeschke, H., Farhood, A., and Smith, C.W. (1990). Neutrophils contribute to ischemia/reperfusion injury in rat liver in vivo. FASEB J. *4*, 3355–3359.

Jaeschke, H., Bautista, A.P., Spolarics, Z., and Spitzer, J.J. (1992). Superoxide generation by neutrophils and Kupffer cells during in vivo reperfusion after hepatic ischemia in rats. J. Leukoc. Biol. *52*, 377–382.

Jaeschke, H., Farhood, A., Bautista, A.P., Spolarics, Z., and Spitzer, J.J. (1993). Complement activates Kupffer cells and neutrophils during reperfusion after hepatic ischemia. Am. J. Physiol. *264*, G801-809.

Jaeschke, H., Fisher, M.A., Lawson, J.A., Simmons, C.A., Farhood, A., and Jones, D.A. (1998). Activation of caspase 3 (CPP32)-like proteases is essential for TNF-alpha-induced hepatic parenchymal cell apoptosis and neutrophil-mediated necrosis in a murine endotoxin shock model. J. Immunol. *160*, 3480–3486.

Jaeschke, H., Knight, T.R., and Bajt, M.L. (2003). The role of oxidant stress and reactive nitrogen species in acetaminophen hepatotoxicity. Toxicol. Lett. *144*, 279–288.

Jaeschke, H., Williams, C.D., and Farhood, A. (2011). No evidence for caspase-dependent apoptosis in acetaminophen hepatotoxicity. Hepatol. *53*, 718–719.

Jaeschke, H., McGill, M.R., and Ramachandran, A. (2012a). Oxidant stress, mitochondria, and cell death mechanisms in drug-induced liver injury: lessons learned from acetaminophen hepatotoxicity. Drug Metab. Rev. *44*, 88–106.

Jaeschke, H., Williams, C.D., Ramachandran, A., and Bajt, M.L. (2012b). Acetaminophen hepatotoxicity and repair: the role of sterile inflammation and innate immunity. Liver Int. *32*, 8–20.

Jain, S., Lee, S.H., Korneszczuk, K., Culberson, C.R., Southard, J.H., Berthiaume, F., Zhang, J.X., Clemens, M.G., and Lee, C.Y. (2008). Improved preservation of warm ischemic livers by hypothermic machine perfusion with supplemented University of Wisconsin solution. J. Investig. Surg. *21*, 83–91.

James, L.P., Letzig, L., Simpson, P.M., Capparelli, E., Roberts, D.W., Hinson, J.A., Davern, T.J., and Lee, W.M. (2009). Pharmacokinetics of acetaminophen-protein adducts in adults with acetaminophen overdose and acute liver failure. Drug Metab. Dispos. *37*, 1779–1784.

Jassem, W., Koo, D.D.H., Cerundolo, L., Rela, M., Heaton, N.D., and Fuggle, S.V. (2003). Cadaveric versus living-donor livers: differences in inflammatory markers after transplantation. Transplantation *76*, 1599–1603.

Jin, X., Ye, Y.-F., Chen, S.-H., Yu, C.-H., Liu, J., and Li, Y.-M. (2009). MicroRNA expression pattern in different stages of nonalcoholic fatty liver disease. Dig. Liver Dis. *41*, 289–297.

Jossé, R., Aninat, C., Glaise, D., Dumont, J., Fessard, V., Morel, F., Poul, J.-M., Guguen-Guillouzo, C., and Guillouzo, A. (2008). Long-term functional stability of human HepaRG hepatocytes and use for chronic toxicity and genotoxicity studies. Drug Metab. Dispos. *36*, 1111–1118.

Kanebratt, K.P., and Andersson, T.B. (2008). HepaRG cells as an in vitro model for evaluation of cytochrome P450 induction in humans. Drug Metab. Dispos. *36*, 137–145.

Karimian, N., Westerkamp, A.C., and Porte, R.J. (2014). Biliary complications after orthotopic liver transplantation. Curr. Opin. Organ Transplant. *19*, 209–216.

Karmen, A., Wroblewski, F., and Ladue, J.S. (1955). Transaminase activity in human blood. J. Clin. Invest. *34*, 126–131.

Kim, J., Kim, H.-Y., and Lee, S.-M. (2013). Protective Effects of Geniposide and Genipin against Hepatic Ischemia/Reperfusion Injury in Mice. Biomol. Ther. *21*, 132–137.

Kim, J.S., He, L., Qian, T., and Lemasters, J.J. (2003). Role of the mitochondrial permeability transition in apoptotic and necrotic death after ischemia/reperfusion injury to hepatocytes. Curr. Mol. Med. *3*, 527–535.

Ko, H.M., Joo, S.H., Jo, J.H., Park, W.S., Jung, W.Y., Shin, J.H., and Ahn, H.J. (2017). Liver-Wrapping, Nitric Oxide-Releasing Nanofiber Downregulates Cleaved Caspase-3 and Bax Expression on Rat Hepatic Ischemia-Reperfusion Injury. Transplant. Proc. *49*, 1170–1174.

