

ACETAMINOPHEN HEPATOTOXICITY IN HUMANS AND MICE

BY

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MITCHELL R. MCGILL

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Committee Chair, Hartmut Jaeschke, PhD

Wen-Xing Ding, PhD

Jed N. Lampe, PhD

Gregory A. Reed, PhD

Steven A. Weinman, MD, PhD

Date Defended: 03-28-2013

The Dissertation Committee for Mitchell R. McGill
certifies that this is the approved version of the following dissertation:

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Committee Chair, Hartmut Jaeschke, PhD

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ABSTRACT

Acetaminophen (APAP) is a popular analgesic and antipyretic. Most of a therapeutic dose is glucuronidated or sulfated and excreted. A small amount is converted by cytochromes P450 to the reactive electrophile N-acetyl-p-benzoquinone imine (NAPQI). Fortunately, NAPQI can be detoxified by conjugation with glutathione (GSH). However, after an overdose the glucuronidation and sulfation pathways are overwhelmed, resulting in formation of excess NAPQI which depletes GSH and binds proteins. This causes mitochondrial dysfunction and oxidative stress. Oxidative stress activates the c-Jun N-terminal kinase, which translocates to mitochondria and exacerbates the injury. The result is hepatocyte death. Though well-established in mice, less work has been done with human models. Our goal was to further investigate the role of mitochondria in mice and to begin studying the mechanisms of hepatotoxicity in humans. A comparison of rats and mice supported the role of mitochondria in mice. Using the human liver cell line HepaRG, we found that protein binding, loss of mitochondrial potential, and oxidative stress preceded injury. Finally, using novel mechanistic plasma biomarkers, we have provided evidence that mitochondrial damage may also occur in APAP overdose patients, leading to oncotic necrosis.

Recently, it was proposed that serum APAP-protein adducts can be used to diagnose APAP overdose. However, little work has been done to characterize the dose-response and timecourse of this parameter. We found that liver GSH depletion isn't required for protein binding in mice and that binding occurred without toxicity. Importantly, APAP-

protein adducts could be measured in plasma without liver injury. The mechanism by which this occurs likely involves secretion of proteins adducted within hepatocytes, though other mechanisms couldn't be ruled out. Finally, liver injury caused by ischemia-reperfusion increased APAP-protein adducts in mouse plasma after a subtoxic dose. Our data support the use of APAP-protein adducts in plasma, but urge consideration of potential confounding factors.

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... and the whole family.

Reality often astonishes theory.

- Ray Magliozzi (Click and Clack, the Tappet Brothers)

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- 3) **McGill MR***, Williams CD*, Xie Y, Ramachandran A, Jaeschke H. (2012) Acetaminophen-induced liver injury in rats and mice: comparison of protein adducts, mitochondrial dysfunction, and oxidative stress in the mechanisms of toxicity. *Toxicol Appl Pharmacol*. 264, 387-94.
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- 13) Ni HM, Boggess N, **McGill MR**, Lebofsky M, Borude P, Apte U, Jaeschke H, Ding WX. (2012) Liver specific loss of Atg5 protects against acetaminophen-induced liver injury. *Toxicol Sci.* 127, 438-50.
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*Co-first authors.

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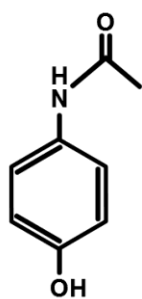
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1. INTRODUCTION

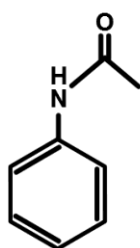
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1.1 HISTORY OF ACETAMINOPHEN

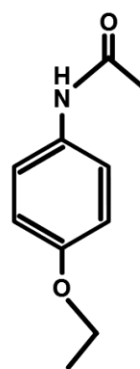
Acetaminophen (APAP) is the only survivor of a class of drugs known as the coal tar analgesics, so-named because they are structural derivatives of aniline which is abundant in coal tar. The first among these was acetanilide (Fig. 1.1.1). Although synthesized by French chemist Charles Gerhardt (better known for his role in the development of aspirin) in 1852, the clinical potential of this drug was not known for another 35 years. In a happy accident, interns Arnold Cahn and Paul Hepp, working under German physician Adolf Kussmaul at the University of Strassburg in France, were dispensed acetanilide by a pharmacist who thought he was giving them naphthalene, which they were testing in a patient as a treatment for intestinal worms. Although the drug did not rid their subject of parasites, it did have a significant antipyretic effect (Cahn and Hepp, 1886; David Josephy, 2005; Fürstenwerth, 2011). When more was ordered from the same pharmacy, the new batch lacked this property but was effective against the worms. A sample of the original preparation was sent to chemists at the German company Kalle, who identified it as acetanilide and began to market it as Antifebrin. With continued use, the analgesic effect of the drug also became known. The unique properties of this novel therapeutic were largely attributed to the acetyl moiety that distinguished it from the precursor aniline. As a result, acetylation of existing compounds became a popular approach to the synthesis of new pharmaceuticals (Fürstenworth, 2011). This trend may have contributed to the development of the next commercially successful analgesic. In an early example of industrial recycling, chemists at Bayer acetylated the primary amine of *p*-aminophenol, an unwanted by-product of



APAP



Acetanilide



Phenacetin

Figure 1.1.1. The structures of the coal-tar analgesics.

dye synthesis, and turned the hydroxyl group into an ethyl ether to produce *p*-ethoxyacetanilide, better known as phenacetin. Phenacetin was also found to have strong analgesic and antipyretic effects and rapidly grew in popularity.

While effective, acetanilide and phenacetin had a number of adverse effects, including methemoglobinemia. The search for a safer alternative led to the first clinical trial of APAP. Although it had been synthesized in 1878 by the German-trained American chemist Harmon Northrop Morse at Johns Hopkins University (Morse, 1878), APAP was not used medically for another ten years. About the same time that antifebrin and phenacetin came to market, the physician Joseph von Mering, another student of Kussmaul, became the first to administer APAP to humans (von Mering, 1893; Bertolini et al., 2006). The therapeutic effects were similar to phenacetin, but so too were the side effects. Several of the patients receiving the new drug developed methemoglobinemia. As a result, and because the earlier aniline derivatives were already successful, APAP was not developed further. Investigation of the drug stagnated as the popularity of phenacetin and another Bayer drug, aspirin, grew. In the 1940's, research of the mechanism of acetanilide-induced methemoglobinemia led to the discovery that APAP is a metabolite of the earlier drug (Greenberg and Lester, 1946; Lester and Greenberg, 1947; Greenberg and Lester, 1947). (Similar results had been published earlier by the Swedish chemist Karl Mörner (Mörner, 1889; David Josephy, 2005), but were largely overlooked.) These data were confirmed by Bernard Brodie and Julius Axelrod (1948), who used improved techniques to demonstrate that metabolism of acetanilide and other aniline derivatives can convert the drugs back to

aniline and that the levels of this compound correlated well with measurements of methemoglobinemia. These data indirectly showed that APAP was unlikely to be the cause of the adverse effect observed by von Mering, which may have been due to an impurity. Importantly, they also demonstrated that APAP is the metabolite responsible for the analgesic effect of the earlier drugs (Brodie and Axelrod, 1948; Flinn and Brodie, 1948). With increasing concern regarding the renal toxicity of phenacetin, APAP was warmly received by pharmaceutical companies in the U.S. The first APAP-containing product was a barbiturate combination dubbed Algoston, marketed by McNeil Laboratories. The brand name Tylenol, an abbreviation of N-acetyl-para-aminophenol, was introduced in 1955. Analgesia without risk of methemoglobinemia or adverse gastrointestinal effects quickly made the drug a popular alternative to the analgesics of the day. The last product to contain phenacetin was removed from the market in the 1980's.

1.2 METABOLISM AND DISPOSITION OF ACETAMINOPHEN

1.2.1 Absorption and Phase II Metabolism of Acetaminophen

APAP is a weak acid with $pK_a \approx 9.5$. Thus, at physiological pH it is almost entirely neutral and is therefore rapidly absorbed from the duodenum. Because of this, measurement of plasma APAP levels following ingestion is a convenient way to assess gastric emptying rates in clinical studies (Heading et al., 1973; Nimmo et al., 1973) and has been used for decades for this purpose. In humans, the half-life of APAP in blood is

1.5 – 3 h (Nelson and Morioka, 1963; Cummings et al., 1967). Elimination occurs in the liver, where the majority of the drug is either glucuronidated or sulfated and then excreted in the urine (Fig. 1.2.1). APAP-glucuronide accounts for 50-70% of the administered drug after a therapeutic dose. Glucuronidation is catalyzed by UDP-glucuronosyl transferases (UGT). These enzymes transfer the glucuronosyl group of uridine 5'-diphospho-glucuronic acid (UDP-glucuronic acid) to target molecules, making them more water-soluble. A number of UGTs have been described in humans and rodents, belonging to four families (UGT1, UGT2, UGT3, and UGT8) (Mackenzie et al., 2005). It was shown in the 1980s that Gunn rats, which are known to be poor bilirubin glucuronidators, were more susceptible to APAP hepatotoxicity than other strains, which were completely resistant (de Morais and Wells, 1989). The increase in injury was probably due to increased formation of the downstream reactive metabolite (de Morais and Wells, 1989). These findings were extended to humans in a clinical study comparing APAP glucuronidation and bioactivation in UGT-deficient Gilbert's syndrome patients and normal volunteers (de Morais et al., 1992). It was later found that the primary defect in Gilbert's syndrome is in the promoter for UGT1A1 (Monaghan et al., 1996; Clarke et al., 1997). There is now evidence for involvement of several UGTs in APAP glucuronidation from both *in vitro* and *in vivo* experiments (de Morais et al., 1992; Court et al., 2001; Navarro et al., 2004). The strongest evidence from humans suggests that 1A1 and 1A6 are critical. The role of 1A1 has been questioned on the basis of conflicting data from other work with individuals with Gilbert's syndrome (Ullrich et al., 1987; Rauchschalbe et al., 2004). However, some of the discrepancy may be due to

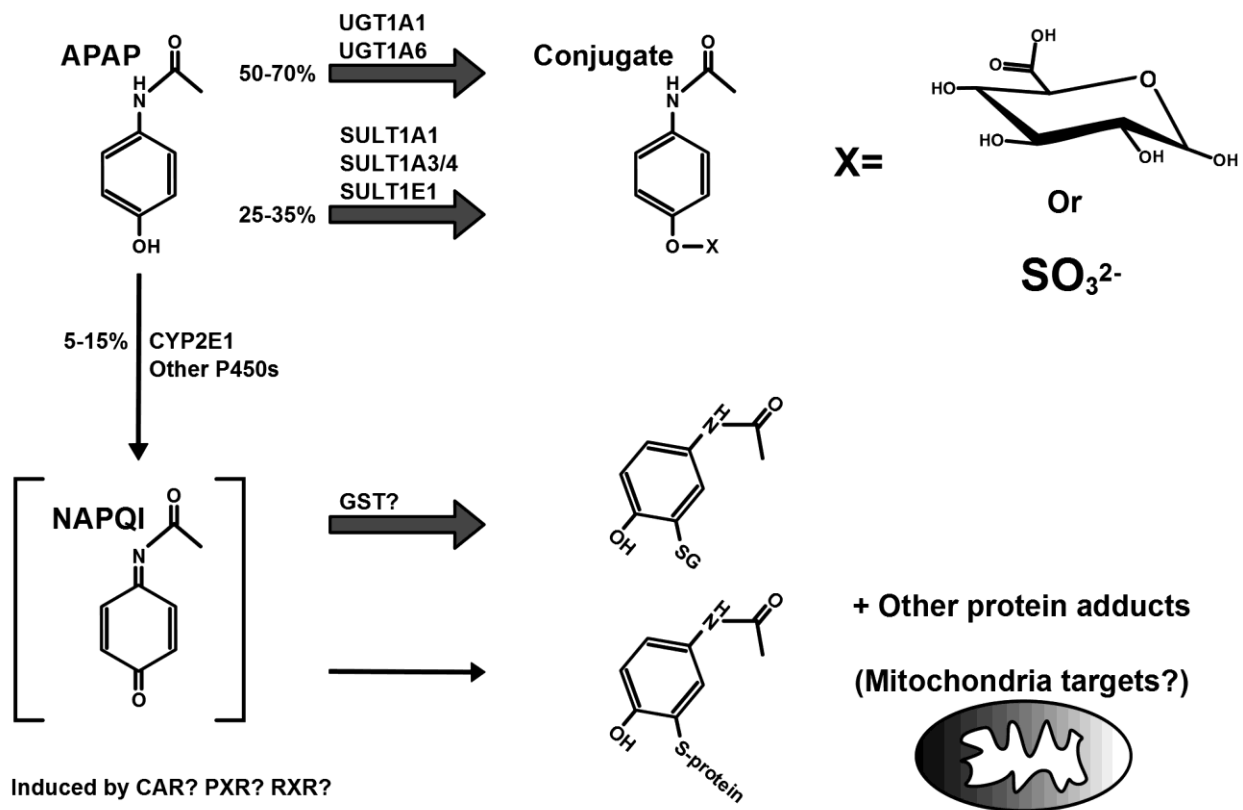


Figure 1.2.1. Metabolism of acetaminophen.

differences in experimental design, including patient selection criteria and normalization of the dose of APAP (de Morais et al., 1992). It has also been suggested that concurrent mutations in other UGTs associated with 1A1 through linkage disequilibrium in some Gilbert's syndrome patients could account for the differences (Rauchschwalbe et al., 2004). Interestingly, it was recently shown that obese mice with steatosis have higher expression of UGTs than wildtype controls, and samples from these animals had higher concentrations of APAP-glucuronide (Xu et al., 2012; Aubert et al., 2012). It is not yet known how this is regulated. A trend toward increased expression of certain UGTs has also been observed in humans with non-alcoholic fatty liver disease (Hardwick et al., 2012). However, this did not achieve significance for any isoform and there was no difference in APAP-glucuronidation activity compared with controls (Hardwick et al., 2012).

Relatively less work has been done to understand APAP sulfation. It is known that 25-35% of a therapeutic dose of APAP is recovered as APAP-sulfate (Fig. 1.2.1).

Interestingly, it has been shown that mice lacking NaS1, a kidney transporter that is involved in reabsorption of inorganic sulfate (SO_4^{2-}), are more susceptible to APAP hepatotoxicity, and NaS1 polymorphisms are known to occur in humans (Lee et al., 2006). Sulfation is catalyzed by sulfotransferase (SULT) enzymes. Generally, these enzymes transfer a sulfo group from 3'-phosphoadenosine-5'-phosphosulfate (PAPS) to an acceptor, like APAP. PAPS is synthesized from sulfate derived from diet. At least thirteen SULT isoforms are known in humans and are organized into four families (Lindsay et al., 2008). Sulfation of xenobiotics, in particular, is usually catalyzed by

cytosolic SULTs (the other major group, Golgi membrane-associated SULTs, act on larger substrates, including proteins) (Lindsay et al., 2008). Using platelet preparations as surrogates for xenobiotic metabolism in the liver, it was shown that human SULT1A1 and 1A3/4 (thermostable and thermolabile sulfotransferases, respectively) can catalyze APAP sulfation (Reiter and Weinshilboum, 1982). This was recently confirmed through *in vitro* assays using fetal human liver samples, and expanded to include SULT1E1 (Adjei et al., 2008). Moreover, increased protein levels of SULT1A1 have been observed in pregnant mice with a corresponding increase in APAP-sulfation activity in liver fractions (Wen et al., 2012). Studies of APAP pharmacokinetics in humans with polymorphisms in these SULTs would be helpful to determine which isoforms are clinically relevant. Interestingly, new data have shown that SULT1A1 protein is significantly increased in liver from humans with steatosis, and microsomal fractions from these samples had higher APAP-sulfation activity (Hardwick et al., 2012).

1.2.2 Phase I Metabolism of Acetaminophen

After a therapeutic dose of APAP, about 5-15% is excreted in urine as a mercapturic acid or cysteine conjugate. This is due to conversion of APAP to a reactive intermediate which can bind to the cysteine thiol of GSH (Fig. 1.2.1). While the glucuronide and sulfate conjugates of APAP are directly excreted in urine, APAP-GSH is initially excreted in bile, degraded in other organs including the kidney (Fischer et al., 1985;

Newton et al., 1986), and the degradation products are excreted in urine after returning to the liver (Wong et al., 1981).

The metabolic activation of APAP is principally catalyzed by cytochrome P450 enzymes (Potter et al., 1973) and the reactive metabolite of greatest relevance for hepatotoxicity is generally believed to be N-acetyl-p-benzoquinone imine (NAPQI) (Dahlin et al., 1984). NAPQI is a soft electrophile that reacts readily with nucleophilic sulfhydryl groups. Overdose of APAP results in formation of excess NAPQI, which can deplete GSH levels and bind to proteins (Jollow et al., 1973). Evidence for GSH depletion and protein binding is not limited to rodents. In patients given bromosulphthalein, the plasma concentration of the GSH conjugate of this drug was decreased after APAP overdose (Davis et al., 1975). More convincingly, increasing therapeutic doses of APAP were found to increase the turnover of GSH in volunteer subjects (Lauterburg and Mitchell, 1987). The observation that alcohol and isoniazid could affect APAP-induced liver injury led to the hypothesis that CYP2E1 is the major P450 responsible for conversion of APAP to NAPQI (McClain et al., 1980; Sato et al., 1981; Sato and Lieber, 1981; Zand et al., 1993; Nolan et al., 1994; Thummel et al., 2000). Accordingly, Cyp2e1 knockout mice were found to be less susceptible to APAP-induced liver injury (Lee et al., 1996). In addition, beta-catenin gene-deficient mice show almost complete elimination of Cyp2e1 and Cyp1a2 protein levels, which correlated with resistance to APAP hepatotoxicity (Sekine et al., 2006). CYP1A2, 2D6, and 3A4 have also been shown to activate APAP in various model systems (Thummel et al., 1993; Patten et al., 1993; Dong et al., 2000). However, Cyp1a2 $-/-$ mice were not protected against APAP toxicity (Tonge et al.,

1998). Moreover, the finding that Cyp2e1 $-/-$ mice were resistant to the hepatotoxicity caused by high doses of APAP, while the same knockout mice transgenically expressing human CYP2E1 were susceptible (Cheung et al., 2005) indicates that CYP2E1 is indeed the main P450 enzyme involved in APAP activation. Data from humans support this conclusion (Sarich et al., 1997; Manyike et al., 2000). One flaw in the latter studies is reliance upon pharmacological CYP inducers and inhibitors which may or may not be specific. More importantly, only low doses of APAP could be given and there is evidence from mice that other P450s, including CYP1A2, become important with increasing exposure (Zaher et al., 1998). Consistent with this, we have observed APAP toxicity in human HepaRG cells (McGill et al., 2011), which express relatively low levels of CYP2E1 (Antherieu et al., 2010). Together, the data suggest that CYP2E1 is the primary enzyme responsible for conversion of APAP to its reactive intermediate, but a role for other P450 enzymes cannot be ruled out.

Induction of some cytochrome P450 enzymes is known to occur after APAP treatment (Fig. 1.2.1). Limited evidence suggests that the constitutive androstane receptor (CAR), the pregnane X receptor (PXR), and the retinoid X receptor alpha (RXR α) are activated during APAP toxicity and can potentiate APAP hepatotoxicity through upregulation of P450 enzymes or altered GSH homeostasis (Zhang et al., 2002; Guo et al., 2004; Wu et al., 2004; Dai et al., 2005; Cheng et al., 2009). However, the results of these studies should be interpreted with caution. CAR and PXR increase expression of P450 enzymes other than Cyp2e1; isoforms with only a minor contribution to NAPQI formation. In fact, CAR activation was found to modestly decrease Cyp2e1 (Zhang et

al., 2002). Thus, the proposed mechanisms in these papers may not be correct (Nelson et al., 2003). In contrast to these nuclear receptors, activation of the peroxisome proliferator-activated receptor alpha (PPAR α) reduces APAP-induced injury, though this may not involve changes in metabolism (Manautou et al., 1996; Chen et al., 2000). Similarly, farnesoid X receptor (FXR) and liver X receptor (LXR) activation appear to protect by decreasing expression of phase I enzymes and/or increasing expression of protective and detoxifying enzymes (Lee et al., 2010; Saini et al., 2011). However, these data were limited at best.

Recent reports indicated that deficiency of natural killer T cell (NKT) in mice (CD1d $^{-/-}$ and J α 18 $^{-/-}$ mice) increased Cyp2e1 protein expression and enzyme activities, which enhanced metabolic activation of APAP and aggravated hepatotoxicity (Martin-Murphy 2012). It was concluded that increased ketone body formation during starvation in NKT-deficient mice was responsible for the induction of Cyp2e1 (Martin-Murphy, 2012). However, another paper using the same mice (J α 18 $^{-/-}$ mice) without starvation reported protection of NKT cell deficient mice against APAP toxicity (Down et al., 2012). The authors concluded that increased hepatic GSH levels in these mice were the cause of protection (Down et al., 2012). These publications are representative of many immunological studies in which conflicting results have been reported using the same animals and where an apparently minor change in the experimental design (fed vs. starved) has a profound impact on the results.

Though glutathionylation is a phase II reaction, in the context of APAP it only occurs after phase I metabolism. As mentioned, the reactive metabolite of APAP, N-acetyl-p-benzoquinone imine (NAPQI) (Dahlin et al., 1989), can bind to the cysteine thiol of GSH. This is a critical mechanism of detoxification. The reaction of NAPQI with GSH has been shown to occur both spontaneously and enzymatically (Coles et al., 1988). Enzymatic GSH conjugation is catalyzed by a group of enzymes called the glutathione-S-transferases (GST). It was thought that GST-Pi was most likely responsible for the enzymatic conjugation of APAP and GSH (Coles et al., 1988). However, Gst-Pi knockout mice actually had reduced injury after APAP treatment (Henderson et al., 2000). It was later found that this effect may have been due to upregulation of cytoprotective genes as a result of constitutive JNK activation in the knockout mice (Elsby et al., 2003). GSTM-null mice were also be resistant to APAP (Arakawa et al., 2012). While a recent study showed that altered function of GSTT and GSTM are associated with idiosyncratic hepatotoxicity (Lucena et al., 2008), another group failed to identify a similar association between GST isoforms and prothrombin time or outcome in APAP overdose patients (Buchard et al., 2012). Unfortunately, the latter study relied on a very small cohort with few negative outcomes. More work is needed to understand the role of GSTs in APAP hepatotoxicity.

1.2.3 Phase III Metabolism of Acetaminophen (Transport)

The conjugates of APAP that result from phase I and II metabolism must be eliminated. However, unlike the parent drug, these metabolites require transporters (Fig. 1.2.2). Using transporter-deficient rodent models and canonical inducers, it was found that biliary excretion of both APAP-glucuronide and APAP-sulfate is largely dependent on Mrp2 and Bcrp in the canalicular hepatocyte membrane (Xiong et al., 2000; Xiong et al., 2002; Zamek-Gliszczynski et al., 2005; 2006b; Lee et al., 2009) while basolateral excretion of APAP-glucuronide involves Mrp3 (Xiong et al., 2000; Xiong et al., 2002; Zamek-Gliszczynski et al., 2005; 2006a; Manautou et al., 2005). The basolateral transporters involved in APAP-sulfate excretion are less clear, but Mrp3 and Mrp4 both appear to play a role (Zamek-Gliszczynski et al., 2006a). Additional work confirmed these findings and revealed that the biliary excretion of APAP-GSH also requires Mrp2 (Chen et al., 2003). Despite significant species differences, it is interesting that induction of basolateral and canalicular efflux transporters has been shown in both APAP-treated rodents (Ghanem et al., 2004; Aleksunes, et al., 2005) and APAP overdose patients (Barnes et al., 2007). MRP2, BCRP, MRP4 and MRP5 protein levels were shown to be increased in samples from APAP overdose patients (Gu and Manautou, 2010; Barnes et al., 2007). Unfortunately, information regarding the time post-ingestion was not available in this study. Apparently, the samples were obtained from patients who required liver transplant (Barnes et al., 2007), and this is likely when most of the samples were taken. Thus, the time points measured were probably several days after APAP overdose and onset of injury. It is possible that some early changes in transporter expression were missed, while changes occurring secondary to injury were detected.