Kochhar, G., Parungao, J.M., Hanouneh, I.A., and Parsi, M.A. (2013). Biliary complications following liver transplantation. World J. Gastroenterol. *19*, 2841–2846.

Kodali, S., and McGuire, B.M. (2015). Diagnosis and Management of Hepatic Encephalopathy in Fulminant Hepatic Failure. Clin. Liver Dis. *19*, 565–576.

Kon, K., Kim, J.-S., Jaeschke, H., and Lemasters, J.J. (2004). Mitochondrial permeability transition in acetaminophen-induced necrosis and apoptosis of cultured mouse hepatocytes. Hepatol. *40*, 1170–1179.

Kota, J., Chivukula, R.R., O'Donnell, K.A., Wentzel, E.A., Montgomery, C.L., Hwang, H.-W., Chang, T.-C., Vivekanandan, P., Torbenson, M., Clark, K.R., et al. (2009). Therapeutic microRNA delivery suppresses tumorigenesis in a murine liver cancer model. Cell *137*, 1005–1017.

Krauskopf, J., Caiment, F., Claessen, S.M., Johnson, K.J., Warner, R.L., Schomaker, S.J., Burt, D.A., Aubrecht, J., and Kleinjans, J.C. (2015). Application of high-throughput sequencing to

circulating microRNAs reveals novel biomarkers for drug-induced liver injury. Toxicol. Sci. *143*, 268–276.

Ku, N.-O., Strnad, P., Zhong, B.-H., Tao, G.-Z., and Omary, M.B. (2007). Keratins let liver live: Mutations predispose to liver disease and crosslinking generates Mallory-Denk bodies. Hepatol. *46*, 1639–1649.

Lackner, C. (2011). Hepatocellular ballooning in nonalcoholic steatohepatitis: the pathologist's perspective. Expert Rev. Gastroenterol. Hepatol. *5*, 223–231.

Ladeiro, Y., Couchy, G., Balabaud, C., Bioulac-Sage, P., Pelletier, L., Rebouissou, S., and Zucman-Rossi, J. (2008). MicroRNA profiling in hepatocellular tumors is associated with clinical features and oncogene/tumor suppressor gene mutations. Hepatol. *47*, 1955–1963.

Lambert, C.B., Spire, C., Renaud, M.-P., Claude, N., and Guillouzo, A. (2009). Reproducible chemical-induced changes in gene expression profiles in human hepatoma HepaRG cells under various experimental conditions. Toxicol. Vitro *23*, 466–475.

Lanford, R.E., Hildebrandt-Eriksen, E.S., Petri, A., Persson, R., Lindow, M., Munk, M.E., Kauppinen, S., and Ørum, H. (2010). Therapeutic silencing of microRNA-122 in primates with chronic hepatitis C virus infection. Science *327*, 198–201.

Larson, A.M. (2007). Acetaminophen hepatotoxicity. Clin. Liver Dis. *11*, 525–548, vi.

Laterza, O.F., Lim, L., Garrett-Engele, P.W., Vlasakova, K., Muniappa, N., Tanaka, W.K., Johnson, J.M., Sina, J.F., Fare, T.L., Sistare, F.D., et al. (2009). Plasma MicroRNAs as sensitive and specific biomarkers of tissue injury. Clin. Chem. *55*, 1977–1983.

Lee, W.M. (2012). Acute liver failure. Semin. Respir. Crit. Care Med. *33*, 36–45.

Lee, W.M. (2013). Drug-induced acute liver failure. Clin. Liver Dis. *17*, 575–586, viii.

Lee, R.C., Feinbaum, R.L., and Ambros, V. (1993). The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. Cell *75*, 843–854.

Lee, S.S., Buters, J.T., Pineau, T., Fernandez-Salguero, P., and Gonzalez, F.J. (1996). Role of CYP2E1 in the hepatotoxicity of acetaminophen. J. Biol. Chem. *271*, 12063–12067.

Leers, M.P., Kölgen, W., Björklund, V., Bergman, T., Tribbick, G., Persson, B., Björklund, P., Ramaekers, F.C., Björklund, B., Nap, M., et al. (1999). Immunocytochemical detection and mapping of a cytokeratin 18 neo-epitope exposed during early apoptosis. J. Pathol. *187*, 567–572.

Lemasters, J.J., DiGuiseppi, J., Nieminen, A.L., and Herman, B. (1987). Blebbing, free Ca2+ and mitochondrial membrane potential preceding cell death in hepatocytes. Nature *325*, 78–81.

Lemasters, J.J., Nieminen, A.L., Qian, T., Trost, L.C., and Herman, B. (1997). The mitochondrial permeability transition in toxic, hypoxic and reperfusion injury. Mol. Cell. Biochem. *174*, 159–165.

Lentsch, A.B. (2012). Regulatory mechanisms of injury and repair after hepatic ischemia/reperfusion. Scientifica *2012*, 513192.

Leslie, B.R., Head, L.H., and Scharfenberg, J.C. (1989). Ischemic hepatitis from aortic dissection. Ann. Intern. Med. *110*, 495.

Li, S., Chen, X., Zhang, H., Liang, X., Xiang, Y., Yu, C., Zen, K., Li, Y., and Zhang, C.-Y. (2009). Differential expression of microRNAs in mouse liver under aberrant energy metabolic status. J. Lipid Res. *50*, 1756–1765.

Linder, S., Olofsson, M.H., Herrmann, R., and Ulukaya, E. (2010). Utilization of cytokeratin-based biomarkers for pharmacodynamic studies. Expert Rev. Mol. Diagn. *10*, 353–359.

Manyike, P.T., Kharasch, E.D., Kalhorn, T.F., and Slattery, J.T. (2000). Contribution of CYP2E1 and CYP3A to acetaminophen reactive metabolite formation. Clin. Pharmacol. Ther. *67*, 275–282.

Marrone, A.K., Tryndyak, V., Beland, F.A., and Pogribny, I.P. (2016). MicroRNA Responses to the Genotoxic Carcinogens Aflatoxin B1 and Benzo[a]pyrene in Human HepaRG Cells. Toxicol. Sci. *149*, 496–502.

Marsman, W.A., Wiesner, R.H., Rodriguez, L., Batts, K.P., Porayko, M.K., Hay, J.E., Gores, G.J., and Krom, R.A. (1996). Use of fatty donor liver is associated with diminished early patient and graft survival. Transplantation *62*, 1246–1251.

Marubayashi, S., Dohi, K., Ochi, K., and Kawasaki, T. (1986). Role of free radicals in ischemic rat liver cell injury: prevention of damage by alpha-tocopherol administration. Surgery *99*, 184–192.

Mathurin, P., Durand, F., Ganne, N., Mollo, J.L., Lebrec, D., Degott, C., Erlinger, S., Benhamou, J.P., and Bernuau, J. (1995). Ischemic hepatitis due to obstructive sleep apnea. Gastroenterology *109*, 1682–1684.

McGill, M.R. (2016). The past and present of serum aminotransferases and the future of liver injury biomarkers. EXCLI J. *15*, 817–828.

McGill, M.R., and Jaeschke, H. (2014). Mechanistic biomarkers in acetaminophen-induced hepatotoxicity and acute liver failure: from preclinical models to patients. Expert Opin. Drug Metab. Toxicol. *10*, 1005–1017.

McGill, M.R., and Jaeschke, H. (2015). MicroRNAs as Signaling Mediators and Biomarkers of Drug- and Chemical-Induced Liver Injury. J. Clin. Med. *4*, 1063–1078.

McGill, M.R., Yan, H.-M., Ramachandran, A., Murray, G.J., Rollins, D.E., and Jaeschke, H. (2011). HepaRG cells: a human model to study mechanisms of acetaminophen hepatotoxicity. Hepatology *53*, 974–982.

McGill, M.R., Sharpe, M.R., Williams, C.D., Taha, M., Curry, S.C., and Jaeschke, H. (2012). The mechanism underlying acetaminophen-induced hepatotoxicity in humans and mice involves mitochondrial damage and nuclear DNA fragmentation. J. Clin. Invest. *122*, 1574–1583.

McGill, M.R., Lebofsky, M., Norris, H.-R.K., Slawson, M.H., Bajt, M.L., Xie, Y., Williams, C.D., Wilkins, D.G., Rollins, D.E., and Jaeschke, H. (2013). Plasma and liver acetaminophen-protein adduct levels in mice after acetaminophen treatment: dose-response, mechanisms, and clinical implications. Toxicol. Appl. Pharmacol. *269*, 240–249.

McKeown, C.M., Edwards, V., Phillips, M.J., Harvey, P.R., Petrunka, C.N., and Strasberg, S.M. (1988). Sinusoidal lining cell damage: the critical injury in cold preservation of liver allografts in the rat. Transplantation *46*, 178–191.

Meyers, L.L., Beierschmitt, W.P., Khairallah, E.A., and Cohen, S.D. (1988). Acetaminophen-induced inhibition of hepatic mitochondrial respiration in mice. Toxicol. Appl. Pharmacol. *93*, 378–387.

Mitchell, J.R., Jollow, D.J., Potter, W.Z., Gillette, J.R., and Brodie, B.B. (1973). Acetaminophen-induced hepatic necrosis. IV. Protective role of glutathione. J. Pharmacol. Exp. Ther. *187*, 211–217.

Moll, R., Divo, M., and Langbein, L. (2008). The human keratins: biology and pathology. Histochem. Cell Biol. *129*, 705–733.

Molnar, A., Haybaeck, J., Lackner, C., and Strnad, P. (2011). The cytoskeleton in nonalcoholic steatohepatitis: 100 years old but still youthful. Expert Rev. Gastroenterol. Hepatol. *5*, 167–177.