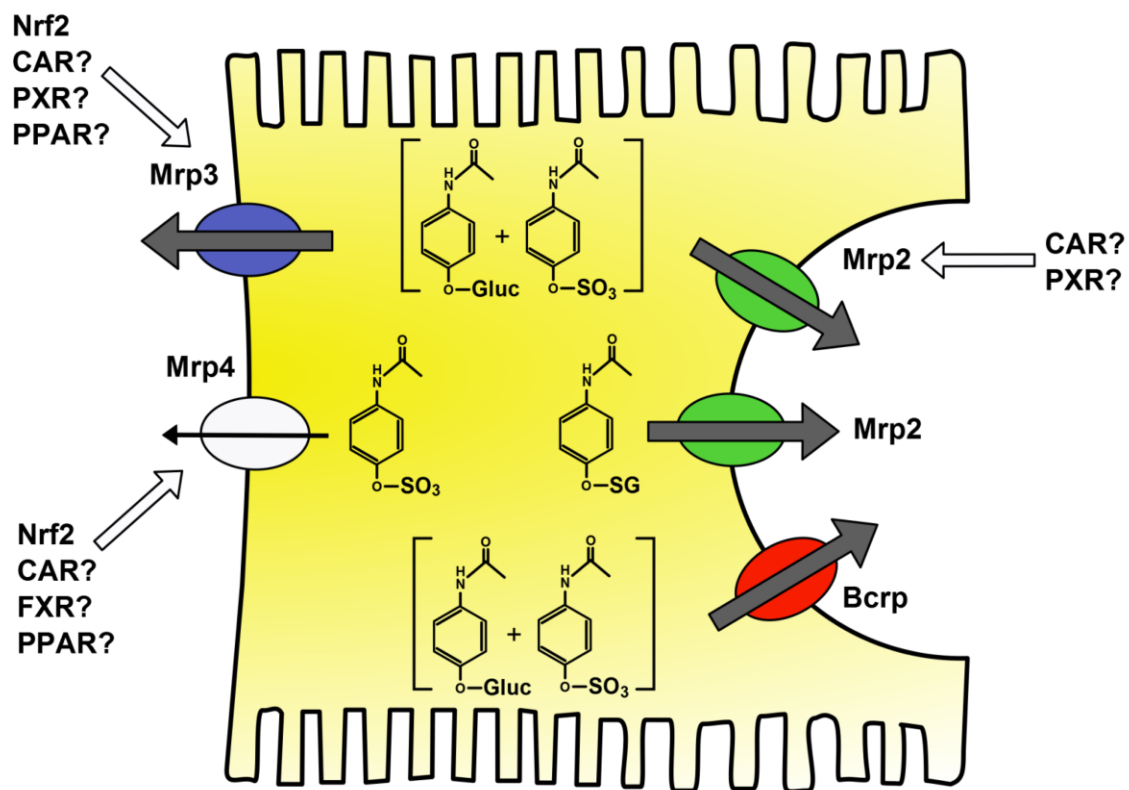


Figure 1.2.1. Transport of acetaminophen metabolites.

Therefore, the results, while interesting, must be interpreted carefully. In any case, altered transporter expression after APAP treatment seems to involve transcription factors and nuclear receptors. Some changes in transporter expression in mice (Mrp3 and Mrp4) after APAP are dependent on Nrf2 (Aleksunes et al., 2008), and may be influenced by Kupffer cell-derived cytokines (Campion et al., 2008). There is also evidence that nuclear receptors that have been shown to play a role in APAP toxicity can induce expression of several of these transporters under certain conditions (Gu and Manautou, 2010).

1.3 TOXICITY OF ACETAMINOPHEN

Exactly forty years ago, Jim Gillette's group at the national institutes of health showed that the hepatotoxicity of APAP begins with binding of a reactive metabolite to hepatocellular proteins (Mitchell et al., 1973a; 1973b; Potter et al., 1973; Jollow et al., 1973). Initially, it was thought that indiscriminate binding to a large number of proteins could lead to widespread cell dysfunction and death. However, it was shown that certain other xenobiotics, the non-hepatotoxic isomer of APAP 3'-hydroxyacetanilide (AMAP) for example, do not cause liver injury after doses that lead to similar levels of protein binding (Tirmenstein and Nelson, 1989). Data such as these provided the rationale for attempts to identify specific protein targets of NAPQI. Though a number have been found, there is no mechanistic evidence to suggest that any of those currently known are responsible for the injury (Jaeschke et al., 2012a). Most are considered non-vital

proteins or are only minimally affected by adduction. The most promising targets appear to be mitochondrial proteins. Although total protein binding was similar in their APAP and AMAP-treated mice, Tirmenstein and Nelson (1989) found that the reactive metabolites of AMAP did not bind to mitochondrial proteins as much. These data are consistent with earlier work which found that toxic doses of APAP alter mitochondrial morphology (Placke et al., 1987), inhibit mitochondrial respiration (Meyers et al., 1988) and cause oxidative stress within the organelle (Jaeschke, 1990; Cover et al., 2005). It seems likely that binding to specific mitochondrial proteins is a necessary precedent of mitochondrial dysfunction and cell death after APAP overdose. Efforts to demonstrate that mitochondrial protein binding is critical and to discover which specific mitochondrial proteins are most important in the mechanism of APAP hepatotoxicity are ongoing.

Regardless of the outcome of these ongoing studies, it is clear that mitochondrial dysfunction and mitochondrial oxidative stress play major roles in APAP-induced liver injury. Treatment with toxic doses of APAP leads to the mitochondrial membrane permeability transition (MPT), resulting in loss of mitochondrial membrane potential (Kon et al., 2004; Reid et al., 2005), and inhibition of the MPT prevents APAP-induced injury in rodent models both *in vitro* and *in vivo* (Kon et al., 2004; Reid et al., 2005; Masubuchi et al., 2005; Ramachandran et al., 2011a). Importantly, oxidative stress appears to be a critical development leading to this mitochondrial dysfunction.

Scavenging of reactive oxygen species (ROS) protects against APAP hepatotoxicity (Knight et al., 2002; Bajt et al., 2003; Bajt et al., 2004) while impairment of the mitochondrial antioxidant enzyme Mn²⁺ SOD (MnSOD or SOD2) increases the injury

(Fujimoto et al., 2009; Ramachandran et al., 2011b). Interestingly, very high doses of APAP (e.g. 600 mg/kg) can induce the MPT independent of the cyclophilin D (CypD)-regulated MPT pore as a result of extreme oxidative stress (LoGuidice and Boelsterli, 2011), though the hepatotoxicity after doses at or below 350 mg/kg is responsive to cyclophilin D inhibition (Masubuchi et al., 2005; Ramachandran et al., 2011a). The mechanisms by which much higher doses cause the MPT and the exact role of CypD at the lower still-toxic doses are controversial. In any case, mitochondrial oxidative stress leads to the MPT, ultimately resulting in cell death. Translocation of Bax into mitochondria, together with the MPT, results in release of the mitochondrial endonucleases Endonuclease G (EndoG) and Apoptosis-Inducing Factor (AIF). These proteins translocate into the nucleus and cause nuclear DNA fragmentation (Bajt et al., 2006; 2008; 2011).

A major link between the extreme oxidative stress after APAP and mitochondrial dysfunction is the mitogen activated protein kinase (MAPK) c-Jun N-terminal Kinase (JNK). It appears that an initial oxidative stress leads to JNK activation (Gunawan et al., 2006; Nakagawa et al., 2008; Saito et al., 2010a), probably by upstream activation of Apoptosis Signal-regulating Kinase 1 (ASK1) (Nakagawa et al., 2008) and Mixed Lineage Kinase 3 (MLK3) (Sharma et al., 2012) which then phosphorylate JNK through their respective MAPK cascades. The cause of the initial oxidative stress and the means by which it activates these MAPKs are not well understood (though it is hypothesized that the former is caused by mitochondrial protein binding). However, in the case of ASK1, evidence suggests that the enzyme is normally held inactive by the

redox-sensitive protein thioredoxin, which dissociates with increasing exposure to ROS (Nakagawa et al., 2008). Once activated, JNK translocates to the mitochondria (Hanawa et al., 2008; Ramachandran et al., 2011b), where it amplifies the mitochondrial oxidative stress (Saito et al., 2010a; Bajt et al., 2011), leading to the MPT (LoGuidice and Boelsterli, 2011). Though some steps remain controversial, the complete pathway to APAP hepatotoxicity as it is currently understood is illustrated in Figure 1.3.1.

Although liver injury is by far the most common and serious adverse effect of APAP, toxicity in other organs has been documented (Boyer and Rouff, 1971; Placke et al., 1987; Price et al., 1991). Of these, kidney damage seems to be the most frequently reported. Following on the earlier work in the liver, it was observed that APAP covalently binds to renal proteins in mice (Mudge et al., 1978). In a rat model, it was found that APAP is converted to p-aminophenol by deacetylation in the kidney and that this is likely metabolized to form a new reactive metabolite responsible for APAP-induced nephrotoxicity (Carpenter and Mudge, 1981; Newton et al., 1982; Newton et al., 1985). However, the later discovery that an antibody directed against the N-acetyl moiety of APAP-cysteine (APAP-CYS) could label APAP-protein adducts in the kidneys of mice revealed a critical species difference: covalent protein binding in the murine kidney is dependent on P450s, similar to the liver (Emeigh Hart et al., 1991). More recent work has shown that the renal toxicity caused by APAP may be exacerbated by free, non-protein-derived APAP-CYS (Stern et al., 2005a; 2005b). Free APAP-CYS is one of the aforementioned breakdown products of APAP-GSH. This degradation is catalyzed by γ -glutamyltransferase (γ GT), an enzyme that is highly expressed in the kidney. γ GT also

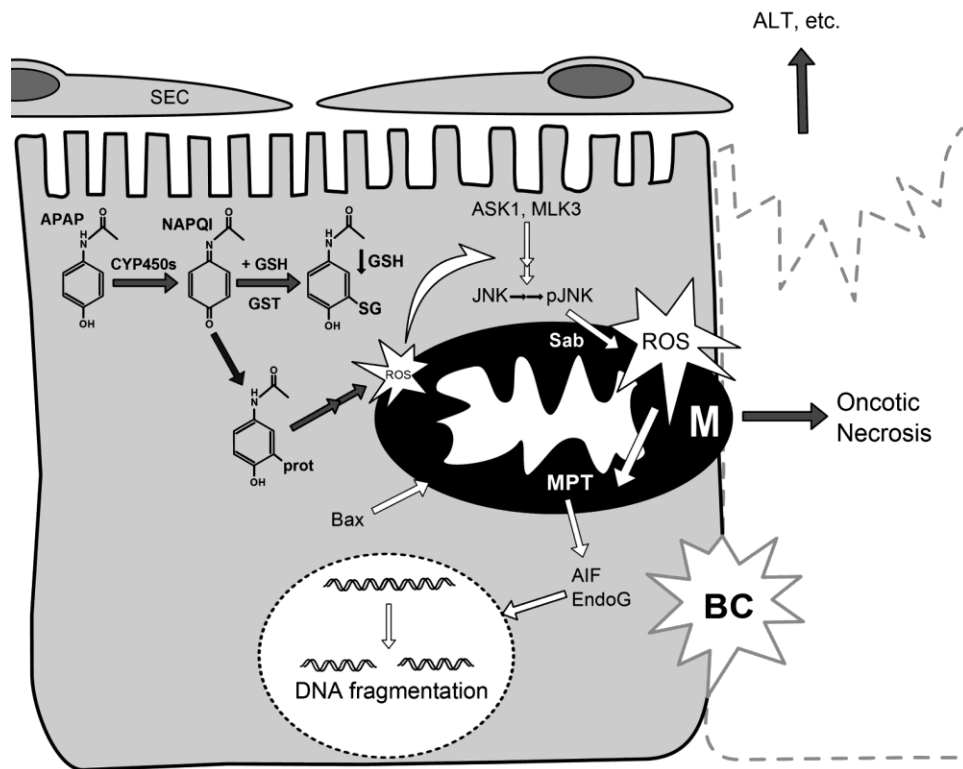


Figure 1.3.1. Mechanisms of acetaminophen hepatotoxicity.

catalyzes the transfer of the γ -glutamyl moiety of GSH to various acceptors, including free APAP-CYS (Stern et al., 2005b). In this way, free APAP-CYS depletes renal GSH, resulting in the loss of the primary defense against NAPQI and ROS.

Recently, it has been suggested that APAP can cause lung injury. Several epidemiological studies have found that frequent use of APAP is associated with asthma (Shaheen et al., 2000; McKeever et al., 2005; Shaheen et al., 2008; Beasley et al., 2008; Thomson et al., 2008; Perzanowski et al., 2010; Kelkar et al., 2012) and chronic obstructive pulmonary disease (COPD) (McKeever et al., 2005). It has been suggested that depletion of lung GSH after APAP leaves the tissue vulnerable to oxidative stress generated by inflammatory cells (Shaheen et al., 2000; Henderson and Shaheen, 2013). However, minimal loss of GSH is observed in the lungs in rodent models after APAP treatment, even at very high doses (Chen et al., 1990; Micheli et al., 1994). Other hypotheses include NAPQI-mediated activation of Transient Receptor Potential Ankyrin-1 (TRPA1) resulting in airway inflammation, and effects on cyclooxygenases (COX) (Henderson and Shaheen, 2013). Unfortunately, data to support these potential mechanisms are scarce. The link between APAP and asthma remains controversial (Henderson and Shaheen, 2013).

1.4 THERAPEUTIC MECHANISMS OF ACETAMINOPHEN

APAP has both analgesic and antipyretic effects, which are generally believed to result from the same physiological mechanisms. Unfortunately, these mechanisms are still not

well understood. Despite the paucity of data, several possibilities have been proposed. Initially, it was thought that APAP could inhibit cyclooxygenase (COX)-1 and 2, similar to the non-steroidal anti-inflammatory group of drugs (NSAIDs) that includes aspirin and ibuprofen among others. Consistent with this, early experiments showed that APAP can reduce prostaglandin synthesis in the brain (Flower and Vane, 1972). A number of studies since have provided more direct evidence that APAP inhibits these enzymes under certain conditions, particularly when peroxide concentrations are low (Bertolini et al., 2006). This would explain why APAP is not anti-inflammatory, as peroxides are present at higher concentrations in peripheral tissues and sites of inflammation. However, while this remains one of the most likely hypotheses, conflicting data exist from different experimental models (Graham and Scott, 2005; Bertolini et al., 2006).

One of the more recent ideas, which enjoyed brief but widespread popularity in the 2000's, is that APAP inhibits a unique isoform of the COX family (Chandrasekharan et al., 2002). In addition to the well-characterized enzymes COX-1 and 2, it was shown that a third cyclooxygenase exists in dogs. Dubbed COX-3, this enzyme is the product of alternative splicing of COX-1, the only difference being inclusion of coding from intron 1 of the COX-1 gene. It was reported that this canine COX variant was uniquely sensitive to inhibition by APAP and that COX-3 could be the pharmacological target of APAP for pain relief in humans (Chandrasekharan et al., 2002). Less than half a year after publication, it was pointed out that the same intron of human and rodent COX-1 is one nucleotide longer, resulting in a frameshift (Dinchuk et al., 2003). The senior author of the original study responded to this criticism by noting that COX-3 antibodies

recognized several proteins by western blot and that this recognition could be prevented by antigen competition with the intron 1 peptide. This suggested that, although out of frame, the sequence somehow produces a protein coded in part by intron 1 (Simmons, 2003). Whether or not COX-3 exists as a functional enzyme in humans, inhibition of the canine form by APAP was relatively weak (Chandrasekharan et al., 2002). In light of these data, COX-3 cannot fully account for the pharmacological effects of the drug. Definitive mechanistic data supporting a role for any of the COX enzymes in APAP analgesia or antipyresis are lacking.

Other possible mechanisms include activation of serotonergic signaling pathways and of cannabinoid receptors, either directly or indirectly (Tjølsen et al., 1991; Pini et al., 1996; Sandrini et al., 1999; Ottani et al., 2006; Bertolini et al., 2006; Dani et al., 2007). One particularly interesting study revealed that binding of NAPQI to TRPA1 in sensory neurons reduces ion currents in these cells (Andersson et al., 2011). However, most of the work supporting these hypotheses has been done with extremely high doses of APAP (100 – 1,000 mg/kg) and antinociception was assessed through behavioral measures. It is well known in the field of APAP toxicity that mice and rats become sluggish and non-responsive after treatment with such high doses. It seems likely then that some of the behavioral results from these studies are confounded by the off-target effects of supratherapeutic doses, making the quality and reproducibility of these data uncertain. Additional studies using lower doses are needed to confirm these findings.

1.5 USE OF ACETAMINOPHEN AND DEMOGRAPHICS OF OVERDOSE

APAP is one of the most commonly used drugs in the U.S. (Kaufman et al., 2002). In 2008 alone, more than 24.6 billion doses were sold (Krenzelok, 2009). Despite a number of case reports claiming otherwise, there is no convincing evidence from well-controlled studies that APAP can cause hepatotoxicity at therapeutic doses (Prescott, 2000; Kuffner et al., 2006; Dart and Bailey, 2007). However, overdose is the leading cause of acute liver failure in many Western countries (Bernal, 2003; Gow et al., 2004; Larson et al., 2005; Canbay et al., 2009). Much work has been done to assess the frequency of drug overdose in various populations. The consensus from these studies and from our own data is that Caucasians, females and younger people overdose on APAP more often than other races, males and older people, respectively, and that most overdoses are intentional (Makin et al., 1995; Larson et al., 2005; Myers et al., 2007; Forde et al., 2009; Manthripragada et al., 2011; Craig et al., 2011) (Fig. 1.5.1). Although female mice are much less susceptible to APAP-induced liver injury, there is currently no evidence for a gender difference in toxicity in humans (Larson et al., 2005). It is interesting that women overdose on APAP more often than men, despite the much higher overall suicide rates among males in most countries. A likely reason for this gender disparity is the greater use of more conclusive methods of self-harm among men than women (Hee Ahn et al., 2012). Like gender, there is no evidence for racial or ethnic differences in outcome after APAP overdose (Forde et al., 2009). In contrast to these findings, several studies have shown that while APAP overdose is more common among young people, elderly people are more likely to develop fulminant hepatic failure

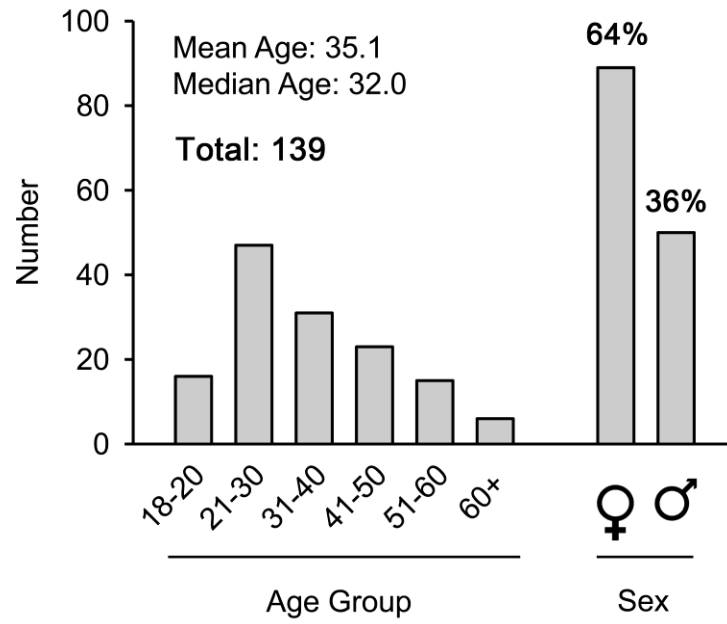


Figure 1.5.1. Demographics of acetaminophen overdose in our study. Data are from patients with probable APAP-induced liver injury only.

and mortality after very high doses (O'Grady et al., 1989; Bateman et al., 2003; Schmidt, 2005). Age-related changes in drug metabolism cannot explain this: aside from a relative shift toward sulfation in children, there appears to be little difference in APAP metabolism across different age groups, at least after therapeutic doses (Miller et al., 1976). There are a number of other possible explanations for the increased mortality in older populations. Expression or activity of electrophile and antioxidant defense systems may be lower in the elderly, or this population may have limited liver regeneration capacity. Some evidence suggests that the difference may be related to lifestyle. For example, chronic alcohol abuse may be more common in older APAP overdose patients (Schmidt, 2005).

1.6 CURRENT TREATMENT OF ACETAMINOPHEN HEPATOTOXICITY

Currently, the only effective treatment strategy for APAP overdose is replenishment of liver GSH levels. Clinically, this is accomplished by giving the patient N-acetyl-cysteine (NAC, a.k.a. Mucomyst). The increased liver GSH can scavenge NAPQI and prevent protein binding (Mitchell et al., 1973b). GSH is also an effective antioxidant and can reduce oxidative stress in the liver after APAP (Knight et al., 2002; Bajt et al., 2003; 2004). NAC may also have effects apart from GSH. Some data suggest that it can delay gastric emptying, resulting in lower APAP absorption rates (Whitehouse et al., 1981). There is strong evidence that NAC may even support mitochondrial function by acting as an energy substrate (Saito et al., 2010b). However, it must be administered within 24

h of overdose in order to have the greatest possible effect. New drugs for patients presenting beyond this time point would be beneficial.

Aside from NAC, a few studies have shown that activated charcoal or emetic agents may be beneficial after APAP overdose (Spiller and Sawyer, 2007). However, because of the very rapid absorption of APAP from the GI tract these compounds likely need to be given very soon after ingestion in order to be effective. Unfortunately, many APAP overdose patients present relatively late – during or after the peak of injury (Singer et al., 1995). Dialysis and extracorporeal adsorbent devices have also been used in the clinic to remove APAP from the blood of overdose patients and to enhance clearance of potentially toxic non-drug metabolites that accumulate during liver failure (de Geus et al., 2010; Wittebole and Hantson, 2011). However, the data supporting the use of extracorporeal liver assist devices are incomplete. Considerably more work is needed in this area.

1.7 ADDITIONAL BACKGROUND AND SIGNIFICANCE

1.7.2 Mitochondria in Acetaminophen Hepatotoxicity in Mice and Humans

The Role of Mitochondria in Mice. As mentioned, the prevailing idea regarding the initiation of APAP-induced liver injury is binding of NAPQI to mitochondrial proteins. This is based on data from a comparison of APAP and AMAP that revealed higher concentrations of protein adducts in mitochondrial fractions from mouse livers after

treatment with APAP, despite similar total protein binding (Tirmenstein and Nelson, 1989). This was reinforced when several mitochondrial proteins were identified during the later search for specific protein targets of NAPQI (Halmes et al., 1996; Qiu et al., 1998). However, these data are correlative at best. No mechanistic studies have demonstrated a direct connection between binding to mitochondrial proteins and liver injury. Moreover, the work by Tirmenstein and Nelson was recently challenged by experiments with cultured liver slices that revealed that AMAP can be toxic in hepatocytes from some species under certain conditions (Hadi et al., 2012). Although the authors did not measure mitochondrial protein adducts in their study, this suggested that mitochondrial protein binding may not be as critical as once thought.

To further explore the relationship between mitochondrial protein binding and the hepatotoxicity of APAP, we compared mitochondrial APAP-protein adducts in rats and mice after treatment with doses of APAP that caused similar total protein binding. We hypothesized that these mitochondrial adducts would be lower in rats, which are less susceptible to APAP-induced liver injury. These experiments provide a more direct comparison, using only APAP, than the earlier work with APAP and AMAP.

The Role of Mitochondria in Humans. Although mitochondrial dysfunction has been known to play a role in APAP hepatotoxicity in mice for decades, it has never been investigated clinically. To fill this gap in knowledge, we adopted two approaches: 1) experiments with the metabolically competent human liver cell line HepaRG and 2) the development of plasma markers of mitochondrial damage and measurement of these

markers in samples from overdose patients. We hypothesized that mitochondrial dysfunction and oxidative stress do develop in human hepatocytes during APAP toxicity.

HepaRG cells are a relatively new model for the study of drug metabolism and toxicity in humans. This cell line was isolated and grown from the liver of a female patient with hepatocellular carcinoma subsequent to chronic hepatitis C virus infection (Gripon et al., 2002; Parent et al., 2004). The initial popularity of these cells was due to their unique susceptibility to hepatitis B infection (Gripon et al., 2002) and because they have the unusual property of bipotency, being able to differentiate into both mature hepatocytes and biliary epithelial cells (Parent et al., 2004). A wealth of literature now attests to the drug metabolizing capabilities of HepaRG cells (Anthérieu et al., 2012) and the cell line is becoming a widely used model for drug studies. Because there is no diagnostic benefit from liver biopsy in cases of APAP overdose and due to coagulopathy and risk of bleeding after liver injury, it is difficult to obtain tissue from APAP overdose patients for detailed mechanistic studies. Instead, we chose to use HepaRG cells. We hypothesized that the MPT and mitochondrial oxidative stress develop in these human liver cells after treatment with high concentrations of APAP and that these events precede injury.

Although it is not possible to obtain tissue from APAP overdose patients, venipuncture is minimally invasive and medically routine. To determine whether or not mitochondrial dysfunction occurs in humans, we developed plasma markers which may be specific for

mitochondrial damage and measured these in plasma from overdose patients. We hypothesized that these markers would be elevated in samples from humans with APAP-induced liver injury. Because mitochondrial dysfunction and mitochondrial protein release lead to apoptosis in some models, we also measured markers of caspase activation in these samples.

1.7.3 Clinical Application of Protein-derived APAP-cysteine Adducts in Serum

The early dose-response studies comparing liver APAP-protein binding and GSH levels seemed to show that depletion of approximately 70% of liver GSH is necessary for protein binding to begin and that this could only occur after toxic doses (Mitchell et al., 1973b). Shortly after the development of the first method to specifically measure APAP-cysteine (APAP-CYS) in the liver (Roberts et al., 1987), it was found that cysteine adducts on proteins can also be detected in serum during APAP hepatotoxicity (Pumford et al., 1990). In addition, protein adducts appeared to be reduced in necrotic areas of the liver at later time points (Roberts et al., 1991). Because these adducts were only found in liver after toxic doses and could only be measured when ALT was elevated, it was believed that they were released into serum as a result of necrosis and cell contents release. With the advent of more sensitive and accurate techniques, it has been suggested that serum APAP-CYS can be used as a diagnostic marker for APAP overdose in cases of liver injury in which the cause is unknown or uncertain (Davern et al., 2006). This is an intriguing possibility. Prior to this, definitive diagnosis depended on

the measurement of the parent drug in serum, along with an accurate patient history. The short serum half-life of APAP made this problematic. Confident use of this parameter required that the patient presented soon after ingestion of the drug and the approximate time of ingestion could be established. APAP-protein adducts persist much longer in serum, making this a much better option. The half-life was found to be 1-2 days after an overdose for both children and adults (James et al., 2009).

Accurate diagnosis of the cause of liver injury can affect how patients are treated. For example, early decisions regarding the necessity of liver transplant may be based in part on etiology (Simpson et al., 2009). Furthermore, when intentional overdose is suspected, the clinician can ensure that the patient receives proper psychiatric treatment. Thus, the measurement of serum APAP-CYS could be a major step forward in patient care, if it is adopted clinically. This could become another example of a significant clinical advance made through basic research in the APAP hepatotoxicity field. However, because these adducts can be detected in serum after therapeutic doses, selection of a sensitive but specific threshold concentration is critical. A combination of $\geq 1.1 \mu\text{M}$ APAP-CYS peak concentration and $> 1,000 \text{ U/L}$ ALT has been proposed (James et al., 2009; Heard et al., 2011). Unfortunately, comprehensive dose-response and time course studies have not been done and the effects of polypharmacy and co-incidental liver injury after ingestion of therapeutic doses of APAP has not been investigated.

2. ACETAMINOPHEN HEPATOTOXICITY IN MICE AND RATS: MITOCHONDRIAL PROTEIN BINDING AND OXIDATIVE STRESS

Portions of this section are adapted from McGill and Williams et al. (2012), *Toxicol Appl Pharmacol*, 264, 387-94, with permission from the publisher.

2.1 INTRODUCTION

When used as directed, acetaminophen (APAP) is a safe and effective analgesic and fever reducer. However, large doses of APAP can cause serious liver injury. In fact, APAP overdose is the primary cause of acute liver failure in many countries throughout the West (Bernal, 2003; Gow et al., 2004; Larson et al., 2005), responsible for more than 70,000 hospitalizations each year in the U.S. alone (Budnitz et al., 2011).

Research on the mechanism of APAP-induced liver injury began four decades ago, following the first published report of this toxicity in humans (Davidson and Eastham, 1966). Though many important questions have yet to be answered, the mechanism of APAP toxicity has been well investigated in rodents (Jaeschke et al., 2011; 2012a) and progress is now being made in humans (Antoine et al., 2012; Atoniades et al., 2012; Davern et al., 2006; McGill et al., 2012a) and with *in vitro* human models (McGill et al., 2011). This profusion of data likely makes APAP the best characterized hepatotoxicant.

Because APAP-induced liver injury is clinically relevant, well studied, and can be rapidly induced *in vivo* with a single dose, it has become a standard model in the pharmacology and toxicology literature. In particular, APAP overdose in rodents is frequently used to test the hepatoprotective potential of herbal therapeutics. While this can be a valid approach, a number of concerns have been raised (Jaeschke et al., 2010; 2011; 2012b). For example, one of the most common issues in the complementary and alternative medicine literature is the use of rats to evaluate protection against APAP injury. It has been known since the early 1970s that rats are resistant to the liver-

damaging effects of APAP (Mitchell et al., 1973a). Doses which far exceed the LD50 for mice cause only minimal necrosis in rat liver. The reason for this difference in susceptibility is not well understood. In mice, APAP hepatotoxicity begins with metabolism of the parent compound to the reactive electrophile *N*-acetyl-*p*-benzoquinone imine (NAPQI). NAPQI depletes glutathione (GSH) and binds to proteins, primarily to the amino acid cysteine (Nelson, 1990; Cohen et al., 1997). Differences in APAP metabolism and protein binding could account for the difference between mice and rats. However, while protein binding appears to be a necessary first step toward injury, it is not sufficient to directly cause cell death (Jaeschke et al., 2012a). 3'-hydroxyacetanilide (AMAP), a non-hepatotoxic isomer of APAP, also binds to proteins (Tirmenstein and Nelson, 1989). Moreover, toxicity develops only after the onset of oxidative stress and mitochondrial dysfunction, and preventing these phenomena protects against APAP (Kon et al., 2004; Cover et al., 2005; Ramachandran et al., 2011a; 2011b). Moreover, activation and mitochondrial translocation of c-Jun N-terminal kinase (JNK) have repeatedly been shown to play a role in APAP toxicity in the liver (Gunawan et al., 2006; Latchoumycandane et al., 2007; Hanawa et al., 2008; Saito et al., 2010a). Thus, mitochondrial dysfunction, oxidative stress, and/or JNK activation may also be different between the two species.

A better understanding of the differences between rats and mice will not only aid future researchers in selection of the best model for their experiments, it may provide important new mechanistic insights into APAP toxicity. Therefore, the objective of the present study was to investigate potential differences in the mechanism of APAP-

induced liver injury between rats and mice with emphasis on protein adduct formation, oxidative stress, and JNK activation.

2.2 MATERIALS AND METHODS

Animals. Male C57Bl/6 mice (Jackson Laboratories, Bar Harbor, ME), Fischer 344 and Sprague- Dawley rats (Harlan Laboratories, Indianapolis, IN) between 8-12 weeks of age were kept in a temperature controlled facility with a 12 hour light/dark cycle and free access to food and water. For all experiments, food was withdrawn 12-15 hours prior to treatment with APAP. The drug was administered i.p. or p.o. at the indicated doses in one of two metabolically inert vehicles: warm saline (mice) or 20% Tween-80 (rats). Saline and solutions of Tween-80 are popular vehicles for the mouse and rat models, respectively. At various times, the animals were sacrificed by cervical dislocation (mice) or exsanguination (rats) under anesthesia. Blood was drawn from the caudal vena cava and centrifuged to obtain serum. After taking blood, livers were excised and portions were flash frozen for determination of glutathione (GSH), APAP-cysteine adducts on proteins (APAP-CYS), and western blotting, or fixed in 10% phosphate-buffered formalin for histology. For organelle isolation, fresh liver tissues were minced and gently homogenized on ice with 20-30 passes using a tight-fitting motor-driven Teflon pestle in a glass mortar. Subcellular fractions were collected by differential centrifugation. Briefly, cell debris was removed with 2,500 g spin for 10 min. The supernatant was then centrifuged at 20,000 g for 10 min to collect mitochondria and again at 110,000 g for 1 h

to pellet the mixed microsomes and light membranes. The new supernatant was then collected as the cytosolic fraction.

Biochemical assays. Serum alanine aminotransferase (ALT) was measured using a kit (Pointe Scientific) and glutamate dehydrogenase (GDH) activity was determined as described (McGill et al., 2012a). Liver GSH levels were measured using a modified Tietze assay as described (Jaeschke and Mitchell, 1990).

APAP-CYS protein adducts. APAP-CYS adducts were measured using the high pressure liquid chromatography with electrochemical detection (HPLC-ED) method of Muldrew et al. (2002) with previously described modifications (Ni et al., 2012b). For total liver adducts, tissues were homogenized with a blade-type homogenizer in 10 mM sodium acetate buffer (pH 6.5), filtered through Bio-Spin 6 columns (Bio-Rad) to remove low molecular weight compounds with the potential to interfere in the assay, and digested overnight with proteases to free the APAP-CYS. For measurement of adducts in the mitochondrial fraction, the pellets were resuspended in small volumes of sodium acetate buffer and subjected to 3 cycles of freeze-thaw to homogenize. The debris was then pelleted by centrifugation and supernatants were filtered and digested as above. Protein was measured using the bicinchoninic acid assay (BCA).

Histology and immunohistochemistry. Liver sections were stained with hematoxylin and eosin for assessment of tissue necrosis. Nitrotyrosine staining was performed as previously described (Knight et al., 2002) using a rabbit polyclonal anti-nitrotyrosine

antibody (Life Technologies, Grand Island, NY) and the Dako LSAB peroxidase kit (Dako, Carpinteria, CA).

Statistical methods. One-way analysis of variance (ANOVA) was used to assess statistical significance between three or more groups. When a difference was detected, the Student Newman-Keul's test was used for multiple comparisons. For non-normally distributed data, the Kruskal-Wallis test was used. $p < 0.05$ was considered significant.

2.3 RESULTS

APAP toxicity in rats. For our initial studies, two strains of rats were chosen based on previously published data reporting liver injury after APAP overdose: Fischer (F344) and Sprague-Dawley rats. The rats were treated orally with 1, 1.5, or 2 g APAP per kg bodyweight and sacrificed 24 h later. Doses were chosen based on the literature (Mitchell et al., 1973a) and on the limit of solubility of APAP. Though Fischer rats did have significantly elevated plasma ALT activity after the 1.5 g/kg dose, both strains showed considerable resistance (Table 2.3.1). Administering the drug i.p. at 1 g/kg in Fischer (F344) rats did not change these findings (Fig. 2.3.1A). For direct comparison, time course studies with mice (300 mg/kg) and rats (1 g/kg) were executed in parallel. While mice had a dramatic increase in both plasma ALT and GDH over time, rat liver enzyme levels in plasma remained low despite receiving a much higher dose (Fig. 2.3.1A,B). Moreover, we observed large areas of necrosis in mouse liver by histology,

Table 2.3.1: APAP Hepatotoxicity in Rats

Strain	Dose	ALT
Fischer (F344)	Control	35 ± 11
Fischer (F344)	1 g/kg	39 ± 4
Fischer (F344)	1.5 g/kg	*101 ± 7
Sprague-Dawley	Control	30 ± 1
Sprague-Dawley	1 g/kg	41 ± 11
Sprague-Dawley	2 g/kg	31 ± 4

Two strains of rats were treated p.o. with the indicated doses of APAP for 24 h. The animals were then sacrificed and ALT activities were measured in serum. Data represent mean ± SE of n = 4 animals per group. *P < 0.05 (compared to control).

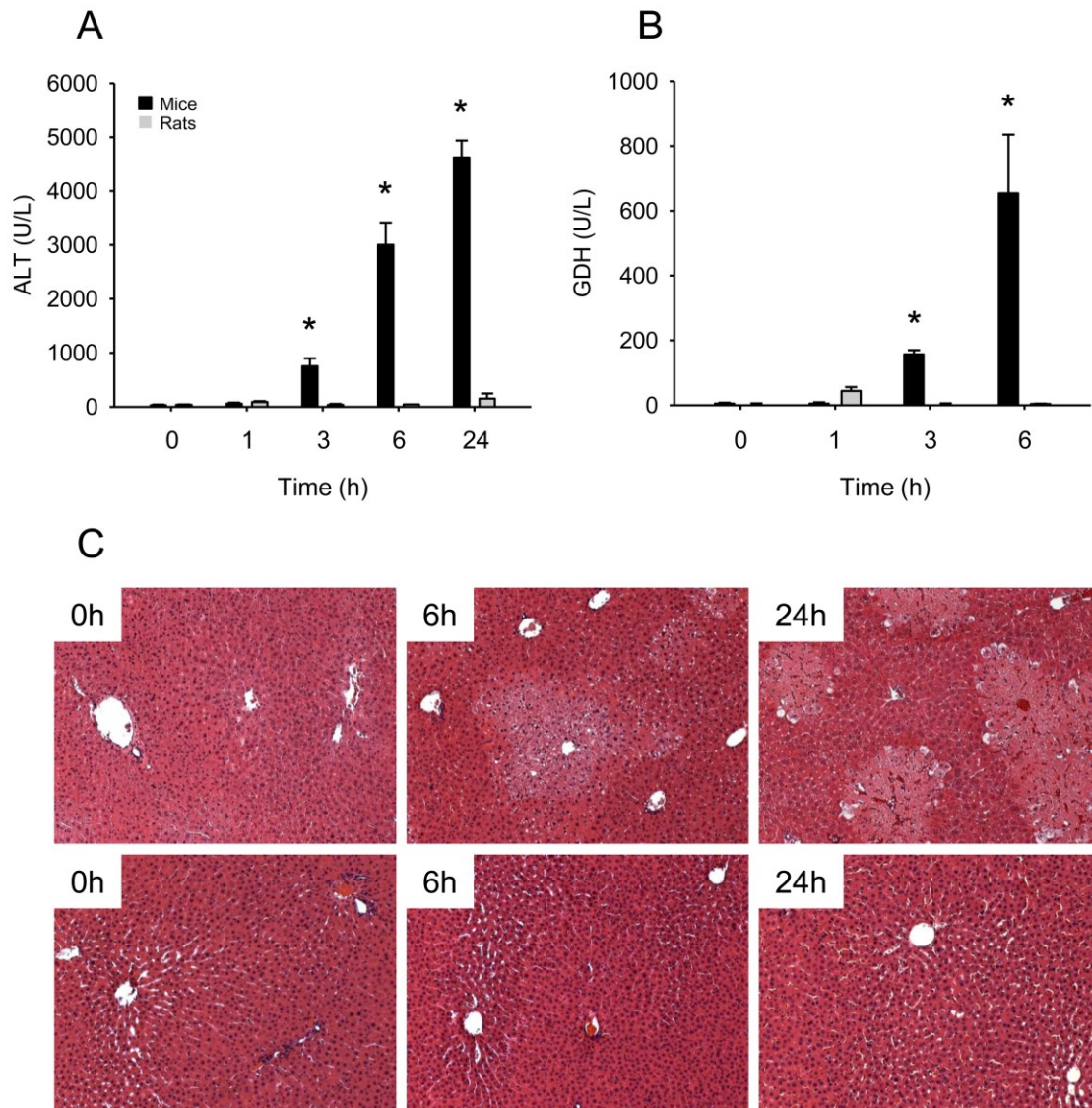


Figure 2.3.1. Liver injury in mice and rats treated with APAP. Mice and Fischer 344 rats were treated i.p. with 300 mg APAP/kg body weight or 1 g APAP/kg, respectively. At various times, the animals were euthanized and serum was collected. (A) Time course of ALT activity in serum from mice and rats after APAP. (B) Time course of glutamate dehydrogenase (GDH) activity in serum from mice and rats. (C) Representative H&E stained liver sections from mice (top row) and rats (bottom row) treated with APAP. Data are expressed as mean \pm SEM for n=3–4 animals per group. *P < 0.05 (compared to t=0).

but little or no injury could be seen in rat samples (Fig. 2.3.1C). These results are confirm that rats are highly resistant to APAP-induced liver injury.

APAP metabolism in mice and rats. APAP toxicity depends upon the formation of the reactive metabolite NAPQI, which will deplete GSH and bind to proteins (Nelson, 1990). Protein binding is known to be the initiating event in the mechanism of injury. To determine whether or not there is a difference in metabolism in the liver between mice and rats, we measured liver GSH and total liver APAP-protein binding (Fig. 2.3.2A, 2.3.3A). GSH concentrations were significantly reduced after 1 h in the livers of mice treated with the toxic dose of 300 mg/kg and remained low until 3 h and then started to recover (Fig. 2.3.2). In rats treated with 1 g/kg, there was a modest delay in GSH depletion so that the lowest concentrations were achieved at 3 h. Interestingly, there was no recovery of liver GSH in the rats even as late as 24 h after APAP treatment (Fig. 2.3.2A). This is partially because the much higher dose took longer to clear, but it is likely also due to differences in GSH resynthesis. In mice, there was significant induction of the glutamyl-cysteine ligase catalytic subunit (Gclc) after APAP treatment, but this was not seen in rats (Fig. 2.3.2C). Consistent with the delayed GSH depletion, the concentration of APAP-protein adducts increased more slowly in rat liver homogenates than in mouse samples, though similar levels were measured in both species by 6 h (Fig. 2.3.3A). The fact that similar overall protein binding was achieved in rats given a dose of APAP more than threefold higher shows that formation of NAPQI occurs much less readily in these animals. Further, the fact that rats given this much higher dose were resistant to injury despite similar levels of protein binding when

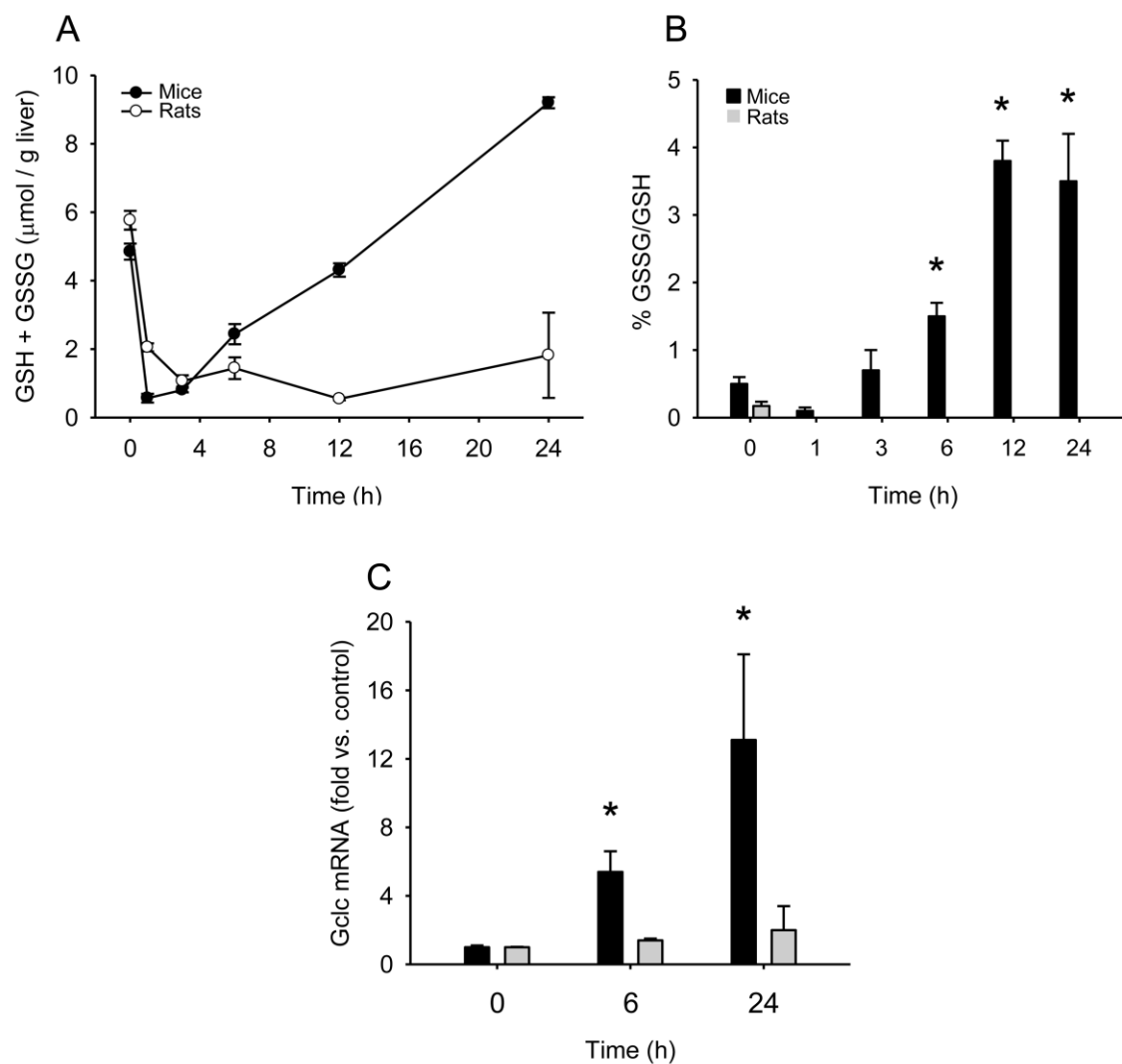


Figure 2.3.2. Liver glutathione (GSH) and glutathione disulfide (GSSG) in mice and Fischer 344 rats after APAP treatment. Mice and rats were treated i.p. with 300 mg APAP/kg body weight or 1 g APAP/kg, respectively. At the indicated times, the animals were euthanized and liver samples were flash frozen for later analysis of GSH and GSSG. (A) Total GSH levels. (B) GSSG-to-GSH ratio shown as a percentage. (C) mRNA levels of glutamate-cysteine ligase (Gclc). Data are expressed as mean \pm SEM for n=3–4 animals per group. *P < 0.05 (compared to t=0).

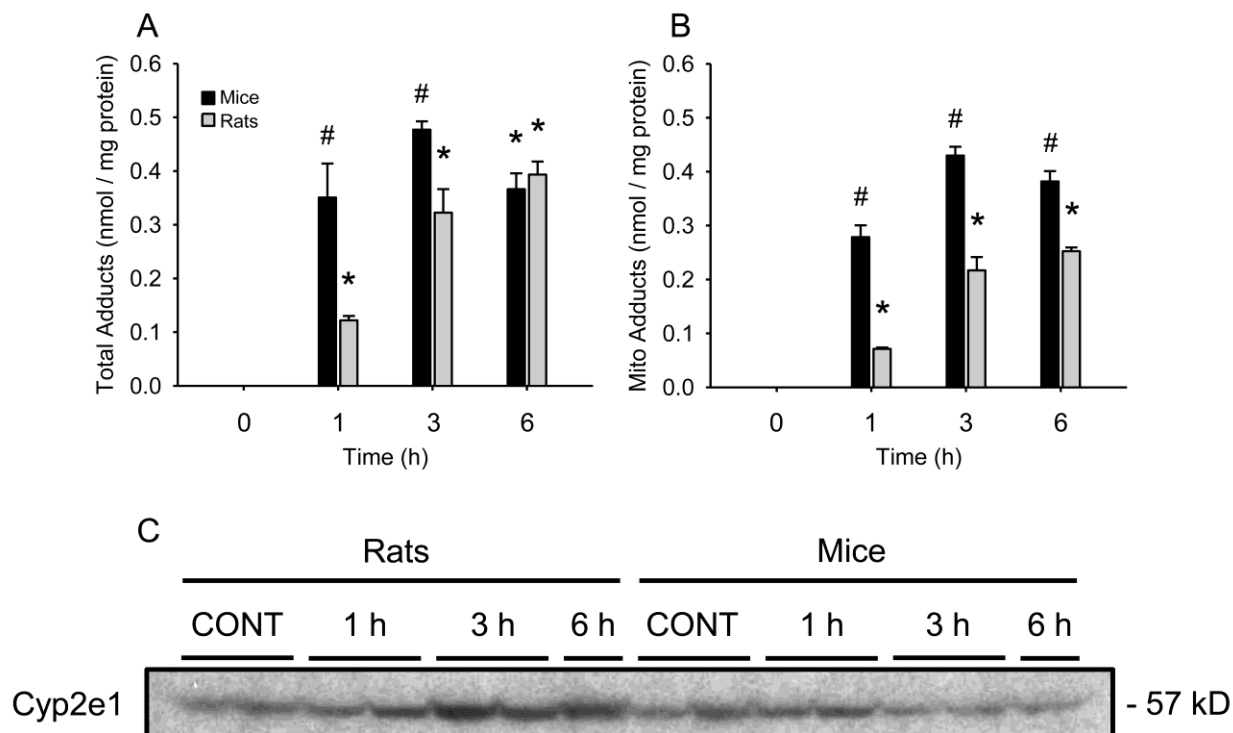


Figure 2.3.3. Total liver and mitochondrial APAP-protein adducts in mice and rats. Mice and rats were treated i.p. with 300 mg APAP/kg body weight or 1 g APAP/kg, respectively. At various times, the animals were euthanized and livers were excised. One lobe from each was immediately homogenized for subcellular fractionation by differential centrifugation. The remaining tissue was flash frozen for later analysis of total liver adducts. (A) Total liver APAP-CYS time courses. (B) Liver mitochondria APAP-CYS time courses. (C) Western blot of Cyp2e1 in mitochondrial fractions. Data are expressed as mean \pm SEM for n=3–4 animals per group. *P < 0.05 (compared to t=0). #P < 0.05 vs. rats.

compared with mice suggests that there are factors other than total protein binding to consider.

Mitochondrial protein adducts, oxidative stress and JNK activation in mice and rats.

Mitochondrial dysfunction is known to play a role in APAP hepatotoxicity in both mice (Meyers et al., 1988; Jaeschke, 1990) and humans (McGill et al., 2012b). It is well-established that NAPQI binds to mitochondrial proteins (Tirmenstein and Nelson, 1989) and it is generally accepted that this is an important early event in the mitochondrial dysfunction and associated oxidative stress seen after APAP overdose. For this reason, although there was little difference in total liver protein binding, mitochondrial APAP-protein adducts were evaluated using heavy membrane (“mitochondrial”) fractions from the rodent livers (Fig. 2.3.3B). The increase in mitochondrial adduct levels from rats paralleled the increase seen in mice. Importantly, however, at all time points the levels were significantly lower in the samples from rats. This was observed even at 6 h when the total adduct levels between the two species were similar (Fig. 2.3.3A,B). It is possible that the lower mitochondrial protein binding is responsible for the resistance to injury. Cyp2e1 is the major P450 responsible for APAP metabolism in rodents (Lee et al., 1996) and there is evidence that mitochondrial 2e1 can contribute to, and may itself be sufficient for, the formation of mitochondrial APAP-protein adducts and oxidative stress (Knockaert et al., 2011). To determine whether or not this species difference in mitochondrial protein binding could be due to differences in mitochondrial P450 expression we immunoblotted for Cyp2e1 in mitochondria from livers of both species before and after APAP treatment (Fig. 2.3.3C). There was no difference in basal

mitochondrial Cyp2e1 between mice and rats. In fact, we observed an increase in rats at later time points after APAP treatment. Thus, the lower mitochondrial APAP-protein adduct levels are not due to differences in mitochondrial Cyp2e1. To further examine the importance of mitochondrial protein binding in the mechanism of hepatotoxicity, we treated mice with multiple doses of APAP and measured the mitochondrial APAP-protein adducts in mitochondrial fractions (Fig. 2.3.4). After treatment with a subtoxic dose of APAP (75 mg/kg) for 2 h we could not detect any mitochondrial protein adducts. Interestingly, however, high concentrations of adducts were detectable in mitochondrial fractions from the livers of mice treated with a toxic dose (either 150 or 300 mg/kg). These dose-response data provide additional evidence suggesting that mitochondrial protein binding is central to the mechanism of APAP toxicity in mice.