Munoz, S.J., Stravitz, R.T., and Gabriel, D.A. (2009). Coagulopathy of acute liver failure. Clin. Liver Dis. *13*, 95–107.

Murakami, Y., Yasuda, T., Saigo, K., Urashima, T., Toyoda, H., Okanoue, T., and Shimotohno, K. (2006). Comprehensive analysis of microRNA expression patterns in hepatocellular carcinoma and non-tumorous tissues. Oncogene *25*, 2537–2545.

Musso, G., Gambino, R., Cassader, M., and Pagano, G. (2011). Meta-analysis: natural history of non-alcoholic fatty liver disease (NAFLD) and diagnostic accuracy of non-invasive tests for liver disease severity. Ann. Med. *43*, 617–649.

Nace, G.W., Huang, H., Klune, J.R., Eid, R.E., Rosborough, B.R., Korff, S., Li, S., Shapiro, R.A., Stolz, D.B., Sodhi, C.P., et al. (2013). Cellular-specific role of toll-like receptor 4 in hepatic ischemia-reperfusion injury in mice. Hepatol. *58*, 374–387.

Noack, K., Bronk, S.F., Kato, A., and Gores, G.J. (1993). The greater vulnerability of bile duct cells to reoxygenation injury than to anoxia. Implications for the pathogenesis of biliary strictures after liver transplantation. Transplantation *56*, 495–500.

Northup, P.G., and Caldwell, S.H. (2013). Coagulation in liver disease: a guide for the clinician. Clin. Gastroenterol. Hepatol. *11*, 1064–1074.

Oliveros, F.H., Santamaría, M.L., Gámez, M., Murcia, J., Leal, N., Frauca, E., Hierro, L., Camarena, C., de la Vega, A., Bortolo, G., et al. (2005). Comparative study between living and cadaveric donors in pediatric liver transplantation. Transplant. Proc. *37*, 3936–3938.

Omary, M.B., Ku, N.-O., Strnad, P., and Hanada, S. (2009). Toward unraveling the complexity of simple epithelial keratins in human disease. J. Clin. Invest. *119*, 1794–1805.

Padgett, K.A., Lan, R.Y., Leung, P.C., Lleo, A., Dawson, K., Pfeiff, J., Mao, T.K., Coppel, R.L., Ansari, A.A., and Gershwin, M.E. (2009). Primary biliary cirrhosis is associated with altered hepatic microRNA expression. J. Autoimmun. *32*, 246–253.

Papatheodoridis, G.V., Hadziyannis, E., Tsochatzis, E., Chrysanthos, N., Georgiou, A., Kafiri, G., Manolakopoulos, S., Tiniakos, D.G., Giannousis, I., Manesis, E.K., et al. (2008). Serum apoptotic caspase activity as a marker of severity in HBeAg-negative chronic hepatitis B virus infection. Gut *57*, 500–506.

Parent, R., Marion, M.-J., Furio, L., Trépo, C., and Petit, M.-A. (2004). Origin and characterization of a human bipotent liver progenitor cell line. Gastroenterology *126*, 1147–1156.

Perez-Daga, J.A., Santoyo, J., Suárez, M.A., Fernández-Aguilar, J.A., Ramírez, C., Rodríguez-Cañete, A., Aranda, J.M., Sánchez-Pérez, B., Montiel, C., Palomo, D., et al. (2006). Influence of degree of hepatic steatosis on graft function and postoperative complications of liver transplantation. Transplant. Proc. *38*, 2468–2470.

Pesonen, E.J., Höckerstedt, K., Mäkisalo, H., Vuorte, J., Jansson, S.E., Orpana, A., Karonen, S.L., and Repo, H. (2000). Transhepatic neutrophil and monocyte activation during clinical liver transplantation. Transplantation *69*, 1458–1464.

Pessayre, D., Fromenty, B., Berson, A., Robin, M.-A., Lettéron, P., Moreau, R., and Mansouri, A. (2012). Central role of mitochondria in drug-induced liver injury. Drug Metab. Rev. *44*, 34–87.

Pine, J.K., Aldouri, A., Young, A.L., Davies, M.H., Attia, M., Toogood, G.J., Pollard, S.G., Lodge, J.P.A., and Prasad, K.R. (2009). Liver transplantation following donation after cardiac death: an analysis using matched pairs. Liver Transplant. *15*, 1072–1082.

Powell, R.D., Swet, J.H., Kennedy, K.L., Huynh, T.T., McKillop, I.H., and Evans, S.L. (2014). Resveratrol attenuates hypoxic injury in a primary hepatocyte model of hemorrhagic shock and resuscitation. J. Trauma Acute Care Surg. *76*, 409–417.

Qi, R., Weiland, M., Gao, X.-H., Zhou, L., and Mi, Q.-S. (2012). Identification of endogenous normalizers for serum microRNAs by microarray profiling: U6 small nuclear RNA is not a reliable normalizer. Hepatol. *55*, 1640-1642.