Mitochondrial protein binding after APAP treatment is thought to lead to downstream mitochondrial oxidative stress. The major reactive oxygen species in the mechanism of APAP toxicity in mice is superoxide (O_2^-), which dismutates to molecular oxygen and hydrogen peroxide or reacts with nitric oxide (NO) to form peroxynitrite ($ONOO^-$), a potent oxidant and nitrating species. To determine whether or not oxidative stress developed in either species, tissue levels of GSSG were measured and the GSSG-to-GSH ratio calculated. Hepatic GSSG levels in mice were 0.5% of the total in controls and increased significantly after 3 h, reflecting an oxidant stress in mouse livers (Fig. 2.3.2B). In contrast, GSSG levels in rats were 0.2% in controls and remained $<0.1\%$ at all time points after APAP administration. These data indicate a substantial oxidant stress after APAP in mice but not in rats. These data were confirmed by immunostaining

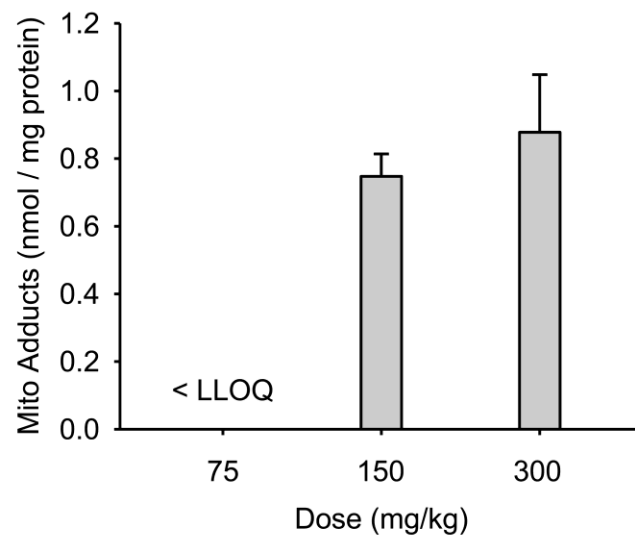


Figure 2.3.4. Mitochondrial acetaminophen-protein adducts dose-response. Mice were treated with the indicated doses of APAP for 2 h and protein-derived APAP-CYS was measured in liver heavy membrane fractions. Data are expressed as mean \pm SEM for $n = 3$ animals per group.

for nitrotyrosine protein adducts (Fig. 2.3.5), which are a footprint of peroxynitrite formation. Extensive centrilobular nitrotyrosine staining was evident in mouse livers but not in rats, suggesting that peroxynitrite formation occurred only in mice (Fig. 2.3.5).

JNK activation has been shown to be critical in the mechanism of APAP-induced liver injury (Gunawan et al., 2006; Latchoumycandane et al., 2007; Hanawa et al., 2008; Saito et al., 2010a). Because JNK activation may be the result of oxidative stress after APAP treatment in mice (Nakagawa et al., 2008; Saito et al., 2010a), we next tested whether or not we could detect JNK phosphorylation (p-JNK) and mitochondrial translocation in liver samples from these two species (Fig. 2.3.6A). Very little phosphorylated JNK could be detected in mitochondrial fractions from rats after APAP, though there was a clear increase in mouse samples at 1 and 3 h post- APAP. The difference in the mitochondrial p-JNK results could have been due to lack of activation or to lack of translocation. To test the latter possibility, we also blotted for p-JNK in the cytosol fractions (Fig. 2.3.6B), as well as for total (non-phosphorylated + phosphorylated) JNK in both fractions (Fig. 2.3.6A,B). Interestingly, non-phosphorylated JNK appears to be constitutively present in mitochondria from rat liver but not from mice. The reason for this is not yet known. In any case, very little p-JNK could be detected in rat samples when compared with samples from mice. Together, these data show that there is no relevant oxidative stress or JNK activation in rats after APAP overdose.

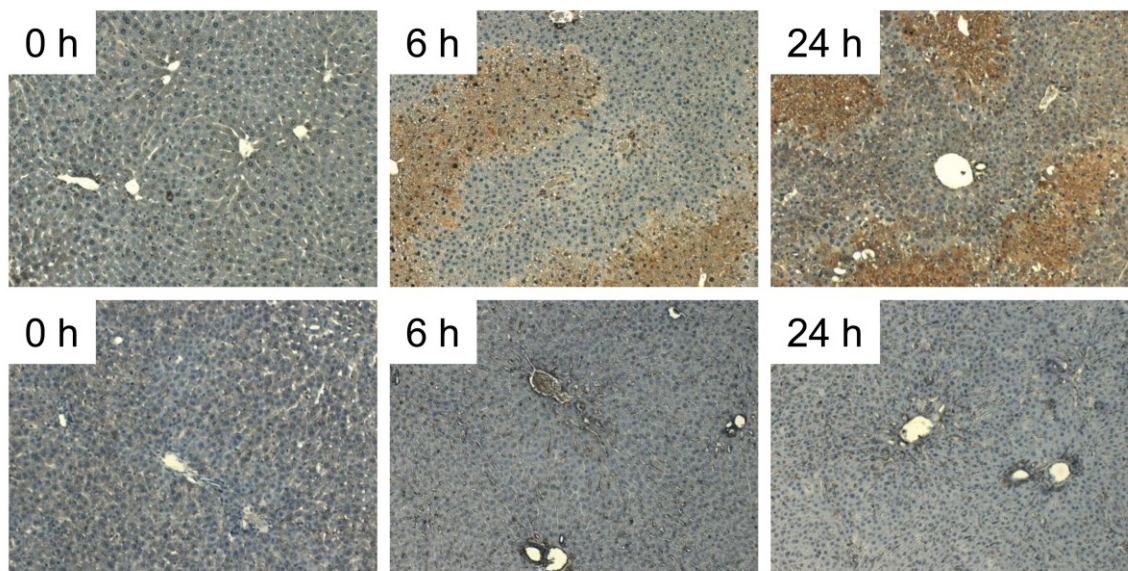


Figure 2.3.5. Nitrotyrosine staining in mice (top row) and rats (bottom row) after APAP treatment. Mice and rats were treated i.p. with 300 mg APAP/kg body weight or 1 g APAP/kg, respectively. At the indicated times, the animals were euthanized and livers were fixed in phosphate-buffered formalin. Sections were stained using an anti-3-nitrotyrosine antibody.

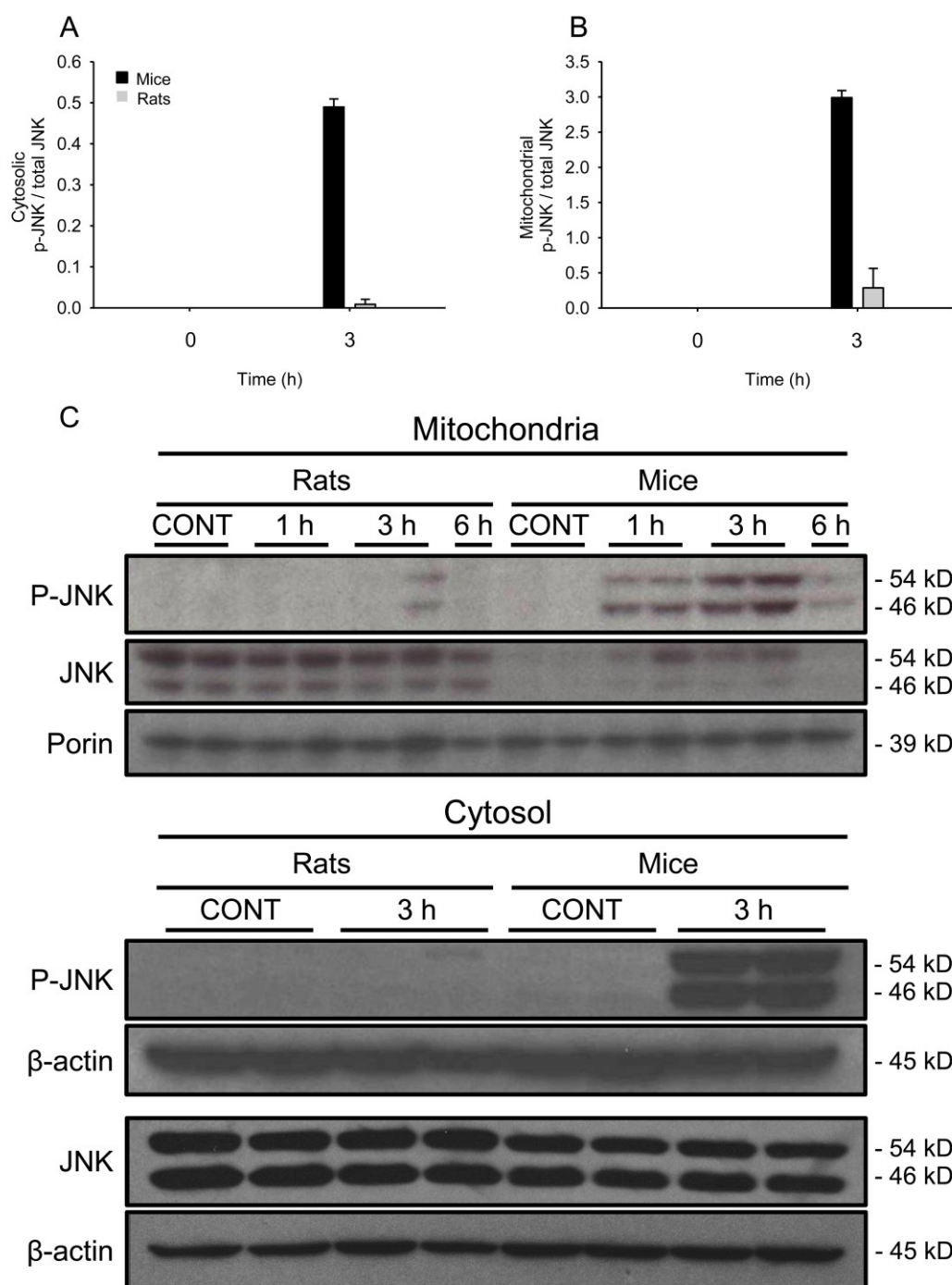


Figure 2.3.6. JNK phosphorylation and mitochondrial translocation in livers from mice and rats after APAP treatment. p-JNK was measured by Western blotting in mitochondrial and cytosolic fractions from mice and rats after treatment with 300 mg APAP/kg body weight or 1 g APAP/kg, respectively for the indicated times (C). Densitometric analysis of p-JNK and total JNK in the cytosol (A) and the mitochondria (B).

2.4 DISCUSSION

The objective of this study was to evaluate potential mechanistic differences of APAP hepatotoxicity between rats and mice. Although it is known that rats are resistant to APAP hepatotoxicity (Mitchell et al., 1973a), many investigators continue to choose this species for their studies of potentially hepatoprotective compounds. This is especially true in the area of herbal therapeutics and natural products (Jaeschke et al., 2011) but it is not limited to this field (Laskin et al., 1995; Miyamoto et al., 2008; Ahmed et al., 2011). Initially, species differences in APAP toxicity were thought to result from different rates of APAP metabolism (Davis et al., 1974). Consistent with this, our data revealed that APAP protein binding in rats was similar to a standard mouse model of toxicity when a threefold higher dose was administered. Moreover, we observed a delay in hepatic GSH depletion and APAP-protein adduct formation in rats compared with mice. In this study, the rats and mice were fasted for approximately the same amount of time (12-16 h) before APAP treatment. It is possible that this was simply insufficient for the rat model due to a difference in GSH turnover between species. Indeed, the starting GSH levels in the livers from fasted rats were on average 1 $\mu\text{mol/g}$ liver higher than in liver from control mice. However, optimization of this value would be difficult due to the fact that the rate of GSH synthesis in rat liver is actually increased during fasting (Lauterburg and Mitchell, 1981). In any case, induction of cytochrome P450 enzymes with phenobarbital or a similar compound is one strategy that has been used to compensate for the apparent difference in metabolism (Mitchell et al., 1973a). However, this usually requires several days of pretreatment. More importantly, many of these

compounds are nuclear receptor activators and the effect of these treatment regimens on the mechanism of toxicity has not been well investigated. The mouse model is more convenient and better characterized. Interestingly, despite the delayed metabolism in rats, at later time points APAP protein binding was similar in both species at the doses used. Thus, it is likely that there are other downstream factors responsible for the difference in susceptibility. After several decades of research on the mechanisms of APAP toxicity, it is now possible to compare some of these downstream events between mice, rats, and even humans, in greater detail.

Mitochondrial dysfunction and oxidative stress in mice and rats. Mitochondrial dysfunction is known to occur after APAP overdose in mice (Jaeschke and Bajt, 2006). Protein adducts in mitochondria are higher after APAP treatment compared with the non-hepatotoxic isomer 3'-hydroxyacetanilide (Tirmenstein and Nelson, 1989). It is generally accepted that this increased mitochondrial protein binding leads to mitochondrial dysfunction and oxidative stress (Jaeschke and Bajt, 2006). APAP overdose inhibits mitochondrial respiration (Meyers et al., 1988) and causes a decrease in hepatic ATP levels in the liver (Jaeschke, 1990). Using electron microscopy, swelling and lysis of mitochondria were also observed (Placke et al., 1987). Evidence for superoxide and peroxynitrite formation selectively in mitochondria has also been found (Jaeschke, 1990; Cover et al., 2005) and it was later discovered that mitochondrial depolarization occurs in primary mouse hepatocytes treated with high concentrations of APAP (Kon et al., 2004; Reid et al., 2005). Importantly, well-characterized inhibitors of the mitochondrial permeability transition (MPT) were protective in this model (Kon et al.,

2004), and mice deficient for the MPT pore regulator cyclophilin D had reduced liver injury *in vivo* (Ramachandran et al., 2011a). However, the MPT is only regulated by cyclophilin D after low but not high overdoses of APAP (LoGuidice and Boelsterli, 2011). Similar to the results with AMAP mentioned above, we saw reduced mitochondrial APAP-protein adducts in rats. Together with the absence of GSSG, nitrotyrosine protein adducts, p-JNK formation or p-JNK translocation to the mitochondria in this species, these data strongly suggest that no mitochondrial dysfunction or oxidative stress occurs in rats after APAP overdose. Moreover, there was no elevation of serum GDH activity, which has been used as a marker of mitochondrial damage (McGill et al., 2012a), though this could be due to the lack of necrosis and enzyme release.

Protein binding, especially mitochondrial protein binding, is necessary for initiation of APAP toxicity (Tirmenstein and Nelson, 1989). A large number of compounds (extracts from natural products) have been claimed to protect against APAP through antioxidant effects or through prevention of mitochondrial damage. However, the metabolic activation of APAP is rarely evaluated. Any reduction in APAP-protein adducts by inhibition of metabolism or scavenging of NAPQI will be protective against APAP-induced liver injury. Without protein binding, downstream events in the mechanism of toxicity (e.g. mitochondrial dysfunction, oxidative stress, JNK activation) will not occur and one could mistakenly conclude that the compound of interest protects by blocking one or more of these events. For this reason, measurement of GSH or APAP-CYS should be the first experiment performed in any test of potentially hepatoprotective compounds relying on the APAP model. In both cases, an early time point (0.5 – 1 h

post-APAP) should be used. Observations later than 1 h may miss early differences in protein adduct formation, and in mice GSH levels begin to recover by 4 - 6 h (Jaeschke et al., 2011).

JNK activation in mice and rats. JNK is phosphorylated and translocates to mitochondria early in APAP hepatotoxicity in mice (Gunawan et al., 2006; Hanawa et al., 2008; Ramachandran et al., 2011b) and this is thought to occur at least partly as a result of an initial oxidative stress (Nakagawa et al., 2008; Saito et al., 2010a; Ramachandran et al., 2011b). Our results confirmed these findings (Fig. 2.3.6). Importantly, inhibition of JNK activation in mice reduces ALT activity in plasma as well as the appearance of necrosis in liver sections, reduces nuclear DNA fragmentation, and prevents the further development of mitochondrial oxidative stress after APAP. In contrast, we could not detect JNK phosphorylation in mitochondria from rats. This supports the conclusion that these animals did not develop mitochondrial dysfunction or oxidative stress. The exact relationship between JNK activity and oxidative stress after APAP intoxication is not fully understood. However, there is evidence that the early oxidant stress is involved in JNK activation, which appears to amplify the mitochondrial oxidant stress (Saito et al., 2010a). Interestingly, non-phosphorylated JNK was present in mitochondria from control rat liver but not from control mice (Fig. 2.3.6A). This may suggest that liver injury involving JNK requires both mitochondrial localization and phosphorylation. Localization or translocation alone is insufficient.

Mechanisms of APAP toxicity in humans. Progress is now being made in the study of APAP toxicity in humans (McGill et al., 2012a; Antoine et al., 2012; Antoniadou et al., 2012). Data from the human cell line HepaRG and from human samples have provided evidence that mitochondrial damage also occurs in humans (McGill et al., 2011; 2012a). APAP selectively causes necrosis of hepatocyte-like cells in HepaRG cultures, and this is preceded by loss of mitochondrial membrane potential and the development of mitochondrial oxidative stress (McGill et al., 2011). In humans, glutamate dehydrogenase (GDH) and mitochondrial DNA (mtDNA) are detectable in plasma during APAP-induced liver injury but are low or nondetectable in samples from overdose patients without serious liver injury or from healthy controls (McGill et al., 2012a). These biomarkers were also elevated in mice after treatment with high doses of APAP but not after treatment with furosemide, a diuretic which can cause similar centrilobular necrosis but without the antecedent mitochondrial dysfunction. These data suggest that GDH and mtDNA in plasma are specific biomarkers for mitochondrial injury and that humans develop this injury after APAP overdose. Because there was lower mitochondrial protein binding in rats and they were resistant to mitochondrial damage in our study, this species is probably not a clinically relevant model for APAP-induced liver injury and the mouse is preferred.

Potential issues with the mouse model. A caveat to our interpretation of these data is that APAP toxicity in some strains of mice has not been as thoroughly studied as in others and there is wide variation in sensitivity to APAP (Harrill et al., 2009). It is also known that female mice are less susceptible to APAP-induced liver injury than male

mice (Guerrero Munoz and Fearon, 1984). It is tempting to speculate that, in addition to differences in expression of cell death genes (Harrill et al., 2009), this may be due in part to variation in mitochondrial protein binding and/or mitochondrial dysfunction and oxidative stress. For our experiments, we chose C57Bl/6 mice. This strain is commonly used for studies of APAP hepatotoxicity and the mechanism of toxicity in these animals is well understood.

While the mouse, in general, appears to be more clinically relevant than the rat model, there may still be important differences between mice and humans. For example, although Cyp2e1-deficient mice are protected against APAP-induced liver injury (Lee et al., 1996), another study found that recombinant human CYP3A4 was much more active than human 2E1 in converting APAP to APAP-GSH (Laine et al., 2009). Further, 2E1 activity is low in the metabolically competent human liver cell line HepaRG (Anthérieu et al., 2010), but these cells metabolize APAP and develop toxicity (McGill et al., 2011). Thus, the enzymes responsible for APAP metabolism may be different in mice and humans.

Summary and conclusions. Rats are much more resistant to APAP hepatotoxicity than mice. This is likely the result of reduced mitochondrial protein binding, which limits mitochondrial dysfunction and prevents the oxidative stress and peroxynitrite formation in rats. These data support the already well-established role of mitochondria in the mechanism of APAP toxicity. Furthermore, because mitochondrial dysfunction occurs in humans and probably leads to the necrosis observed after APAP overdose, rats are not

a human-relevant species for studies using the APAP liver injury model.

3. PROTEIN BINDING, MITOCHONDRIAL DYSFUNCTION, AND OXIDATIVE STRESS PRECEDE CELL DEATH IN HUMAN HepaRG CELLS

Portions of this section are adapted from McGill and Yan et al. (2011), *Hepatology*, 53, 974-82, with permission from the publisher.

3.1 INTRODUCTION

Acetaminophen (APAP) is a widely used over-the-counter analgesic and antipyretic drug, and is a common component of opioid-containing prescription formulations. Though safe at therapeutic levels, overdose of APAP causes liver injury and is the foremost cause of acute liver failure in the US and the UK (Larson et al., 2005). At therapeutic doses, >90% of the drug is glucuronidated or sulfated in the liver and subsequently excreted. The remainder is metabolized by cytochromes P450 (CYP450) to the electrophilic intermediate N-acetyl-*p*-benzoquinoneimine (NAPQI), which can be neutralized by conjugation with glutathione (Nelson, 1990). However, after an overdose of APAP, formation of NAPQI exceeds the detoxification capacity of glutathione, resulting in covalent binding to cellular proteins (Cohen et al., 1997). Although the overall protein binding caused by an overdose of APAP or its isomer 3'-hydroxyacetanilide is similar and many adducted proteins have been identified, toxicity only occurred with APAP, which shows greater binding to mitochondrial proteins (Tirmenstein et al., 1989; Qiu et al., 1998). The subsequent mitochondrial dysfunction leads to inhibition of mitochondrial respiration (Meyers et al., 1988), ATP depletion (Meyers et al., 1988), and formation of reactive oxygen (Jaeschke, 1990) and peroxynitrite (ROS and RNS) (Cover et al., 2005) inside mitochondria. The oxidant stress is involved in activation of the c-jun-N-terminal kinase (JNK) pathway (Hanawa et al., 2008) and eventually triggers the opening of the mitochondrial membrane permeability transition (MPT) pore resulting in collapse of the mitochondrial membrane potential (Kon et al., 2004; Bajt et al., 2004). Mitochondrial matrix swelling and rupture

of the outer membrane causes the release of intermembrane proteins including cytochrome c, endonuclease G and apoptosis-inducing factor (AIF) (Jaeschke and Bajt, 2006; Bajt et al., 2006). Only endonuclease G and AIF translocate to the nucleus and induce DNA fragmentation (Bajt et al., 2006). The severe impairment of aerobic energy metabolism, massive ATP depletion and nuclear DNA damage result in necrotic cell death (Gujral et al., 2002). Despite the release of cytochrome c from mitochondria, no significant activation of caspases has been detected and apoptosis contributes less than 5% to the overall injury in mice (Lawson et al., 1999; Gujral et al., 2002; Jaeschke et al., 2006).

Most of our present knowledge of APAP hepatotoxicity has been learned from rodent studies *in vivo* and in primary culture (Nelson, 1990; Jaeschke and Bajt, 2006).

However, notable differences exist in the time course of injury between rodents and humans. In particular, increased aminotransferase activity can be detected in rodent plasma within 2-6h of administration of a toxic dose of APAP, with peak activity achieved around 12h (Knight et al., 2001). In humans, increased plasma enzyme activity is rarely observed before 12-24h following ingestion and does not peak until 48-72h (Singer et al., 1995). Although such differences between humans and rodents may be mainly due to species differences in metabolic rate and body size, mechanistic dissimilarities cannot be completely ruled out.

In order to bridge this gap between rodents and humans, a human *in vitro* system is needed. Primary human hepatocytes as the gold standard have major drawbacks. The

availability of these cells is limited, and due to significant differences in donor background they can vary considerably in drug response. Moreover, primary human hepatocytes have a limited lifespan, undergoing phenotypic changes and displaying highly variable CYP450 expression as a function of time in culture. In contrast, most hepatoma cell lines are very stable, available in large quantities, and easy to work with. Unfortunately, the majority does not express the CYP450 enzymes necessary for metabolism of drugs and are therefore not useful for studies of drug toxicity (Jover et al., 2001; Rodriguez-Antona et al., 2002).

HepaRG cells were recently isolated and cultured from a hepatoma in a female patient with cirrhosis subsequent to hepatitis C virus infection (HCV) (Gripon et al., 2002). HepaRG cells are bipotent progenitors. Upon differentiation, two morphologically distinct populations become apparent: hepatocyte-like cells and biliary epithelial-like cells (Parent et al., 2004; Cerec et al., 2007). Several studies have demonstrated high expression and activity of xenobiotic metabolizing enzymes in this cell line, comparable to primary human hepatocytes, suggesting their use in drug studies (Aninat et al., 2006; Guillouzo et al., 2007). However, detailed investigations into the mechanisms of drug toxicities have not been performed with this cell line. Therefore, the objective of the current investigation was to assess the value of HepaRG cells as a human system to study APAP hepatotoxicity and to determine if mechanisms of cell death observed in primary mouse hepatocytes are applicable to human hepatocytes.

3.2 MATERIALS AND METHODS

Cell culture. HepaRG cells were obtained from Biopredic International (Rennes, France). The cells were seeded at 1×10^5 undifferentiated cells/cm² in hepatocyte wash medium (Invitrogen Corporation, Carlsbad, CA) containing additives for growth (Biopredic). The cells were cultured at 37°C with 21% O₂ and 5% CO₂ for 14 days before differentiation. Medium was renewed every 3 days. Cell differentiation was induced as described (Gripon et al., 2002). The cells were maintained up to 4 weeks after differentiation for use. HepG2 cells were grown to 90% confluence in DMSO-free Williams' E Medium containing penicillin/streptomycin, insulin, and 10% FBS. For APAP treatment, HepaRG or HepG2 cells were washed with phosphate buffered saline (PBS) and changed to DMSO-free medium containing the desired concentration of APAP. For caspase inhibition, some cells were pretreated for 1h with medium containing 20 µM Z-VD-fmk (generous gift from Dr. S. X. Cai, Epicept Corp., San Diego, CA), then changed to medium containing 20 µM Z-VD-fmk and 20 mM APAP. As a positive control for caspase activation, some cells were treated for 16.5h with 5 mM galactosamine and 100 ng/mL recombinant human TNF (Genzyme, Cambridge, MA). HepaRG cells were used at passages 18, 19, and 20. Within this range, no variation in GSH depletion or in the kinetics of injury was observed after APAP exposure suggesting no relevant change in CYP expression or activity between these passages.

APAP-CYS protein adducts. After protease digestion, APAP-cysteine (APAP-CYS) adducts were measured in cells and in the culture medium by LC-MS/MS as described (McGill et al., 2011).

Biochemical analyses. Cell death was assessed by lactate dehydrogenase (LDH) release, as described in detail (Bajt et al., 2004). LDH release is a more sensitive parameter of cell death because HepaRG cells contain only low levels of alanine aminotransferase activity. The JC-1 Mitochondrial Membrane Potential Kit (Cell Technology, Mountain View, CA) was used according to the manufacturer's instructions (Bajt et al., 2004). Cellular glutathione was measured using a modified Tietze assay, as described (Jaeschke and Mitchell, 1990). For measurement of mitochondrial ROS generation, HepaRG cells were seeded on glass bottom dishes and ROS and peroxynitrite generation was measured using MitoSox Red® and dihydrorhodamine, respectively, as described (Yan et al., 2010). Caspase activity based on Z-VAD-fmk inhibitable Ac-DEVD-AMC fluorescence was measured as described (Jaeschke et al., 1998).

Propidium iodide staining. Cells were seeded on glass bottom dishes and treated with APAP and 30 μ M propidium iodide in DMSO-free, phenol red-free Williams' E Medium with penicillin/streptomycin and 10% FBS. At various time points, the live cells were imaged on a Zeiss Axiovert inverted fluorescence microscope through a Texas Red filter to assess PI uptake. All fluorescence images were taken at the same exposure

and later superimposed on phase contrast images of the same fields using Image J software.

TUNEL imaging. For the terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling (TUNEL) assay, sections of liver were stained with the In Situ Cell Death Detection Kit, AP (Roche Diagnostics, Indianapolis, IN) as described in the manufacturer's instructions.

Statistical methods. All results were expressed as mean \pm SE. Comparisons between multiple groups were performed with one-way ANOVA followed by a post hoc Bonferroni test. If the data were not normally distributed, we used the Kruskal-Wallis Test (nonparametric ANOVA) followed by Dunn's Multiple Comparisons Test; $p < 0.05$ was considered significant.

3.3 RESULTS

Metabolism of Acetaminophen. The first event in the pathogenesis of APAP hepatotoxicity in rodents is metabolism of the drug to the reactive intermediate NAPQI, which can bind to and deplete glutathione. To verify metabolism of APAP in this model, differentiated HepaRG cells were treated with 20 mM APAP and the GSH content of the cells was measured at various time points. Significant glutathione depletion (16% decrease from baseline) could be detected as early as 3h, with only 26% of control GSH levels remaining at 24h (Fig. 3.3.1A). Measurement of APAP-protein adducts in these

cells showed a significant increase as early as 1h, peak levels at 6h and a gradual decline during the subsequent 18h (Fig. 3.3.1B). Protein adducts in culture medium were only detected at 12h (4.38 ± 0.20 ng/ml) and 24h (24.38 ± 1.05 ng/ml), which correlated with the decline in cellular adduct levels at these time points. These results indicate that protein adducts were formed well before cellular GSH levels were exhausted.

Mitochondrial dysfunction and oxidative stress. To explore the role of mitochondrial dysfunction after APAP exposure in HepaRG cells, we examined mitochondrial integrity using the JC-1 assay. In healthy cells, the dye preferentially localizes to mitochondria, where it forms aggregates which fluoresce red. When the mitochondrial membrane potential collapses (e.g. after the membrane permeability transition), the dye can diffuse into the cytosol in monomeric form which fluoresces green. Thus, the ratio of red to green fluorescence reflects mitochondrial membrane integrity. HepaRG cells showed a significant decrease in red/green fluorescence by 12h in the presence of 20 mM APAP, which persisted to at least 24h (Fig. 3.3.1C). As an indicator of cell death, lactate dehydrogenase (LDH) activity was measured in cell lysate and in culture medium. LDH release into the culture medium was not observed up to 15h with 20 mM APAP (Figure 3.3.1D). However, a significant increase was found at 24h (29%), and this continued to rise until at least 48 hours, reaching 62% (Fig. 3.3.1D). Notably, all four parameters discussed (GSH levels, protein adducts, JC-1 fluorescence, and LDH release) exhibited a clear concentration-response (Fig. 3.3.2).

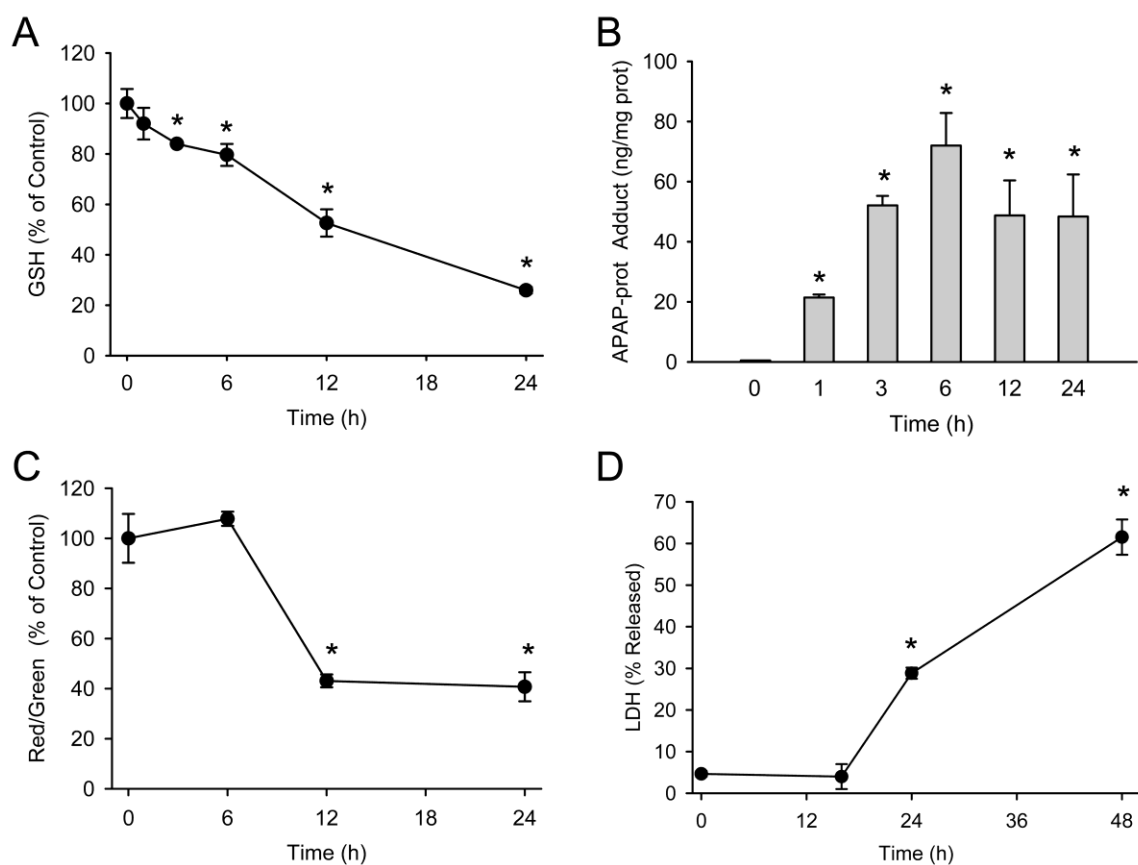


Figure 3.3.1. Time course of APAP toxicity in HepaRG cells. Confluent cells were treated with 20 mM APAP and GSH, APAP-protein adducts, JC-1 fluorescence, and LDH release were measured as described in Materials and Methods. (A) Glutathione content before and 1-24 hours after treatment with APAP. (B) APAP-protein adducts before and 1-24 hours after APAP treatment. (C) Ratio of red to green fluorescence of the JC-1 dye before and 6-24 hours after APAP treatment. (D) Percentage of total LDH activity found in culture medium before and 15-48 hours after APAP treatment. Data are expressed as mean \pm SE of 3-6 independent cell preparations. *P < 0.05 (compared to time 0).

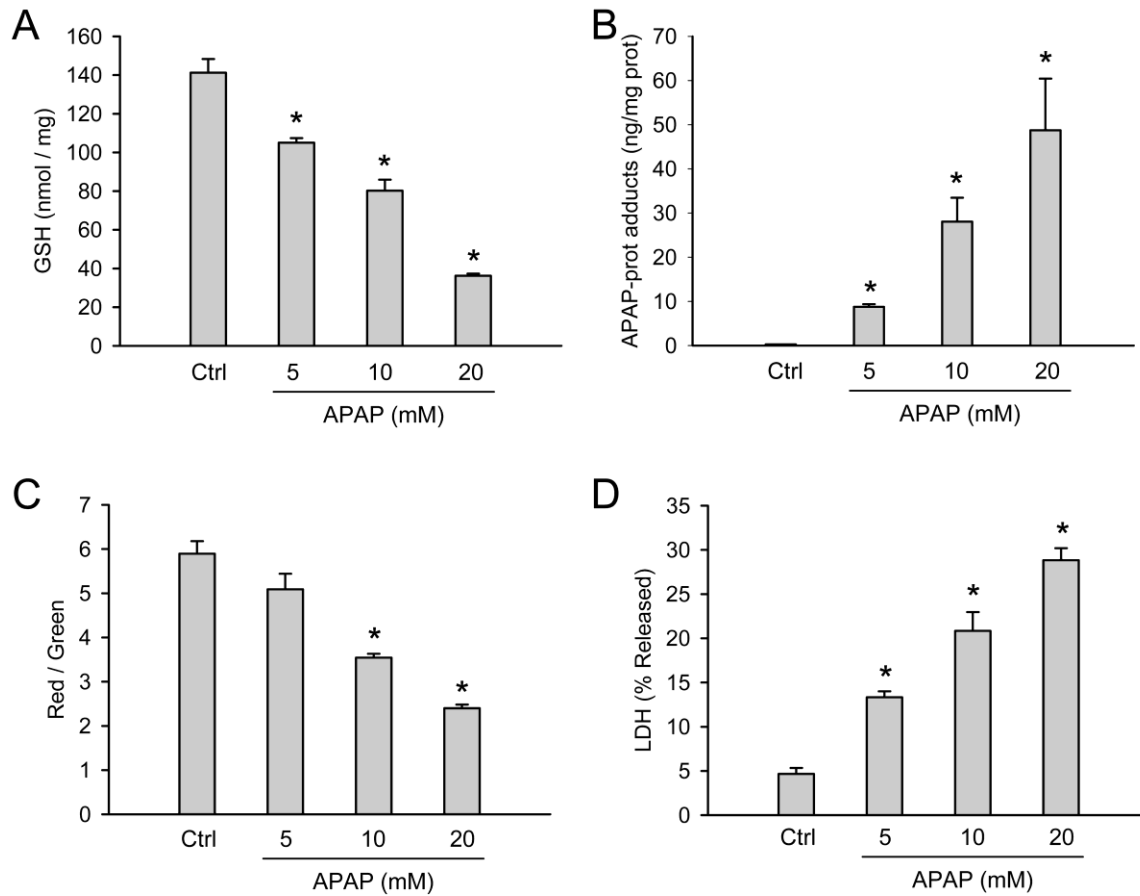


Figure 3.3.2. Dose-response for APAP toxicity in HepaRG cells. Confluent cells were treated with various concentrations of APAP and GSH, APAP-protein adducts, JC-1 fluorescence, and LDH release were measured before and 24 hours after APAP treatment (0-20 mM) as described in Materials and Methods. (A) Cellular glutathione content, (B) APAP-protein adducts, (C) ratio of red to green fluorescence of the JC-1 assay, (D) percentage of total LDH activity found in culture medium. Data are expressed as mean \pm SE of 3-4 independent cell preparations. *P < 0.05 (compared to time control).

To test for mitochondrial ROS in HepaRG cells, cultures were treated with 20 mM APAP and Mitosox Red fluorescence was evaluated. It has been suggested that Mitosox Red detects mainly mitochondrial superoxide (Mukhopadhyay et al., 2007). Compared to control cells there was a clear increase in Mitosox Red fluorescence 6h after APAP (Fig. 3.3.3), which was the time point with the highest fluorescence (data not shown). Higher magnification (inserts) shows the punctate fluorescence characteristic of mitochondrial staining (Fig. 3.3.3). Merging the Mitosox Red fluorescence with phase contrast images demonstrates that the oxidant stress occurred only in hepatocytes (Fig. 3.3.3).

In rodents, RNS such as peroxynitrite are critically involved in the injury mechanism after APAP overdose (Knight et al., 2001; Knight et al., 2002). Dihydrorhodamine (DHR) fluorescence can serve as a marker of peroxynitrite in biological systems (Crow, 1997). The compound is taken up into cells, where it reacts with intracellular RNS resulting in formation of fluorescent rhodamine. To investigate RNS formation in HepaRG cells, DHR fluorescence was measured at several time points during APAP exposure. Rhodamine fluorescence was greater than untreated controls at 6 and 12h post-APAP (Fig. 3.3.4). Like rodent hepatocytes (Yan et al., 2010), rhodamine fluorescence peaked in HepaRG cells (12h) after the peak of Mitosox Red fluorescence (Fig. 3.3.4).

Hepatocyte-specific injury. HepaRG cells are bipotent progenitors which differentiate into two morphologically distinct cell populations (Parent et al., 2004; Cerec et al., 2007). The hepatocyte-like cells have a characteristic granular appearance and grow in small clusters or “hepatocyte islands” (Fig. 3.3.4). Surrounding these islands are flatter,

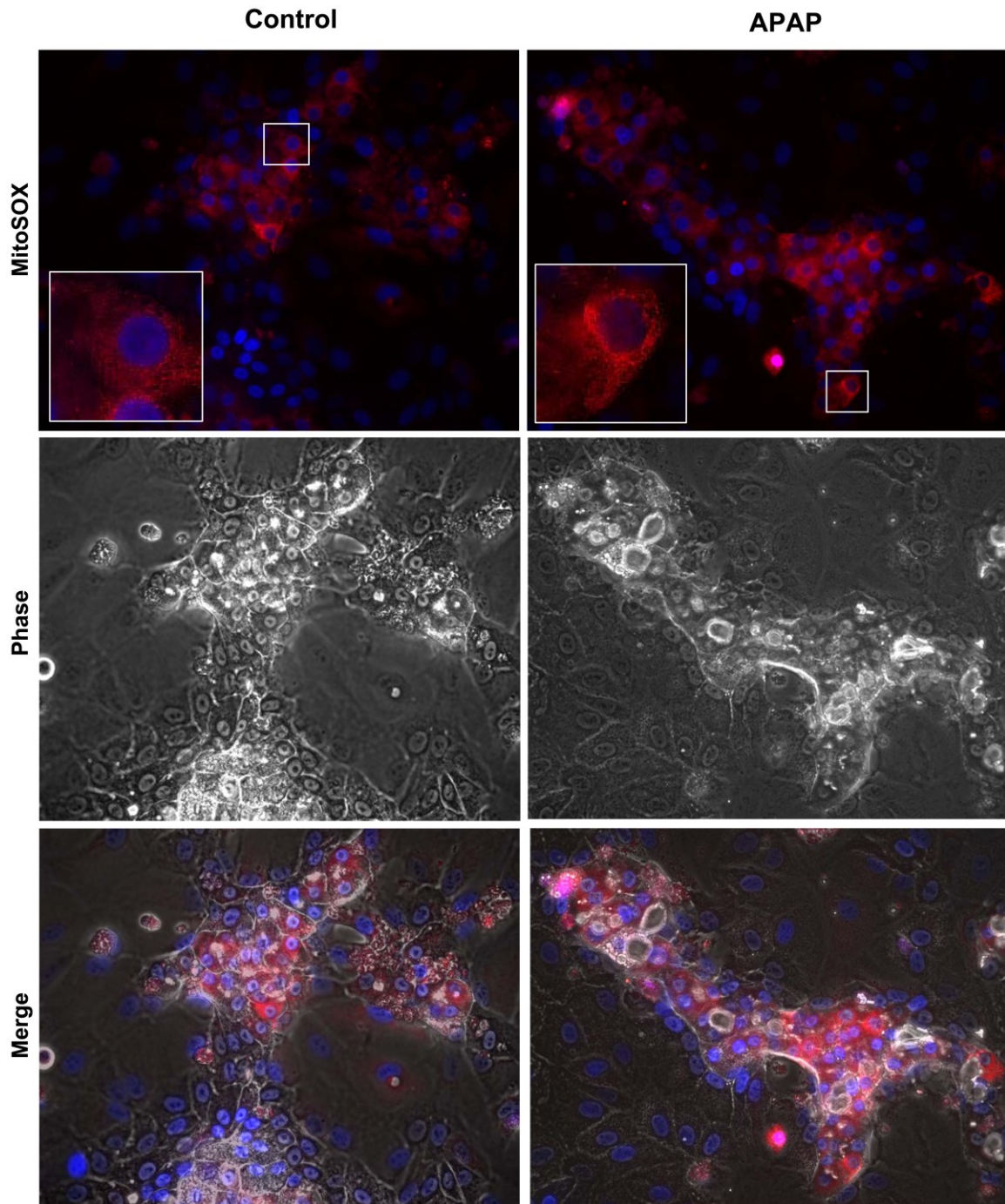


Figure 3.3.3. Mitochondrial reactive oxygen species generation after APAP exposure in HepaRG cells. Confluent HepaRG cells were treated with 20 mM APAP as described in Materials and Methods. Six hours following APAP, cells were loaded with Mitosox Red and live cell imaging carried out. The top panels show MitoSOX fluorescence in control and APAP treated cells (400x), with the boxed areas digitally magnified in the inserts to demonstrate mitochondrial localization of the signal. The bottom panel is a merge of fluorescence and phase contrast images to demonstrate that the majority of MitoSOX signal is from hepatocytes.

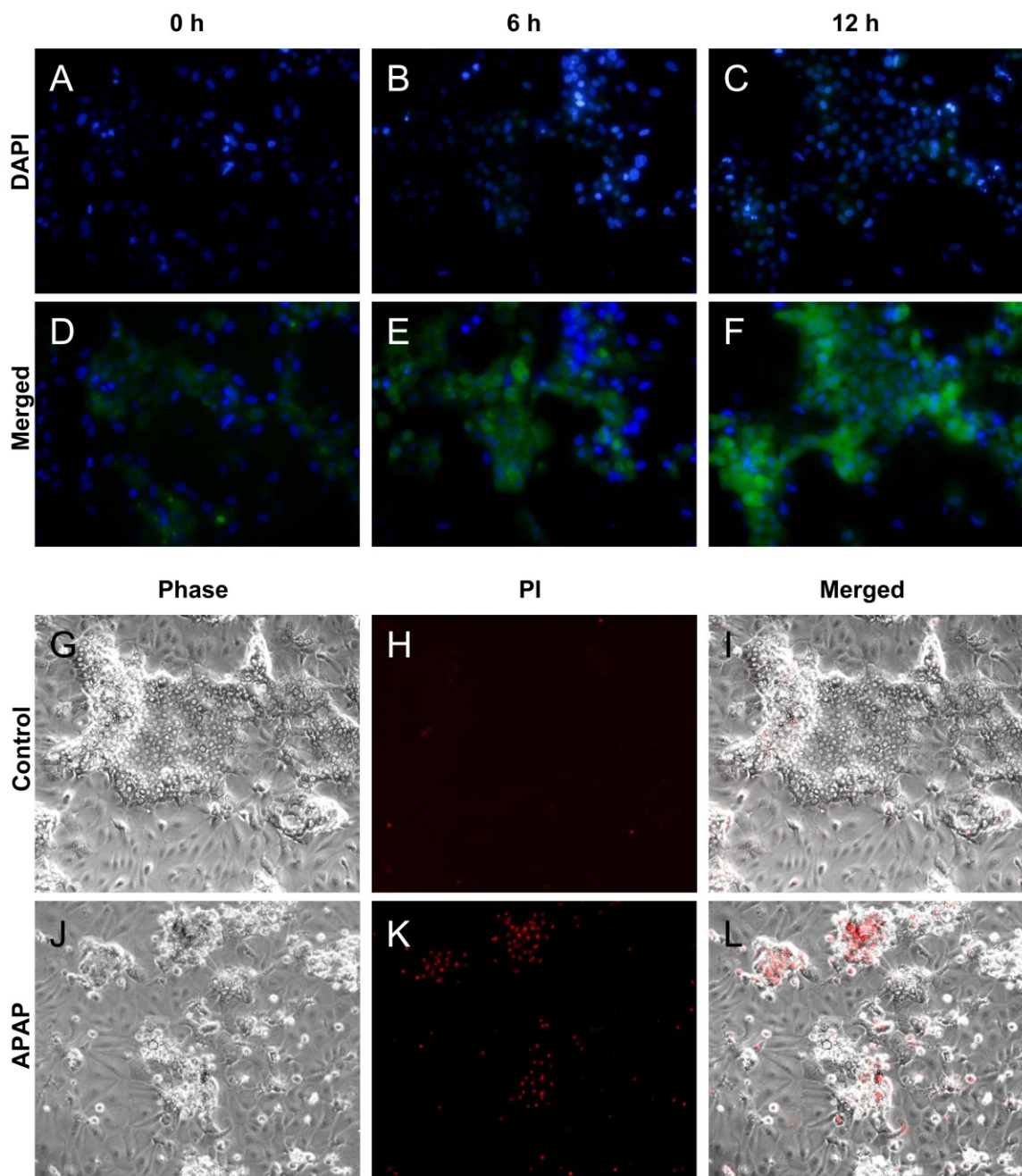


Figure 3.3.4. Reactive oxygen and reactive nitrogen species and cell type-specific death after APAP. (A-C) HepaRG cells were treated with 20 mM APAP. At various timepoints the cells were washed and loaded with 10 μ M dihydrorhodamine (DHR) and 300 nM DAPI for imaging. Merged images showing DAPI (blue) and DHR (green) fluorescence at 0, 6, and 12 hours. (D-F) HepaRG cells were cotreated with 20 mM APAP and 30 μ M propidium iodide in phenol red-free medium and imaged at 24 hours. (D) Phase contrast, (E) PI fluorescence, and (F) merged images of cells 24 hours after APAP treatment. Images A-C: 200x. Images D-F:

clearer biliary epithelial-like cells (Fig. 3.3.4). To assess the contribution of each cell type to our data showing APAP toxicity, APAP-treated cells were exposed to propidium iodide (PI), which stains nuclei of necrotic cells red (Fig. 3.3.4). At 24h, the majority of the PI staining was seen in the hepatocyte-like cells, with very little among the biliary-like cells (Fig. 3.3.4). The distribution was similar at 48h (data not shown). This suggests that APAP mainly affects the hepatocytes. Together, these data indicate that - similar to rodent hepatocytes - cell death in human HepaRG cells is preceded by GSH depletion, protein binding, formation of reactive oxygen and peroxynitrite and mitochondrial dysfunction.

Nuclear DNA fragmentation. In mice, mitochondrial damage leads to release of endonucleases and nuclear DNA fragmentation after treatment with toxic doses of APAP. To determine whether or not this might happen in HepaRG cells, we performed TUNEL labeling after 24 h of treatment with 20 mM APAP (Fig. 3.3.5). We observed a clear increase in TUNEL staining in the APAP-treated cells.

Comparison with other hepatoma lines. To compare HepaRG cells to other hepatoma cell lines, APAP toxicity was evaluated in HepG2 cells. HepG2 cells treated with 20 mM APAP for 24h showed no evidence of GSH depletion, mitochondrial dysfunction (JC-1 assay) or cell injury (LDH release) in response to the toxic dose of APAP (Table 3.3.1). However, low levels of protein adducts were identified despite the absence of toxicity (Table 3.3.1). Thus, the near absence of drug-metabolizing enzymes drastically reduced the metabolic activation of APAP and prevented any toxicity in HepG2 cells.