Qian, T., Nieminen, A.L., Herman, B., and Lemasters, J.J. (1997). Mitochondrial permeability transition in pH-dependent reperfusion injury to rat hepatocytes. Am. J. Physiol. *273*, C1783-1792.

Qiu, Y., Benet, L.Z., and Burlingame, A.L. (1998). Identification of the hepatic protein targets of reactive metabolites of acetaminophen in vivo in mice using two-dimensional gel electrophoresis and mass spectrometry. J. Biol. Chem. *273*, 17940–17953.

Rao, J., Qin, J., Qian, X., Lu, L., Wang, P., Wu, Z., Zhai, Y., Zhang, F., Li, G., and Wang, X. (2013). Lipopolysaccharide preconditioning protects hepatocytes from ischemia/reperfusion injury (IRI) through inhibiting ATF4-CHOP pathway in mice. PloS One *8*, e65568.

Reckendorfer, H., Burgmann, H., and Spieckermann, P.G. (1992). Hepatic energy metabolism during hypothermic storage and reperfusion using different protecting solutions. Eur. Surg. Res. Eur. Chir. Forsch. Rech. Chir. Eur. *24*, 339–348.

Reuben, A., Koch, D.G., Lee, W.M., and Acute Liver Failure Study Group (2010). Drug-induced acute liver failure: results of a U.S. multicenter, prospective study. Hepatol. *52*, 2065–2076.

Rijntjes, P.J., Moshage, H.J., Van Gemert, P.J., De Waal, R., and Yap, S.H. (1986). Cryopreservation of adult human hepatocytes. The influence of deep freezing storage on the viability, cell seeding, survival, fine structures and albumin synthesis in primary cultures. J. Hepatol. *3*, 7–18.

Roderburg, C., Benz, F., Vargas Cardenas, D., Koch, A., Janssen, J., Vucur, M., Gautheron, J., Schneider, A.T., Koppe, C., Kreggenwinkel, K., et al. (2015). Elevated miR-122 serum levels are an independent marker of liver injury in inflammatory diseases. Liver Int. 1172–1184.

Rodríguez-Antona, C., Donato, M.T., Boobis, A., Edwards, R.J., Watts, P.S., Castell, J.V., and Gómez-Lechón, M.-J. (2002). Cytochrome P450 expression in human hepatocytes and hepatoma cell lines: molecular mechanisms that determine lower expression in cultured cells. Xenobiotica *32*, 505–520.

Runge, D., Runge, D.M., Jäger, D., Lubecki, K.A., Beer Stolz, D., Karathanasis, S., Kietzmann, T., Strom, S.C., Jungermann, K., Fleig, W.E., et al. (2000). Serum-free, long-term cultures of human hepatocytes: maintenance of cell morphology, transcription factors, and liver-specific functions. Biochem. Biophys. Res. Commun. *269*, 46–53.

Saito, C., Lemasters, J.J., and Jaeschke, H. (2010). c-Jun N-terminal kinase modulates oxidant stress and peroxynitrite formation independent of inducible nitric oxide synthase in acetaminophen hepatotoxicity. Toxicol. Appl. Pharmacol. *246*, 8–17.

Sanchez-Urdazpal, L., Gores, G.J., Ward, E.M., Maus, T.P., Wahlstrom, H.E., Moore, S.B., Wiesner, R.H., and Krom, R.A. (1992). Ischemic-type biliary complications after orthotopic liver transplantation. Hepatol. *16*, 49–53.

Sassa, S., Sugita, O., Galbraith, R.A., and Kappas, A. (1987). Drug metabolism by the human hepatoma cell, Hep G2. Biochem. Biophys. Res. Commun. *143*, 52–57.

Schotte, P., Declercq, W., Van Huffel, S., Vandenabeele, P., and Beyaert, R. (1999). Non-specific effects of methyl ketone peptide inhibitors of caspases. FEBS Lett. *442*, 117–121.

Schwartz, J., Holmuhamedov, E., Zhang, X., Lovelace, G.L., Smith, C.D., and Lemasters, J.J. (2013). Minocycline and doxycycline, but not other tetracycline-derived compounds, protect liver cells from chemical hypoxia and ischemia/reperfusion injury by inhibition of the mitochondrial calcium uniporter. Toxicol. Appl. Pharmacol. *273*, 172–179.

Scott, T.R., Kronsten, V.T., Hughes, R.D., and Shawcross, D.L. (2013). Pathophysiology of cerebral oedema in acute liver failure. World J. Gastroenterol. *19*, 9240–9255.

Seehofer, D., Eurich, D., Veltzke-Schlieker, W., and Neuhaus, P. (2013). Biliary complications after liver transplantation: old problems and new challenges. Am. J. Transplant. *13*, 253–265.

Snawder, J.E., Roe, A.L., Benson, R.W., and Roberts, D.W. (1994). Loss of CYP2E1 and CYP1A2 activity as a function of acetaminophen dose: relation to toxicity. Biochem. Biophys. Res. Commun. *203*, 532–539.

Soejima, Y., Taketomi, A., Yoshizumi, T., Uchiyama, H., Harada, N., Ijichi, H., Yonemura, Y., Ikeda, T., Shimada, M., and Maehara, Y. (2006). Biliary strictures in living donor liver transplantation: incidence, management, and technical evolution. Liver Transplant. *12*, 979–986.

Song, G.-W., Lee, S.-G., Hwang, S., Kim, K.-H., Ahn, C.-S., Moon, D.-B., Ha, T.-Y., Jung, D.-H., Park, G.-C., Kang, S.-H., et al. (2014). Biliary stricture is the only concern in ABO-incompatible adult living donor liver transplantation in the rituximab era. J. Hepatol. *61*, 575–582.

Starkey Lewis, P.J., Dear, J., Platt, V., Simpson, K.J., Craig, D.G.N., Antoine, D.J., French, N.S., Dhaun, N., Webb, D.J., Costello, E.M., et al. (2011). Circulating microRNAs as potential markers of human drug-induced liver injury. Hepatol. *54*, 1767–1776.

Steven Stockham, and Michael Scott (2002). Fundamentals of Veterinary Clinical Pathology (A Blackwell).

Strnad, P., Paschke, S., Jang, K.-H., and Ku, N.-O. (2012). Keratins: markers and modulators of liver disease. Curr. Opin. Gastroenterol. *28*, 209–216.

Szczeklik, E., Orlowski, M., and Szewczuk, A. (1961). [Activity of serum gamma-glutamylotranspeptidase as a new enzymatic test in liver diseases. Comparison with other enzymatic tests]. Pol. Tyg. Lek. Wars. Pol. 1960 *16*, 503–510.

Tailor, A., Faulkner, L., Naisbitt, D.J., and Park, B.K. (2015). The chemical, genetic and immunological basis of idiosyncratic drug-induced liver injury. Hum. Exp. Toxicol. *34*, 1310–1317.

Taner, C.B., Bulatao, I.G., Perry, D.K., Sibulesky, L., Willingham, D.L., Kramer, D.J., and Nguyen, J.H. (2012). Asystole to cross-clamp period predicts development of biliary

complications in liver transplantation using donation after cardiac death donors. Transpl. Int. *25*, 838–846.

Tang, D., Loze, M.T., Zeh, H.J., and Kang, R. (2010). The redox protein HMGB1 regulates cell death and survival in cancer treatment. Autophagy *6*, 1181–1183.

Tang, D., Billiar, T.R., and Lotze, M.T. (2012). A Janus tale of two active high mobility group box 1 (HMGB1) redox states. Mol. Med. *18*, 1360–1362.

Thawley, V. (2017). Acute Liver Injury and Failure. Vet. Clin. North Am. Small Anim. Pract. *47*, 617–630.

Theruvath, T.P., Zhong, Z., Pediaditakis, P., Ramshesh, V.K., Currin, R.T., Tikunov, A., Holmuhamedov, E., and Lemasters, J.J. (2008). Minocycline and N-methyl-4-isoleucine cyclosporin (NIM811) mitigate storage/reperfusion injury after rat liver transplantation through suppression of the mitochondrial permeability transition. Hepatol. *47*, 236–246.

Thummel, K.E., Lee, C.A., Kunze, K.L., Nelson, S.D., and Slattery, J.T. (1993). Oxidation of acetaminophen to N-acetyl-p-aminobenzoquinone imine by human CYP3A4. Biochem. Pharmacol. *45*, 1563–1569.

Tirmenstein, M.A., and Nelson, S.D. (1989). Subcellular binding and effects on calcium homeostasis produced by acetaminophen and a nonhepatotoxic regioisomer, 3'-hydroxyacetanilide, in mouse liver. J. Biol. Chem. *264*, 9814–9819.

Tomiyama, K., Ikeda, A., Ueki, S., Nakao, A., Stolz, D.B., Koike, Y., Afrazi, A., Gandhi, C., Tokita, D., Geller, D.A., et al. (2008). Inhibition of Kupffer cell-mediated early proinflammatory response with carbon monoxide in transplant-induced hepatic ischemia/reperfusion injury in rats. Hepatol. *48*, 1608–1620.

Trilok, G., Qing, Y.C., and Li-Jun, X. (2012). Hypoxic hepatitis: a challenging diagnosis. Hepatol. Int. *6*, 663–669.

Trotter, J.F. (2009). Practical management of acute liver failure in the Intensive Care Unit. Curr. Opin. Crit. Care *15*, 163–167.

Tsung, A., Sahai, R., Tanaka, H., Nakao, A., Fink, M.P., Lotze, M.T., Yang, H., Li, J., Tracey, K.J., Geller, D.A., et al. (2005). The nuclear factor HMGB1 mediates hepatic injury after murine liver ischemia-reperfusion. J. Exp. Med. *201*, 1135–1143.

Ulukaya, S., Ulukaya, E., Alper, I., Yilmaztepe-Oral, A., and Kilic, M. (2010). Soluble cytokeratin 18 biomarkers may provide information on the type of cell death during early ischemia and reperfusion periods of liver transplantation. Clin. Transplant. *24*, 848–854.