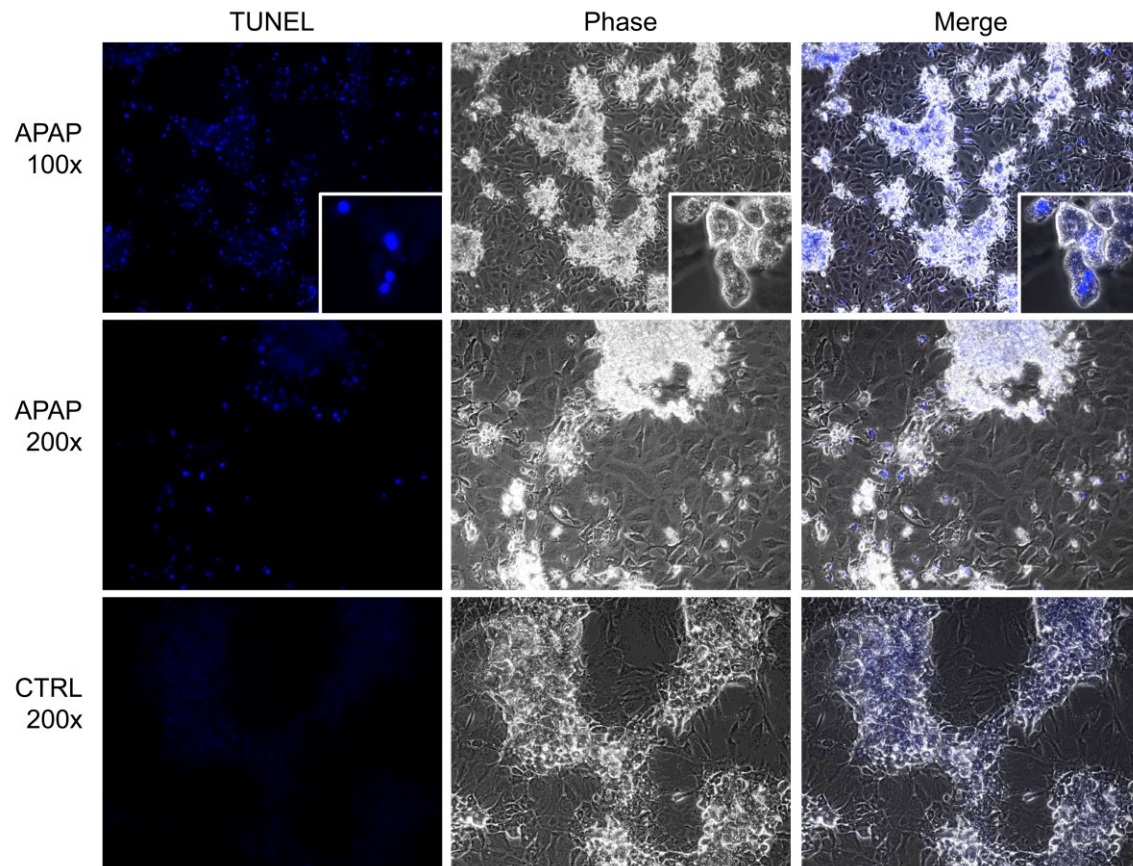


Figure 3.3.5. DNA Fragmentation in HepaRG Cells after APAP Treatment. HepaRG cells were grown and differentiated on glass bottom dishes and treated with 20 mM APAP. After 24 h, the cells were fixed and fluorescent TUNEL labeling was performed. Top row insets: 400x.

Table 3.3.1: APAP Toxicity in HepG2 Cells

Treatment	GSH + GSSG (nmol/mg protein)	JC-1 (red/green ratio)	LDH Released (% of total)	APAP-protein Adducts (nmol / mg prot)
Control	98.5 ± 12.0	4.38 ± 1.0	5.9 ± 2.0	ND
20 mM APAP	90.8 ± 11.0	4.43 ± 0.1	10.3 ± 3.0	3.9 ± 0.8

HepG2 cells were grown to 90% confluence and treated with 20 mM APAP for 24 h. Total glutathione (GSH + GSSG) content, mitochondrial function (JC-1 assay), and LDH release were measured as described in the methods section. Values represent mean ± SE (n = 3 experiments).

Mode of cell death. Loss of mitochondrial membrane integrity can result in release of pro-apoptotic proteins, including the caspase activator cytochrome c, into the cytosol. To determine whether or not APAP toxicity in HepaRG cells involves apoptosis, caspase-3 activity was measured in lysates of cells treated for 24h with APAP. There was no significant increase in caspase activity over control with 20 (Fig. 3.3.6A), 5 or 10 mM APAP (data not shown). In addition, the potent pan-caspase inhibitor Z-VD-fmk had no effect on APAP-induced LDH release at 24h (Fig. 3.3.6B), suggesting that APAP did not cause apoptosis in HepaRG cells. In contrast, caspase activity was significantly increased when cells were exposed to 100 ng/mL human rTNF- α and 5 mM galactosamine for 16.5h as positive control (Fig. 3.3.6A). The caspase inhibitor prevented the increase in caspase activity. This indicates that HepaRG cells do have the capacity to undergo apoptotic cell death in response to an appropriate stimulus.

3.4 DISCUSSION

HepaRG cells as a tool to study hepatotoxicity. The objective of this study was to assess the value of HepaRG cells as a model to investigate mechanisms of APAP toxicity in a human system and to determine if certain key features of toxicity observed in rodents apply to this cell line. Our data suggest that APAP treatment leads to GSH depletion, protein adduct formation, mitochondrial dysfunction and oxidant stress and eventually oncotic necrosis in HepaRG cells, similar to what has been observed in primary mouse hepatocytes but not in typical human hepatoma cells. The basis for this

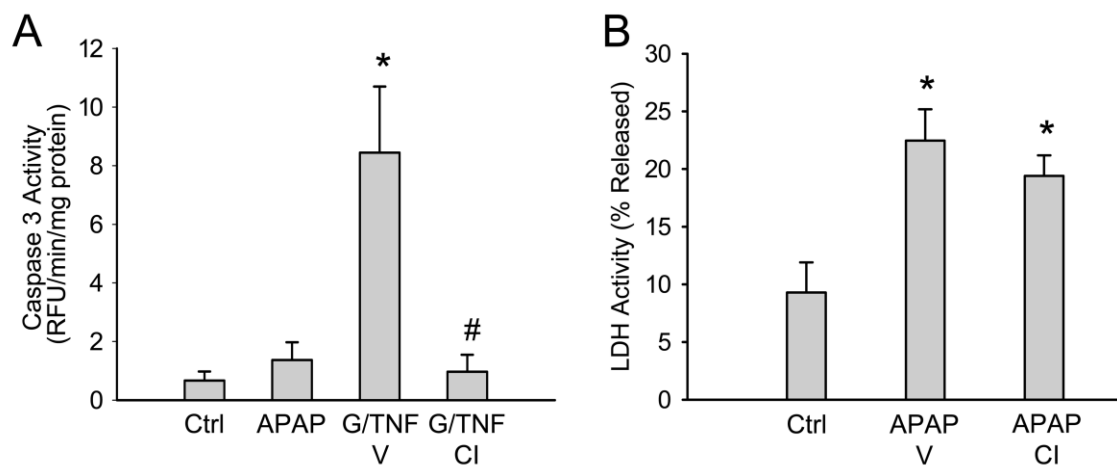


Figure 3.3.6. Effect of a pan-caspase inhibitor on APAP toxicity in HepaRG cells. (A) Caspase-3 activity was measured in cell lysates of untreated cells or 24 hours after APAP treatment (20 mM). As positive control, cells were treated with galactosamine and recombinant human TNF for 16.5 hours in the presence or absence of vehicle (Tris-buffer) or the pan-caspase inhibitor (CI) Z-VD-fmk (20 μ M). (B) LDH release in untreated cells or 24 hours after APAP treatment. Some cells were pretreated for 1 hour with vehicle or CI before addition of 20 mM APAP. Data are expressed as mean \pm SE of 4 independent cell preparations. *P < 0.05 (compared to Ctrl). #P < 0.05 (compared to APAP or G/TNF).

behavior is that HepaRG cells are capable of differentiating into two subpopulations: one with hepatocyte-like morphology and gene expression and one with the appearance of biliary epithelial cells (Gripon et al., 2002; Cerec et al., 2007). The hepatocyte-like cells express a nearly complete complement of drug-metabolizing enzymes, including most of the cytochrome P450 enzymes (Aninat et al., 2006; Guillouzo et al., 2007). HepaRG cells also possess many other characteristics unique to adult differentiated hepatocytes, including hepatocyte-specific transporter expression (Le Vee et al., 2006; Guillouzo et al., 2007), iron-loading capacity (Troadec et al., 2006), and inducibility of CYPs and other proteins (Le Vee et al., 2006; Antherieu et al., 2010). Thus, these cells have the potential to be a useful tool to study mechanisms of drug hepatotoxicity in a human system. The distinct advantage of the HepaRG cell line over primary human hepatocytes is the unlimited availability of identical cells. Nevertheless, they are hepatoma-derived and there is the possibility that certain intracellular signaling mechanisms might be different. It is therefore important to study mechanisms of cell death induced by known hepatotoxicants in these cells.

Mechanisms of acetaminophen-induced liver injury. Acetaminophen hepatotoxicity in rodents depends on the formation of the reactive metabolite NAPQI, which can be detoxified by glutathione. However, after depletion of GSH in the cell, NAPQI binds to cellular proteins, which is considered the initiating event in the toxicity (Nelson, 1990; Jaeschke et al., 2003). Our experiments with HepaRG cells identified depletion of cellular GSH and the formation of protein adducts as the earliest detectable events. This is consistent with mouse studies of APAP hepatotoxicity (Muldrew et al., 2002; Knight et

al., 2001). Evidence for increased GSH turnover and detection of APAP-protein adducts in human plasma after APAP exposure suggests that these events also occur in humans (Lauterburg et al., 1987; Davern et al., 2006). Although our data agree with the general hypothesis of reactive metabolite formation, GSH depletion and protein adduct formation as early response to APAP exposure, the sequence of events is not as previously assumed. Our data clearly show that protein adduct formation occurs parallel to GSH consumption and does not require extensive GSH depletion. In fact, protein adducts were detected in HepaRG cells and in HepG2 cells before significant effects on GSH levels and well before any evidence of mitochondrial dysfunction and cell death. This suggests that not just any protein binding can initiate toxicity and that probably a certain level needs to be reached to trigger the early mitochondrial effects.

More recently, mitochondrial dysfunction and the MPT have emerged as central to the mechanism of APAP-induced cell death in cultured rodent hepatocytes (Kon et al., 2004; Bajt et al., 2004; Hanawa et al., 2008) and in vivo (Jaeschke, 1990; Knight et al., 2001; Cover et al., 2005). Consistent with this, APAP triggered mitochondrial dysfunction in HepaRG cells (indicated by JC-1 fluorescence) before cell death (LDH release). In addition, mitochondrial oxidant stress with peroxynitrite formation is a hallmark of the mechanism of APAP-induced injury in rodents (Jaeschke, 1990; Knight et al., 2002; Cover et al., 2005). and is critically involved in the MPT pore opening and cell death (Ramachandran et al., 2010). Similar evidence for mitochondrial oxidant stress (MitoSox Red) and peroxynitrite (DHR) was detected in the HepaRG cells before massive mitochondrial dysfunction and cell death. Although the specificity of

fluorescence dyes is sometimes questioned, DHR can be directly oxidized by peroxynitrite but not by reactive oxygen without a catalyst (Wardman et al., 2007) and DHR fluorescence has been used as an indicator for peroxynitrite in cell culture (Crow, 1997). Consistent with these findings, we showed the correlation between nitrotyrosine protein adducts and DHR fluorescence as indicators for peroxynitrite formation in mouse hepatocytes (Yan et al., 2010). Thus, the mechanisms of APAP-induced cell death in human HepaRG cells is similar to rodent hepatocytes, involving reactive metabolite formation with GSH depletion, protein adduct formation, mitochondrial oxidant stress and peroxynitrite formation, loss of the mitochondrial membrane potential (MPT), and nuclear DNA fragmentation before cell death (LDH release, PI uptake). Interestingly, however, the time course of cell death resembles more closely what is observed in humans. The discussed events appear to occur almost exclusively in the hepatocyte-like cells as markers of oxidant stress and cell death (PI staining) were only observed in hepatocytes but not in the biliary epithelial-like cells. The fact that none of the events (except very minor protein adduct formation) including cell death are observed in HepG2 cells, which lack relevant P450 activity, indicates that HepaRG cells are a suitable human model to study drug hepatotoxicity that is dependent on metabolic activation.

A limitation of HepaRG cells as with other cultured cells is the absence of nonparenchymal cells. Although the majority of experimental evidence argues against direct cytotoxicity of Kupffer cells, infiltrating neutrophils and macrophages in this model, cytokines derived from nonparenchymal cells may modulate the intracellular signaling

mechanisms and this limitation needs to be kept in mind when extrapolating these data to the in vivo situation (Adams et al., 2010).

Mode of APAP-induced cell death. It is generally accepted that the mode of cell death in APAP hepatotoxicity in primary mouse hepatocytes and in vivo is oncotic necrosis (Gujral, et al., 2002; Kon et al., 2004). Our findings in HepaRG cells indicate that there is no significant caspase activation and a potent pancaspase inhibitor did not prevent APAP-induced cell injury. In addition, loss of cell viability correlated with PI uptake and LDH release, both of which are indicators of necrotic cell death. In contrast, exposure of HepaRG cells to galactosamine/hTNF- α , a well-established system of TNF-mediated apoptosis, induced substantial caspase activation, which was inhibited by the caspase inhibitor. This demonstrated that HepaRG cells have the capability to undergo apoptosis when appropriately stimulated. However, APAP exposure was not able to induce caspases and apoptotic cell death in these cells. The absence of apoptosis in human HepaRG cells is consistent with a case report on APAP overdose where no markers of apoptosis were detectable in plasma (Bechmann et al., 2008).

Summary and conclusions. Our data indicate that APAP overdose causes necrotic cell death in HepaRG cells. The hepatocyte-like cells but not the biliary epithelial cell-like cells are primarily affected. The sequence of cellular events include GSH depletion, APAP-protein adduct formation, oxidant stress and peroxynitrite generation, loss of the mitochondrial membrane potential and ultimately necrotic cell death. Thus, these mechanisms of APAP-induced cell death are the same as were reported for mouse

hepatocytes and mouse liver in vivo. In addition, APAP-induced cell death in HepaRG cells follows a time course similar to that in humans and all intracellular events are also consistent with the limited mechanistic observations in humans. Therefore, we conclude that HepaRG cells are a reliable and useful model to study mechanisms of APAP hepatotoxicity and possibly other drugs in a human system.

4. EVIDENCE THAT THE MECHANISM OF ACETAMINOPHEN HEPATOTOXICITY IN MICE AND HUMANS INVOLVES MITOCHONDRIAL DAMAGE AND NUCLEAR DNA FRAGMENTATION

Portions of this section are adapted from McGill et al. (2012), *J Clin Invest*, 122, 1574-83 with permission from the publisher, and from McGill et al. (2013), in preparation.

4.1 INTRODUCTION

Acetaminophen (APAP) is one of the best selling analgesics in the U.S. (Kaufman et al., 2002; Wilcox et al., 2005; Blazer et al., 2009) and one of the most popular drugs in the world. Although it is safe at therapeutic doses, overdose of APAP can cause severe liver injury. The first reports of APAP hepatotoxicity in humans appeared in the literature in the 1960's (Davidson and Eastham, 1966). Since that time, APAP overdose has become the most common cause of acute liver failure in most Western countries (Bernal et al., 2003; Gow et al., 2004; Larson et al., 2005; Wei et al., 2007). In the U.S. alone, misuse of APAP is responsible for 50,000 – 70,000 emergency room visits, 26,000 hospitalizations, and nearly 500 deaths each year (Larson et al., 2005; Nourjah et al., 2006; Budnitz et al., 2011). Approximately half of these cases are the result of intentional overdosing, with higher proportions in other countries. These patients typically present to the emergency department at the start or near the peak of injury, with rapidly increasing liver enzymes in plasma.

Over the last four decades considerable progress has been made in rodent models toward understanding the mechanisms of APAP hepatotoxicity. The majority of a therapeutic dose (>90%) of APAP is glucuronidated or sulfated and then excreted. A small percentage is metabolized by cytochrome P450 enzymes (CYP) to the reactive intermediate N-acetyl-p-benzoquinone imine (NAPQI), which is readily detoxified by conjugation with glutathione (GSH) (Nelson, 1990). From rodent studies we know that much higher doses saturate the glucuronidation and sulfation pathways, resulting in

formation of excess NAPQI. The additional reactive metabolite depletes liver GSH and binds to proteins (Mitchell et al., 1973a; 1973b; Jollow et al., 1973; Potter et al., 1973). Ultrastructural and biochemical studies demonstrated that toxic doses of APAP could cause changes in the morphology and function of liver mitochondria (Placke et al., 1987; Meyers et al., 1988). While it was clear from these data that mitochondrial injury occurs after treatment with large doses of APAP, it was not known how this developed. Important insight came from two sources. First, a comparison of APAP and the non-hepatotoxic regioisomer 3'-hydroxyacetanilide, which can also bind to proteins, revealed that APAP binds more frequently to mitochondrial proteins (Tirmenstein and Nelson, 1989). Second, it was found that APAP treatment could cause mitochondrial oxidative stress in mice (Jaeschke, 1990). This suggested that NAPQI binding to mitochondrial proteins leads to mitochondrial oxidative stress. It is now known that this causes the mitochondrial membrane permeability transition (MPT) pore opening (Kon et al., 2004; Masubuchi et al., 2005; Ramachandran et al., 2011a), matrix swelling, and outer membrane lysis in rodent models (Placke et al., 1987). The permeabilization and lysis result in the release of apoptosis-inducing factor (AIF) and endonuclease G (EndoG) from mitochondria. These endonucleases translocate to nuclei and cause nuclear DNA fragmentation (Bajt et al., 2006). Proapoptotic proteins, including cytochrome c and Smac/DIABLO, are also released. However, there is no activation of caspases and caspase inhibitors do not protect against APAP toxicity (Gujral et al., 2002; Jaeschke et al., 2006; McGill et al., 2011). Most likely, ATP depletion prevents activation of the

effector caspase-3 by the apoptosome. The end result is centrilobular hepatocyte necrosis and liver failure (Davidson and Eastham, 1966).

In contrast to rodents, understanding of the mechanisms of APAP hepatotoxicity in humans is limited. There is evidence for increased plasma GSH turnover after APAP treatment, suggesting a stress on the hepatic GSH pool (Lauterburg and Mitchell, 1987; Slattery et al., 1987). In addition, APAP protein adducts are present in plasma after APAP overdose (Muldrew et al., 2002; Davern et al., 2006). These data indicate that metabolic activation and protein adduct formation occur in humans in a manner similar to what has been described in rodents. However, the later events, including mitochondrial dysfunction, DNA fragmentation, and mode of cell death have not been investigated in the clinic. Although studies using the metabolically competent human liver cell line HepaRG provided evidence that the mechanisms of injury in human hepatocytes are similar to rodent models (McGill et al., 2011), cell lines may not reflect *in vivo* mechanisms.

Currently, the only effective therapy for APAP overdose is GSH replacement in order to scavenge NAPQI, which is accomplished with N-acetyl-L-cysteine (NAC) and other sulfhydryl donors. However, NAC needs to be given within 12-24 hours of APAP ingestion. Patients presenting later may benefit from increased metabolic flux (Saito et al., 2010b), but the likelihood of a positive outcome is notably decreased (Smilkstein et al., 1988). A better understanding of the later events in human APAP toxicity may lead to the development of more effective interventions for late-presenting patients.

Therefore the purpose of this study was to gain further insight into the mechanisms of APAP-induced liver injury in humans by investigating whether or not mitochondrial injury and nuclear DNA fragmentation occur as part of the mechanism of toxicity. In addition, we sought direct evidence for caspase activation in overdose patients as there are somewhat conflicting data regarding the relevance of caspase-dependent apoptosis in the pathophysiology based on the caspase-dependent cleavage of cytokeratin-18 (Rutherford et al., 2007; Volkmann et al., 2008). We hypothesized that, if mitochondrial injury and nuclear DNA fragmentation occur, mitochondrial contents and DNA fragments lost into the cytosol as a result of the MPT, mitochondrial lysis, and DNA fragmentation should be detectable in plasma when the cells become necrotic. Furthermore, if relevant apoptotic cell death should occur, active fragments of caspase-3 and increased caspase-3 enzyme activity should be detectable in plasma of APAP overdose patients. To further explore the role of mitochondrial dysfunction in APAP-induced liver injury, we also measured acylcarnitines in serum from mice treated with APAP and in plasma from APAP overdose patients.

4.2 MATERIALS AND METHODS

Patient selection and consent. Patients admitted to the University of Kansas Hospital in Kansas City, Kansas or to the Banner Good Samaritan Medical Center in Phoenix, Arizona following APAP overdose were studied prospectively. The study design and protocol were approved by the respective institutional review board (IRB), with regular

follow-up assessment. Patients presented to the emergency department or were admitted to the intensive care unit with evidence of APAP overdose. The diagnosis was made by a physician on site and all participants were required to sign a consent form. The patient population included both acute and chronic overdose patients. The diagnostic criteria were patient-reported history of APAP overdose, high serum APAP levels, and characteristic signs of liver injury including elevated liver enzymes. All of the participants fulfilled at least two of these criteria. All overdose patients received standard of care NAC treatment. For each individual, age and sex were recorded. Serum APAP, alanine aminotransferase (ALT), aspartate aminotransferase (AST), prothrombin time (PT), and total bilirubin levels in serum were monitored. Healthy volunteers were recruited from among workers (physicians and nurses) in the Medical Intensive Care Unit at the University of Kansas Hospital.

Animal Studies. Male C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and bred for a few generations in an animal facility at the University of Kansas Medical Center. The animals were housed in an environmentally controlled room with 12 h light/dark cycle and *ad libitum* access to food and water. All experimental protocols were approved by the Institutional Care and Use Committee. For acetaminophen treatment, some mice were fasted overnight before receiving an i.p. injection of 300 mg/kg APAP in warm saline. Another group of animals were treated i.p. with 400 mg/kg furosemide dissolved in warm phosphate-buffered saline, pH 7.5 – 8.0. All chemicals were purchased from the Sigma Chemical Co. (St. Louis, MO). At the indicated times, animals were killed by cervical dislocation. After blood collection, serum

was obtained by centrifugation. Livers were excised and small sections were fixed in 10% phosphate-buffered formalin for histological analysis.

Clinical assays. ALT levels in serum were measured in-hospital and later confirmed in our laboratory using an ALT kit (Pointe Scientific, Canton, MI). AST, coagulation parameters, and bilirubin were also measured on site using standard protocols.

Glutamate dehydrogenase activity. GDH activity was determined using a modified version of the method of Passonneau and Lowry (Passoneau and Lowry, 1993). Briefly, aliquots of 10-100 μ L plasma were mixed in 700 μ L of 200 mM imidazole buffer with 25 mM ammonium acetate, 200 μ M NADH, 100 μ M ADP, and 0.05% bovine serum albumin, pH 8.0. The disappearance of NADH was monitored at 340 nm to obtain a baseline reading, then 50 μ L of a 2 mM α -ketoglutarate solution were added to begin the GDH reaction. The baseline activity was then subtracted from the GDH activity.

DNA fragmentation. DNA fragmentation was measured using an anti-histone ELISA with an anti-DNA secondary antibody, according to the manufacturer's instruction (Roche, Indianapolis, IN). For the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay, sections of liver were stained with the In Situ Cell Death Detection Kit, AP (Roche Diagnostics, Indianapolis, IN) as described in the manufacturer's instructions

Caspase activation and Keratin 18. Procaspase-3 processing was assessed by western blotting with a human and mouse cross-reactive caspase-3 antibody (Cell Signaling, Danvers, MA). Caspase activity was measured as z-VAD-fmk inhibitable cleavage of

the caspase-3 substrate Ac-DEVD-AMC, as described (Jaeschke et al., 1998). Caspase cleaved and total K18 were determined using the M65 and M30 (apoptosense) ELISA kits according to the manufacturer's instructions and as previously described (Cummings et al., 2007).

Mitochondrial DNA. Mitochondrial DNA (mtDNA) was measured by absolute quantification real-time PCR, as described (Zhang et al., 2010a; 2010b). Total DNA was isolated from plasma samples using a QIAamp Blood and Mini Kit (Qiagen, Valencia, CA). Mitochondrial DNA was determined using two pairs of human primers, one for human NADH dehydrogenase (forward 5'-ATACCCATGGCCAACCTCCT-3' and reverse 5'-GGGCCTTTGCGTAGTTGTAT-3') and one for human cytochrome c oxidase subunit III (forward 5'-ATGACCCACCAATCACATGC-3' and reverse 5'-ATCACATGGCTAGGCCGGAG-3'). For mice, another two primer pairs were designed: mouse NADH dehydrogenase subunit 6 (forward 5'-CACACAAACATAACCACTTTAAC-3' and reverse 5'-GTAGGTCAATGAATGAGTGGTT-3') and mouse cytochrome c oxidase subunit III (5'-ACCAAGGCCACCACACTCCT-3' and 5'-ACGCTCAGAAGAATCCTGCAAAGAA-3'). To construct standard curves, mitochondrial pellets were isolated from either human hepatoma cells (HepaRG) or mouse liver by differential centrifugation. Purity of mitochondrial DNA standards was verified by real-time PCR using primers for both mitochondrial genes and nuclear-encoded β -actin. Serial dilutions of these purified mtDNA samples were prepared and standards were included on each PCR plate for each gene tested. The limit of detection for the assay was <0.01 ng/mL.