Vairetti, M., Richelmi, P., Bertè, F., Currin, R.T., Lemasters, J.J., and Imberti, R. (2006). Role of pH in protection by low sodium against hypoxic injury in isolated perfused rat livers. J. Hepatol. *44*, 894–901.

de Vera, M.E., Lopez-Solis, R., Dvorchik, I., Campos, S., Morris, W., Demetris, A.J., Fontes, P., and Marsh, J.W. (2009). Liver transplantation using donation after cardiac death donors: long-term follow-up from a single center. Am. J. Transplant. *9*, 773–781.

Verdonk, R.C., Buis, C.I., Porte, R.J., and Haagsma, E.B. (2006). Biliary complications after liver transplantation: a review. Scand. J. Gastroenterol. Suppl. 89–101.

Verhoeven, C.J., Farid, W.R.R., de Ruiter, P.E., Hansen, B.E., Roest, H.P., de Jonge, J., Kwekkeboom, J., Metselaar, H.J., Tilanus, H.W., Kazemier, G., et al. (2013). MicroRNA profiles in graft preservation solution are predictive of ischemic-type biliary lesions after liver transplantation. J. Hepatol. *59*, 1231–1238.

Vine, W., Link, J., Thoma, W.J., and Uğurbil, K. (1989). Injury and recovery of the liver from preservation assessed by 31P NMR spectroscopy: the contrast between preservation with Collins' solution and Ringer's lactate solution. NMR Biomed. *2*, 19–26.

van der Vliet, J.A., and Warlé, M.C. (2013). The need to reduce cold ischemia time in kidney transplantation. Curr. Opin. Organ Transplant. *18*, 174–178.

Voinnet, O. (2005). Induction and suppression of RNA silencing: insights from viral infections. Nat. Rev. Genet. *6*, 206–220.

Volkmann, X., Anstaett, M., Hadem, J., Stiefel, P., Bahr, M.J., Lehner, F., Manns, M.P., Schulze-Osthoff, K., and Bantel, H. (2008). Caspase activation is associated with spontaneous recovery from acute liver failure. Hepatol. *47*, 1624–1633.

Wang, K., Zhang, S., Marzolf, B., Troisch, P., Brightman, A., Hu, Z., Hood, L.E., and Galas, D.J. (2009a). Circulating microRNAs, potential biomarkers for drug-induced liver injury. Proc. Natl. Acad. Sci. *106*, 4402–4407.

Wang, K., Zhang, S., Marzolf, B., Troisch, P., Brightman, A., Hu, Z., Hood, L.E., and Galas, D.J. (2009b). Circulating microRNAs, potential biomarkers for drug-induced liver injury. Proc. Natl. Acad. Sci. *106*, 4402–4407.

Ward, J., Kanchagar, C., Veksler-Lublinsky, I., Lee, R.C., McGill, M.R., Jaeschke, H., Curry, S.C., and Ambros, V.R. (2014). Circulating microRNA profiles in human patients with acetaminophen hepatotoxicity or ischemic hepatitis. Proc. Natl. Acad. Sci. *111*, 12169–12174.

Weemhoff, J.L., Woolbright, B.L., Jenkins, R.E., McGill, M.R., Sharpe, M.R., Olson, J.C., Antoine, D.J., Curry, S.C., and Jaeschke, H. (2017). Plasma biomarkers to study mechanisms of liver injury in patients with hypoxic hepatitis. Liver Int. *37*, 377–384.

Wetzel, G., Relja, B., Klarner, A., Henrich, D., Dehne, N., Brühne, B., Lehnert, M., and Marzi, I. (2014). Myeloid knockout of HIF-1 α does not markedly affect hemorrhage/resuscitation-induced inflammation and hepatic injury. Mediators Inflamm. *2014*, 930419.

Wieckowska, A., Zein, N.N., Yerian, L.M., Lopez, A.R., McCullough, A.J., and Feldstein, A.E. (2006). In vivo assessment of liver cell apoptosis as a novel biomarker of disease severity in nonalcoholic fatty liver disease. Hepatol. *44*, 27–33.

Wilkening, S., Stahl, F., and Bader, A. (2003). Comparison of primary human hepatocytes and hepatoma cell line Hepg2 with regard to their biotransformation properties. Drug Metab. Dispos. *31*, 1035–1042.

Williams, C.D., Bajt, M.L., Sharpe, M.R., McGill, M.R., Farhood, A., and Jaeschke, H. (2014). Neutrophil activation during acetaminophen hepatotoxicity and repair in mice and humans. Toxicol. Appl. Pharmacol. *275*, 122–133.

Wojcicki, M., Milkiewicz, P., and Silva, M. (2008). Biliary tract complications after liver transplantation: a review. Dig. Surg. *25*, 245–257.