Acylcarnitines. Acylcarnitines were measured in serum or plasma after cold methanol precipitation of proteins. Briefly, 40 μ L of serum was mixed with 80 μ L of methanol, vortexed, and centrifuged at 16,000 g for 10 min. The supernatants were subjected to UPLC-QTOF mass spectrometry for analysis in both positive and negative mode. The data were collected and processed using MassLynx and MarkerLynx.

Statistical methods. To test for statistical significance, one-way analysis of variance was performed to compare the human data from volunteers, patients without serious injury, and patients with serious liver injury. Unpaired two-tailed Student's t-test was used to assess significance between values from control and experimental animals within treated groups. To test for a correlation between peak ALT and peak mtDNA in patients, Pearson's correlation coefficient was calculated. For all tests, $p < 0.05$ was considered significant.

4.3 RESULTS

Study design and patient information. Samples from forty patients were used in this study: twenty patients with maximum ALT $\geq 1,000$ U/L and maximum prothrombin time (PT) ≥ 18 s (abnormal liver test group) and twenty with maximum ALT $< 1,000$ U/L and maximum prothrombin time < 18 s (defined as the normal LT group), the latter group having no or minimal hepatic injury. Blood samples were obtained from six volunteers as healthy controls. For the abnormal LT cohort, the mean age was 38 years, with a range from 19 to 65 (Table 4.3.1). The percentage of male and female patients in the

Table 4.3.1

Parameter	Abnormal LT group* (n = 20)				Normal LT group# (n = 20)				Healthy volunteers (n = 6)		
	Range	Mean	Parameter	%	Range	Mean	Parameter	%	Range	Mean	Sex (% F)
Age	19 – 65	38			18 – 56	29			23 – 51	36	
Admission APAP (µg/mL) ^A	0 – 136	40	Sex (F)	65	11 – 582	162	Sex (F)	70	ND	ND	66
Peak ALT (U/L) ^B	1,137 – 8,545	5,330			14 – 158	54			14 – 31	25	
Peak AST (U/L) ^B	486 – 26,000	8,527			5 – 300	64			4 – 48	27	
Peak PT ^B	18.7 – 374	59.2	Survived	95	13.1 – 17.5	15.3	Survived	100	ND	ND	
Peak Bilirubin (mg/dL) ^B	0.9 – 25	7.5			0.2 – 1.9	0.9			ND	ND	
Peak Creatinine (mg/dL) ^B	0.6 – 7.8	2.5			0.4 – 2.1	0.9			ND	ND	
Encephalopathy Grade ≥1				6/20				2/20			

*Peak ALT ≥ 1,000 U/L and peak PT ≥ 18 s. #Peak ALT < 1,000 U/L and peak PT < 18 s. ^AWhen available. ^BPeak value achieved during hospital stay.

PT = prothrombin time. ND = Not Determined.

cohorts with normal and abnormal LTs was similar (70% females vs. 65% females, respectively). The average peak ALT value for the group with elevated liver test values was $5,330 \pm 562$ U/L. Among these twenty patients, one did not survive. The remaining patients survived without liver transplant. For the normal LT cohort, the mean age was 29 years with a range from 18 to 56. Survival in this group was 100%. The healthy volunteer group was matched to these two cohorts, 66% females with a mean age of 36 and a range of 23 to 51 (Table 4.3.1). Interestingly, average serum APAP levels were significantly higher than in the normal LT group. This is likely because many of the patients in this group presented earlier, before extensive APAP metabolism. Earlier admission and treatment would explain why these patients did not develop signs of liver injury.

Glutamate dehydrogenase activity in plasma from overdose patients. We reasoned that, if mitochondrial injury occurs in humans as it does in rodents, mitochondrial contents released into the cytosol after the MPT and mitochondrial membrane lysis should become detectable in the plasma of the injured patients after cells become necrotic. With this in mind, we measured the activity of the mitochondrial enzyme glutamate dehydrogenase (GDH) in the plasma obtained from each cohort. The glutamate dehydrogenases are enzymes of the mitochondrial matrix which are highly expressed in the liver (Racine-Samson et al., 1996). These enzymes catalyze the conversion of 2-oxoglutarate to L-glutamate using NADH and NH_4^+ . We observed clear increases in GDH activity over time in patients with abnormal LTs (Fig. 4.3.1A-C). The average GDH activities were 22 ± 7 , 10 ± 3 , and 552 ± 113 U/L for healthy volunteers,

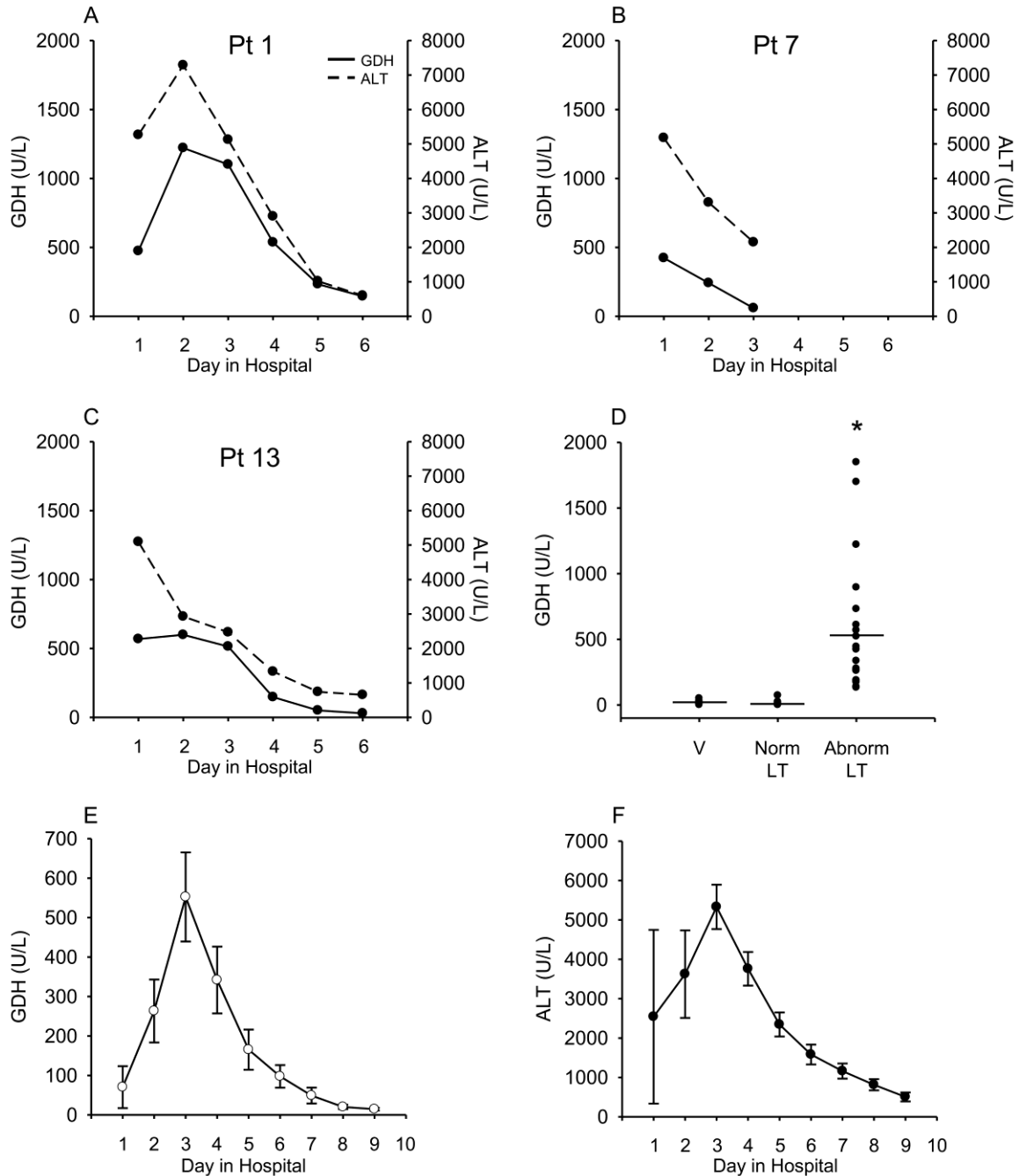


Figure 4.3.1. GDH activity in plasma from APAPoverdose patients. GDH activity was measured in the plasma from APAP-overdose patients throughout their time in the hospital. (A–C). Time course data from 3 representative patients showing both GDH and ALT activity. (D) Plasma GDH activity at the time of peak ALT in healthy volunteers (V), normal LT group (Norm LT), and abnormal LT group (Abnorm LT). (E) Average plasma GDH activity over time for the patients with abnormal LT results. (F) Average plasma ALT activity over time for patients with abnormal LT results. Data are expressed as mean \pm SEM for $n = 20$. * $P < 0.05$ compared with healthy volunteers.

the normal LT group, and the abnormal LT group, respectively (Fig. 4.3.1D). Additionally, the average increase in GDH activity in patients with abnormal LTs correlated with the rise in ALT values (Pearson's coefficient = 0.45, $p < 0.05$) (Fig. 4.3.1E-F). Although the average PT values in patients with liver injury (high ALT) were almost 4-times higher than in patients with no relevant ALT increase, there was no significant correlation between peak GDH activity and PT values in the abnormal LT group (data not shown).

Mitochondrial DNA in plasma from overdose patients. Mitochondrial DNA (mtDNA) has been shown to be released into circulation after hemorrhagic shock in rats (Zhang et al., 2010a) and other physical trauma in humans (Zhang et al., 2010b). In addition to serving as another marker of mitochondrial injury, mtDNA appears to act as a damage-associated molecular pattern (DAMP) capable of activating neutrophils and other cells of the innate immune system via toll-like receptors (TLRs) (Hemmi et al., 2000; Chuang et al., 2002; Zhang et al., 2010a; 2010b). To determine whether or not APAP hepatotoxicity leads to release of mtDNA into circulation in humans, mtDNA concentrations were determined in the plasma of these patients using absolute quantification real-time PCR. Primers were designed for subunits of complex I (NADH dehydrogenase) and complex III (cytochrome c oxidase) of the electron transport chain which are exclusively encoded in mtDNA (Wallace, 1992). The concentration of mtDNA in the plasma of overdose patients with abnormal liver function increased over time (Fig. 4.3.2A-B) and peak levels correlated with peak ALT (Pearson's coefficient = 0.54, $p < 0.02$) (Fig. 4.3.2C). While mtDNA was nearly undetectable in plasma from healthy

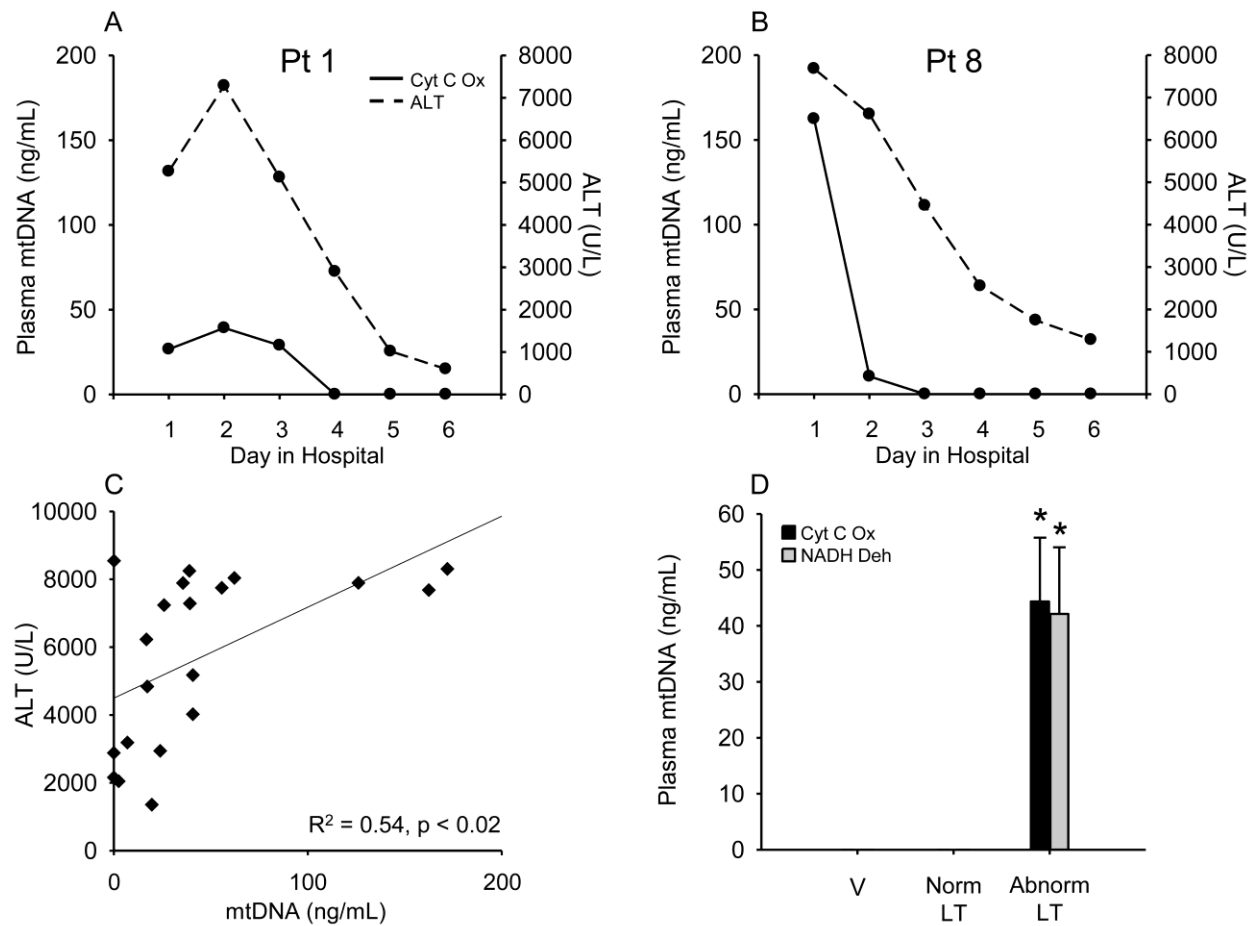


Figure 4.3.2. mtDNA in plasma from APAP-overdose patients. The concentration of mtDNA was determined in the plasma of patients after APAP overdose. Concentrations were measured by absolute quantification real-time PCR using primers for subunits of complex I (NADH deh) and complex IV (Cyt c ox) of the electron transport chain exclusively encoded in mtDNA. (A and B) Time course data from 2 representative patients (Pt) showing both mtDNA concentration (Cyt c ox) and ALT activity in plasma. (C) Scatterplot of peak plasma ALT activity against peak mtDNA (Cyt c ox) concentration in plasma from patients of the abnormal LT group. Pearson's correlation coefficient is shown. (D) Average mtDNA concentrations in plasma from healthy volunteers (n = 6), normal LT group (n = 20), and abnormal LT group (n = 20), expressed as mean \pm SEM. *P < 0.05 compared with healthy volunteers.

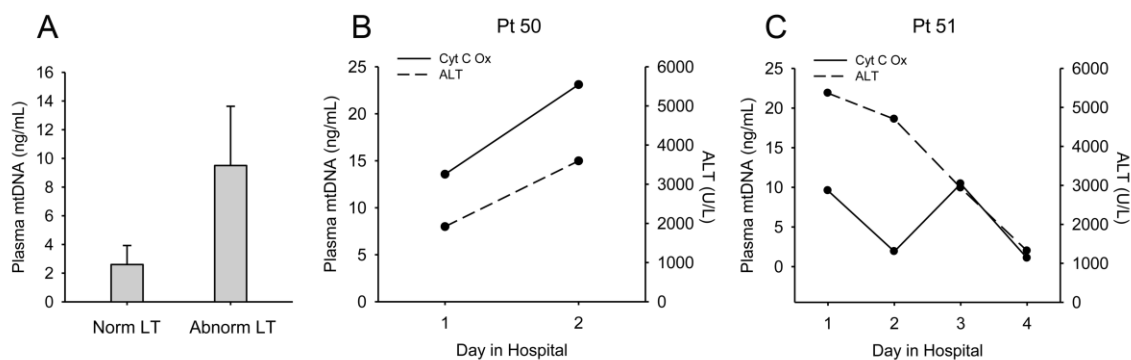


Figure 4.3.3. mtDNA in plasma from APAP-overdose patients. The concentration of mtDNA was determined in the plasma of patients after APAP overdose as before. The anticoagulant used was EDTA. (A) mean \pm SEM of $n = 3-4$ per group. (B-C) Time courses of plasma ALT and mtDNA from representative patients.

volunteers and the normal LT group, the average peak mtDNA concentration was 45 ± 15 ng/mL in plasma from patients with injury (Fig. 4.3.2D). Again, despite the correlation with ALT, there was no significant correlation between mtDNA and PT values in the group with the abnormal LT (data not shown). In these experiments we used plasma anticoagulated with heparin. Although it is claimed the the DNA isolation method that we used prevents any interference from compounds with the potential to affect PCR measurements, which we later found includes the anticoagulant heparin (Qiagen representative Ajinder Pal Kaur, personal communication, 2011), we ran additional samples using EDTA plasma from APAP overdose patients to ensure that this was not an issue. While the much smaller EDTA sample population available at the time of the experiment yielded less impressive results, the overall patterns in the data remained the same (Fig. 4.3.3).

Nuclear DNA fragmentation in plasma from overdose patients. Nuclear DNA fragmentation has been demonstrated in mice following treatment with toxic doses of APAP and is important in the mechanism of hepatotoxicity in those animals (Bajt et al., 2006; Ray et al., 1990). Moreover, because the endonucleases responsible for nuclear DNA fragmentation come from the mitochondrial intermembrane space during mitochondrial dysfunction (Bajt et al., 2006), nuclear DNA fragmentation can be considered an additional indirect marker of mitochondrial dysfunction. We observed an increase in nuclear DNA fragments in plasma over time in the patients of the abnormal LT group using an anti-histone ELISA with an anti-DNA secondary antibody (Fig. 4.3.4). Again, this closely followed ALT activity (Fig. 4.3.4A-C). Together with the GDH and

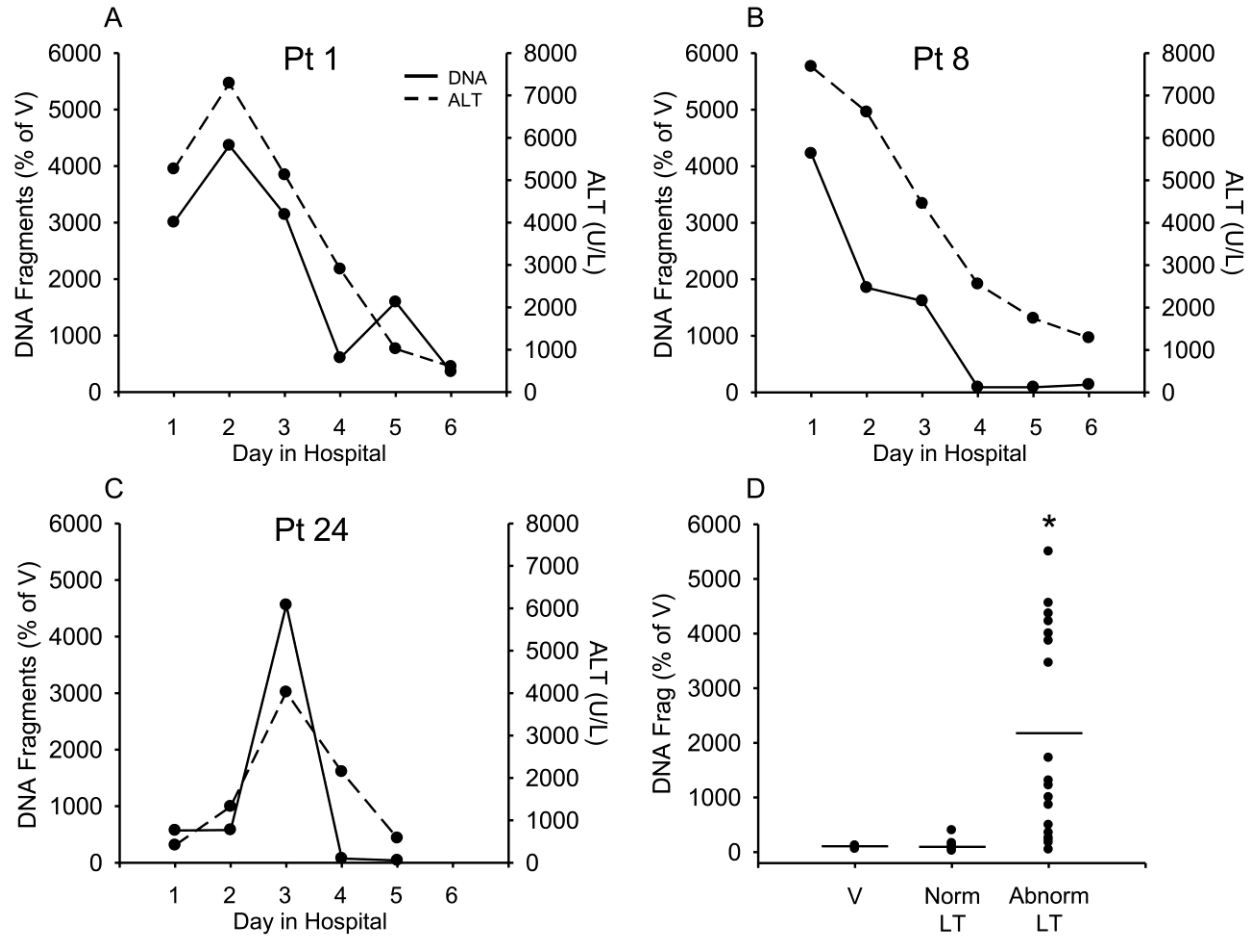


Figure 4.3.4. Nuclear DNA fragments in plasma from APAP-overdose patients. Nuclear DNA fragments were measured by ELISA in plasma from patients after APAP overdose as an indirect marker of mitochondrial injury. (A–C) Time course data from 3 representative patients showing both DNA fragments (expressed as percentage of the average change in OD/min with plasma from healthy volunteers) and ALT. (D) Average DNA fragments, expressed as percentage of the average change in OD/min with plasma from healthy volunteers, for healthy volunteers ($n = 6$), for the normal LT group ($n = 20$), and the abnormal LT group ($n = 20$). Data are expressed as mean \pm SEM. * $P < 0.05$ compared with healthy volunteers.

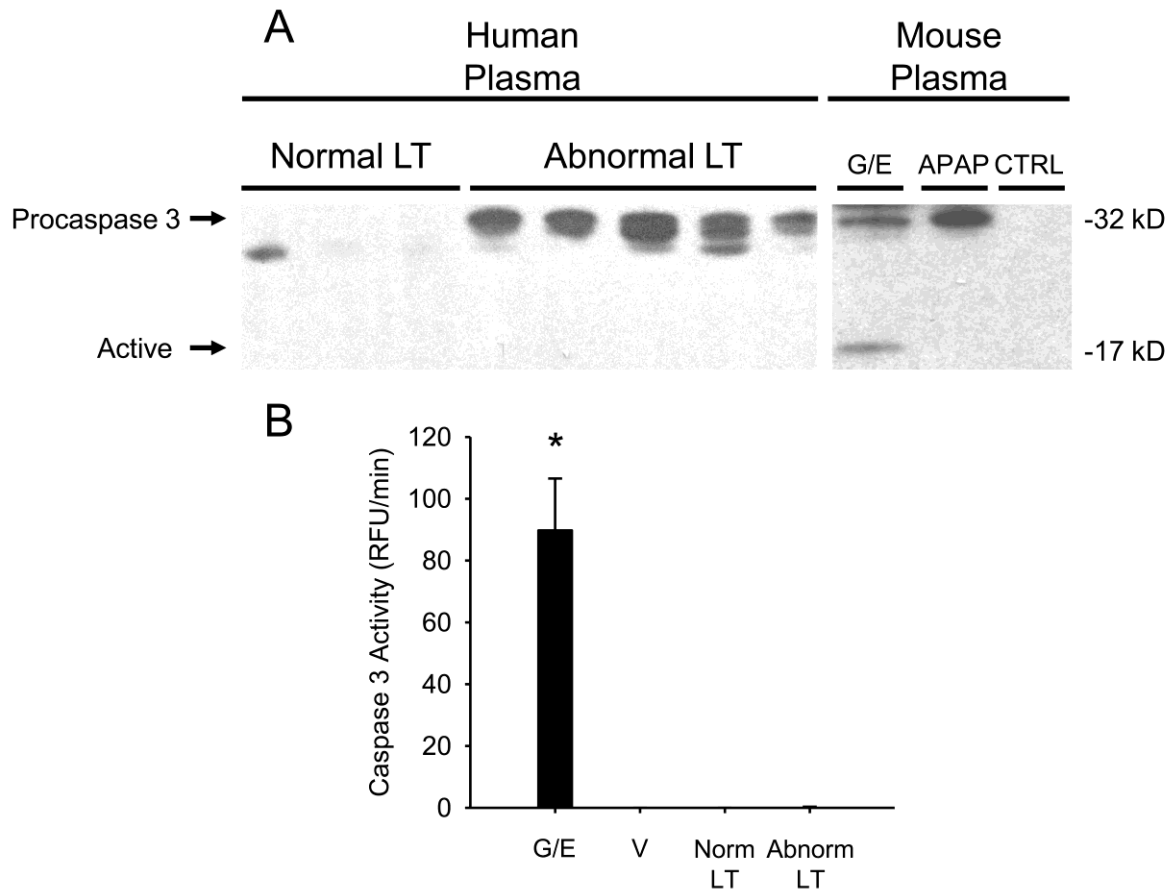


Figure 4.3.5. Caspase-3 activation is not detectable in plasma from APAPoverdose patients. (A) Caspase-3 processing was assessed by immunoblot in plasma from patients or from mice treated with either APAP or G/E or from control mice. The human and mouse samples were run on different gels but probed with the same antibody. Full-length (32 kDa) pro-caspase-3 was detectable in samples from the abnormal LT patient group, as well as from mice treated with APAP or G/E. Cleaved active caspase-3 fragment (17 kDa) was detectable only in plasma from the positive control G/E-treated mice. (B) Average caspase-3 activity in plasma from G/E-treated mice (n = 3), healthy volunteers (n = 6), normal LT group (n = 20), and abnormal LT group (n = 20) based on cleavage of the caspase-3 substrate Ac-DEVD-AMC. Expressed as mean \pm SEM. *P < 0.05 compared with healthy volunteers.

mtDNA data, these results provide evidence for release of mitochondrial contents and nuclear DNA fragments into the cytosol with subsequent release into peripheral blood after cell death.