Woolbright, B.L., Antoine, D.J., Jenkins, R.E., Bajt, M.L., Park, B.K., and Jaeschke, H. (2013). Plasma biomarkers of liver injury and inflammation demonstrate a lack of apoptosis during obstructive cholestasis in mice. Toxicol. Appl. Pharmacol. *273*, 524–531.

Woolbright, B.L., Dorko, K., Antoine, D.J., Clarke, J.I., Gholami, P., Li, F., Kumer, S.C., Schmitt, T.M., Forster, J., Fan, F., et al. (2015). Bile acid-induced necrosis in primary human hepatocytes and in patients with obstructive cholestasis. Toxicol. Appl. Pharmacol. *283*, 168–177.

Woolbright, B.L., McGill, M.R., Yan, H., and Jaeschke, H. (2016). Bile Acid-Induced Toxicity in HepaRG Cells Recapitulates the Response in Primary Human Hepatocytes. Basic Clin. Pharmacol. Toxicol. *118*, 160–167.

Xia, L., Sakban, R.B., Qu, Y., Hong, X., Zhang, W., Nugraha, B., Tong, W.H., Ananthanarayanan, A., Zheng, B., Chau, I.Y.-Y., et al. (2012). Tethered spheroids as an in vitro hepatocyte model for drug safety screening. Biomaterials *33*, 2165–2176.

Xie, Y., McGill, M.R., Dorko, K., Kumer, S.C., Schmitt, T.M., Forster, J., and Jaeschke, H. (2014a). Mechanisms of acetaminophen-induced cell death in primary human hepatocytes. Toxicol. Appl. Pharmacol. *279*, 266–274.

Xie, Y., McGill, M.R., Dorko, K., Kumer, S.C., Schmitt, T.M., Forster, J., and Jaeschke, H. (2014b). Mechanisms of acetaminophen-induced cell death in primary human hepatocytes. Toxicol. Appl. Pharmacol. *279*, 266–274.

Xie, Y., McGill, M.R., Cook, S.F., Sharpe, M.R., Winefield, R.D., Wilkins, D.G., Rollins, D.E., and Jaeschke, H. (2015a). Time course of acetaminophen-protein adducts and acetaminophen metabolites in circulation of overdose patients and in HepaRG cells. Xenobiotica 1–9.

Xie, Y., McGill, M.R., Du, K., Dorko, K., Kumer, S.C., Schmitt, T.M., Ding, W.-X., and Jaeschke, H. (2015b). Mitochondrial protein adducts formation and mitochondrial dysfunction during N-acetyl-m-aminophenol (AMAP)-induced hepatotoxicity in primary human hepatocytes. Toxicol. Appl. Pharmacol. *289*, 213–222.

Yang, H., Hreggvidsdottir, H.S., Palmblad, K., Wang, H., Ochani, M., Li, J., Lu, B., Chavan, S., Rosas-Ballina, M., Al-Abed, Y., et al. (2010). A critical cysteine is required for HMGB1 binding to Toll-like receptor 4 and activation of macrophage cytokine release. Proc. Natl. Acad. Sci. *107*, 11942–11947.

Yang, M., Antoine, D.J., Weemhoff, J.L., Jenkins, R.E., Farhood, A., Park, B.K., and Jaeschke, H. (2014). Biomarkers Distinguish Apoptotic and Necrotic Cell Death During Hepatic Ischemia-Reperfusion Injury in Mice. Liver Transplant. *20*, 1372-1382

Yang, X., Salminen, W.F., Shi, Q., Greenhaw, J., Gill, P.S., Bhattacharyya, S., Beger, R.D., Mendrick, D.L., Mattes, W.B., and James, L.P. (2015). Potential of extracellular microRNAs as biomarkers of acetaminophen toxicity in children. Toxicol. Appl. Pharmacol. *284*, 180–187.

Yoon, E., Babar, A., Choudhary, M., Kutner, M., and Pyrsopoulos, N. (2016). Acetaminophen-Induced Hepatotoxicity: a Comprehensive Update. J. Clin. Transl. Hepatol. *4*, 131–142.

Zamore, P.D., and Haley, B. (2005). Ribo-gnome: the big world of small RNAs. Science *309*, 1519–1524.

Zatloukal, K., French, S.W., Stumptner, C., Strnad, P., Harada, M., Toivola, D.M., Cadrin, M., and Omary, M.B. (2007). From Mallory to Mallory-Denk bodies: what, how and why? Exp. Cell Res. *313*, 2033–2049.

Zhai, Y., Shen, X.-D., Gao, F., Zhao, A., Freitas, M.C., Lassman, C., Luster, A.D., Busuttil, R.W., and Kupiec-Weglinski, J.W. (2008). CXCL10 regulates liver innate immune response against ischemia and reperfusion injury. Hepatol. *47*, 207–214.

Zuckerbraun, B.S., McCloskey, C.A., Gallo, D., Liu, F., Ifedigbo, E., Otterbein, L.E., and Billiar, T.R. (2005). Carbon monoxide prevents multiple organ injury in a model of hemorrhagic shock and resuscitation. Shock *23*, 527–532.