Lack of caspase activation in APAP overdose patients. It is generally believed that the primary mode of cell death in APAP hepatotoxicity in rodents is oncotic necrosis (Jaeschke et al., 2004). To determine whether or not apoptosis could be involved in the pathophysiology in humans, caspase-3 activity and caspase-3 protein levels were measured in plasma. In the normal LT group, no caspase-3 activity or caspase-3 protein was detectable (Fig. 4.3.5A,B). In patients with APAP-induced liver injury, pro-caspase-3 was clearly present in plasma (Fig. 4.3.5A), but neither the active, low-molecular weight fragments nor an increase in enzyme activity were detectable (Fig. 4.3.5A,B). Similar to these findings in patients, no caspase-3 protein was observed in plasma of control mice. APAP-induced liver injury resulted in the selective appearance of the proenzyme in plasma (Fig. 4.3.5A), but again no low molecular weight forms could be seen. In contrast, mice treated with galactosamine and endotoxin (G/E) as a positive control for apoptosis not only had detectable procaspase-3 in plasma, but also an active fragment (Fig. 4.3.5A). Consistent with this observation, a substantial caspase-3 enzyme activity was present (Fig. 4.3.5B). Thus, if there is relevant apoptotic cell death in the liver, as after G/E treatment (Jaeschke et al., 1998), both caspase-3 enzyme activity and active caspase-3 fragments are found in plasma. The absence of these parameters in human and mouse plasma after APAP overdose suggests that apoptosis is not a relevant mechanism of cell death. To further explore this, we engaged in a

collaboration with a team at the University of Liverpool to measure full-length (necrotic) and caspase-cleaved (apoptotic) keratin 18 (K18) in the plasma from several of our patients and from patients in the United Kingdom (Antoine et al., 2012). Although apoptotic K18 was detectable in samples from some of the subjects, in most cases the fraction of total K18 in the caspase-cleaved form was very low. Moreover, in samples from several patients the apoptotic K18 was not at all detectable. Overall, the average percentage of K18 in the apoptotic form for all of the patients with abnormal liver test results was only about 15% (Antoine et al., 2012). Together, these data strongly suggest that apoptosis is not a relevant mode of cell death during APAP hepatotoxicity in humans.

Release of mitochondrial markers is not a secondary effect of cell necrosis. It is possible that mitochondria and mitochondrial contents will be released into blood and become detectable in plasma as a result of oncotic necrosis. To verify that high levels of GDH and mtDNA in plasma reflect the mechanism of APAP hepatotoxicity and are not merely a secondary effect of oncotic cell death, these parameters were measured in a model of liver necrosis not thought to involve mitochondria. Furosemide is a loop diuretic prescribed for cases of hypertension and congestive heart failure. At high doses, furosemide can cause liver injury without affecting mitochondrial function (Wong et al., 2000). Mice treated with this drug had a significant increase in ALT activity in serum at 24 hours (Fig. 4.3.6A) with centrilobular necrosis similar to APAP-induced injury (Fig. 4.3.7). Although there was a minor increase in GDH activity over controls at this time point, the difference was not statistically significant (Fig. 4.3.6A). Similarly, serum

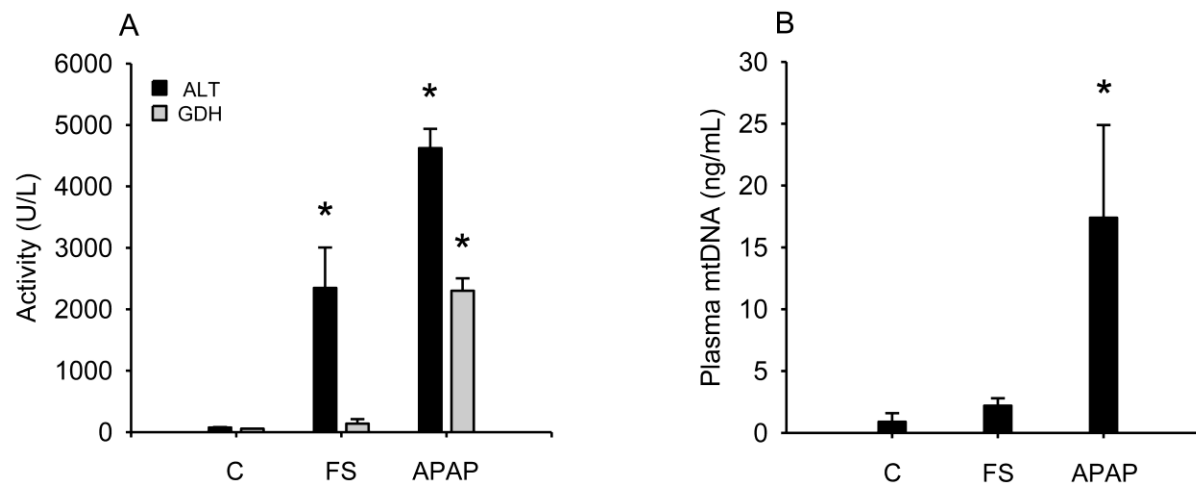


Figure 4.3.6. Release of GDH and mtDNA into blood is not a secondary effect of necrosis. Mice were treated with APAP or furosemide (FS) to cause liver necrosis, and mitochondrial markers were measured in plasma from these animals near the peak of injury for each model (24 hours for FS and 12 hours for APAP). (A) Average GDH and ALT activity. (B) Average mtDNA concentration (Cyt c ox). Data are expressed as mean \pm SEM for n = 3–6. *P < 0.05 versus control.

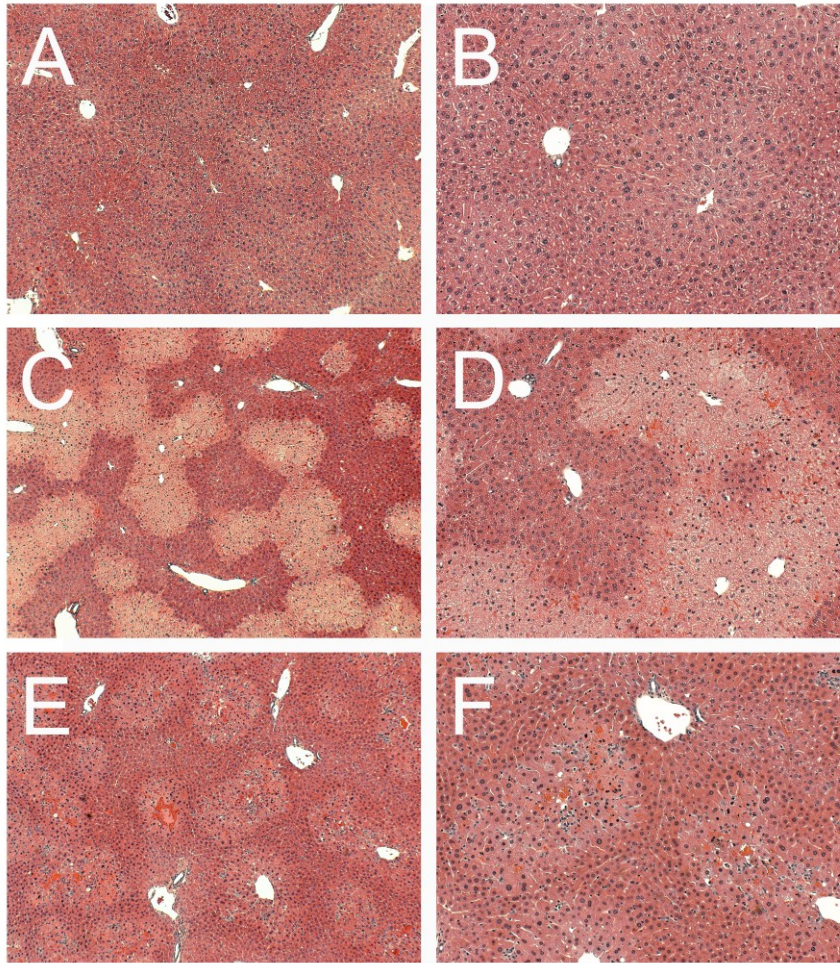


Figure 4.3.7. APAP and furosemide both cause centrilobular necrosis. Mice were treated with 300 mg/kg APAP or 400 mg/kg furosemide to cause liver necrosis, and liver sections were stained with H&E near the peak of injury for each model (24 hours for FS and 12 hours for APAP). (A and B) Representative images of livers from untreated control mice or (C and D) 12 hours after 300 mg/kg APAP or (E and F) 24 hours after 400 mg/kg FS. Lighter staining necrotic areas were only visible in the perivenous regions after both treatments. Magnification: x50 (A, C, and E); x100 (B, D, and F).

mtDNA concentrations were only slightly elevated compared to the extensive increase in APAP-treated mice (Fig. 4.3.6B). These data indicate that high levels of plasma GDH and mtDNA are indicative of the mechanism of APAP toxicity and are not simply a result of tissue necrosis.

Plasma biomarkers and tissue correlation. Because no liver biopsy samples can be obtained from patients during the early phase of liver injury after APAP overdose, we have to rely on plasma biomarkers. To test how well plasma biomarkers reflect events in the tissue, mice were treated with 300 mg/kg APAP. Blood and livers were collected at several time points and plasma biomarkers were assayed. Liver histology revealed a close correlation between the development of areas of necrosis in the tissue and the time course of ALT activity in plasma (Fig. 4.3.8A-B). Similarly, TUNEL staining (for DNA fragmentation) in tissue sections was followed closely by the rise in nuclear DNA fragments in mouse plasma (Fig. 4.3.8C-D). These data show that plasma biomarkers accurately reflect events in the tissue injury after APAP overdose in mice. It seems reasonable to assume that the same relationship between plasma biomarkers and tissue exists in patients.

Serum acylcarnitines in samples from mice and humans. Transfer of the acyl moiety from acyl-coenzyme A (acyl-CoA) to carnitine to form acylcarnitine is an early step in the transport of fatty acids into mitochondria for β -oxidation. Thus, mitochondrial dysfunction is likely to result in accumulation of these fatty acid-carnitine conjugates. Consistent with this, Chen et al. (2009) reported increased concentrations of some

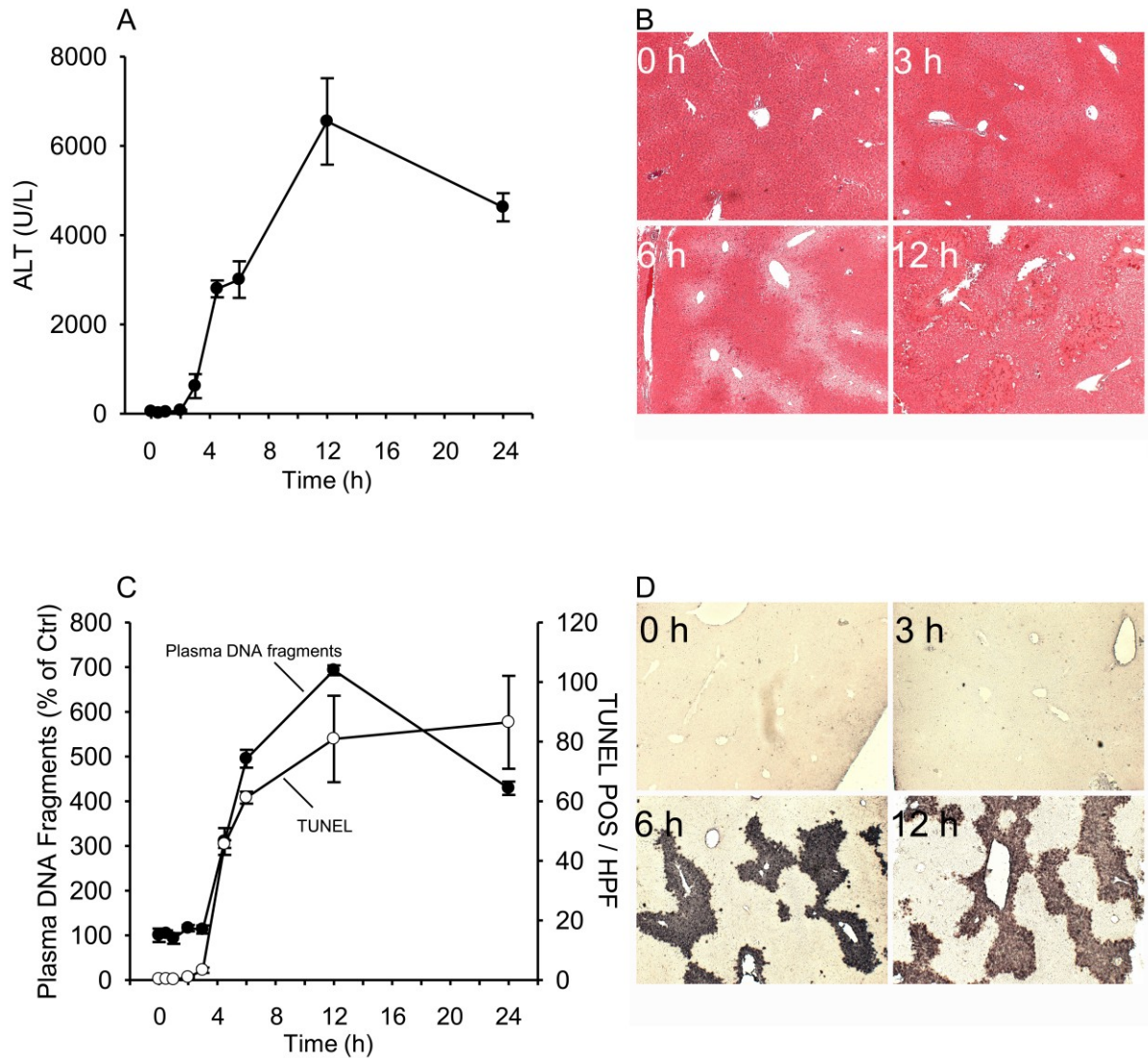


Figure 4.3.8. ALT activity and DNA fragments in plasma correlate with tissue injury. Mice were treated with 300 mg/kg APAP. At various time points, plasma was collected for measurement of ALT and DNA fragments and tissues were collected for histology. **(A)** Time course of ALT activity in plasma from APAP-treated mice. **(B)** H&E sections of liver from APAP-treated mice. Necrosis is evident as pale eosinophilic staining and loss of hepatocyte nuclei. **(C)** Time course of DNA fragments in plasma from APAP-treated mice (closed circles) and of TUNEL staining in tissue (expressed as TUNEL-positive cells per high power field [HPF]). **(D)** Representative sections from TUNEL-stained livers over time. Original magnification, 50x. Data are expressed as mean \pm SEM; $n = 6$ per time point.

acylcarnitines in serum from mice treated with APAP. We obtained similar results from both fasted and fed mice, identifying three (molecular weights 426, 424, and 400) that increased over time (Fig. 4.3.9). Importantly, serum levels of these acylcarnitines did not increase in FS-treated mice (Fig. 4.3.10). Unfortunately, we could not detect a significant increase over controls for any of the three compounds in patient plasma, neither in the first study samples taken after admission nor in those taken near the time of peak ALT (Fig. 4.3.9). However, when we measured acylcarnitines over time in samples from individual patients, we did observe a pattern of increase and decrease over time that correlated with ALT in some subjects (Fig. 4.3.11). Although serum acylcarnitines are unlikely to be clinically useful due to the variation in absolute values, the higher earlier values in some patients before or at the time of peak ALT (Fig. 4.3.11; Pts 1, 28, and 108) may support our hypothesis that mitochondrial damage occurs in humans during APAP hepatotoxicity. A possible partial explanation for the less dramatic effect of APAP overdose on plasma acylcarnitines in humans is treatment with NAC, which is known to support mitochondrial function (Saito et al., 2010b). To test this, we treated mice with NAC 1.5 h post-APAP. NAC treatment did prevent the increase in acylcarnitines in mice (Fig. 4.3.12). However, it is not clear whether this effect was due to enhanced mitochondrial function or reduced metabolic activation of APAP.

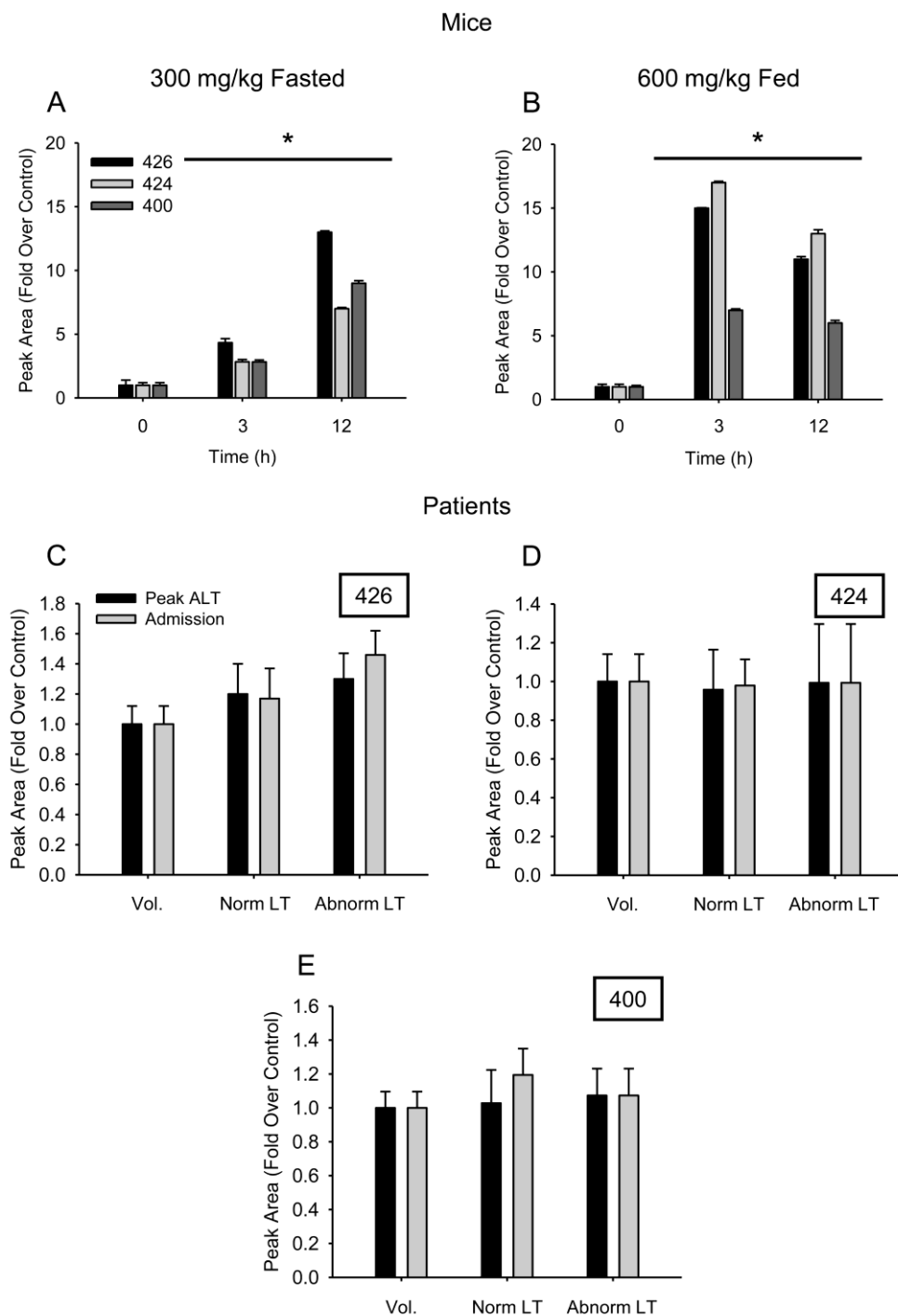


Figure 4.3.9. Serum and plasma acylcarnitines during APAP hepatotoxicity. (A and B) Mice were either fasted or fed overnight and then treated with APAP (300 or 600 mg/kg respectively). The data were normalized to control (0 h) values. (C – E) The same acylcarnitines were measured in plasma samples from APAP overdose patients and normalized to control (Vol.) values. Data are expressed as mean \pm SEM of $n = 3$ (mice and healthy volunteers) or $n = 4 - 6$ (Norm and Abnorm LT groups). * $P < 0.05$ vs. control.

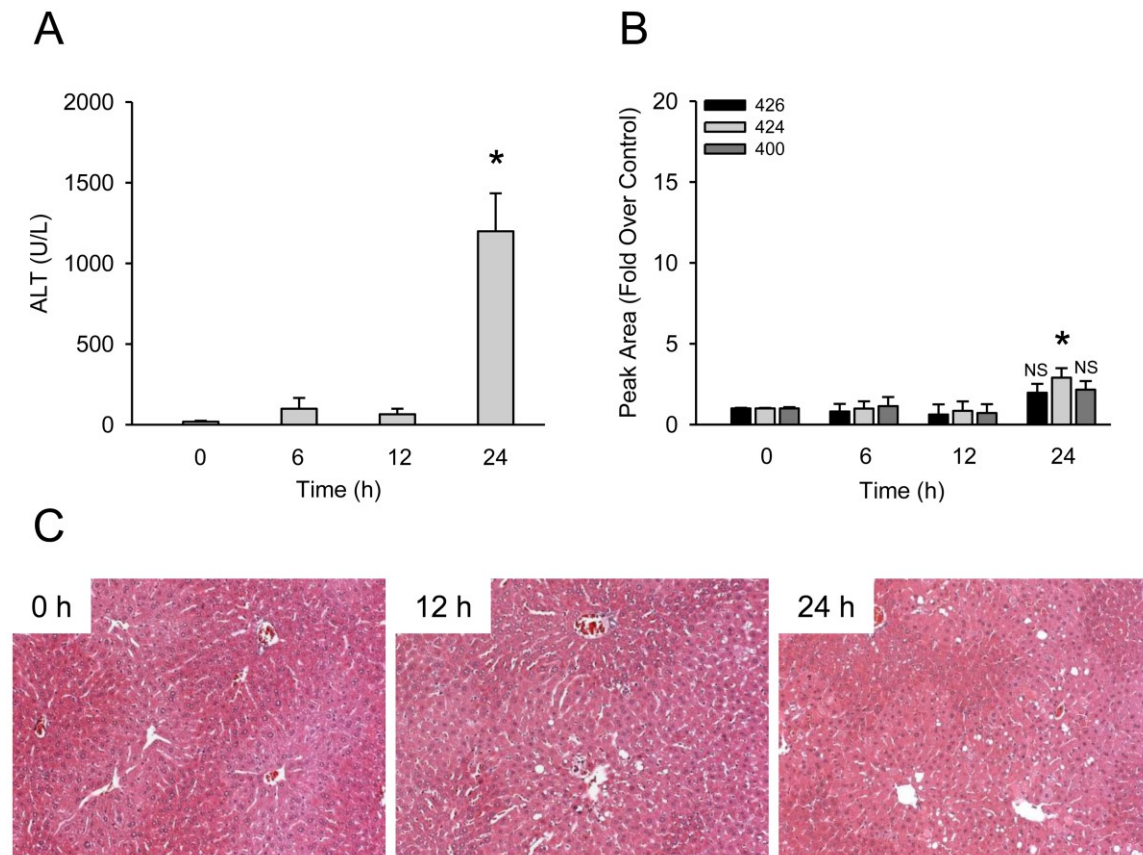


Figure 4.3.10. Serum acylcarnitines during furosemide hepatotoxicity. Fed mice were treated with 500 mg/kg furosemide. (A) ALT was measured in serum. (B) Acylcarnitines in serum, normalized to control (0 h) values. (C) Time course images of H&E-stained liver sections. Data are expressed as mean \pm SEM of $n = 3$. * $p < 0.05$ vs. 0 h.

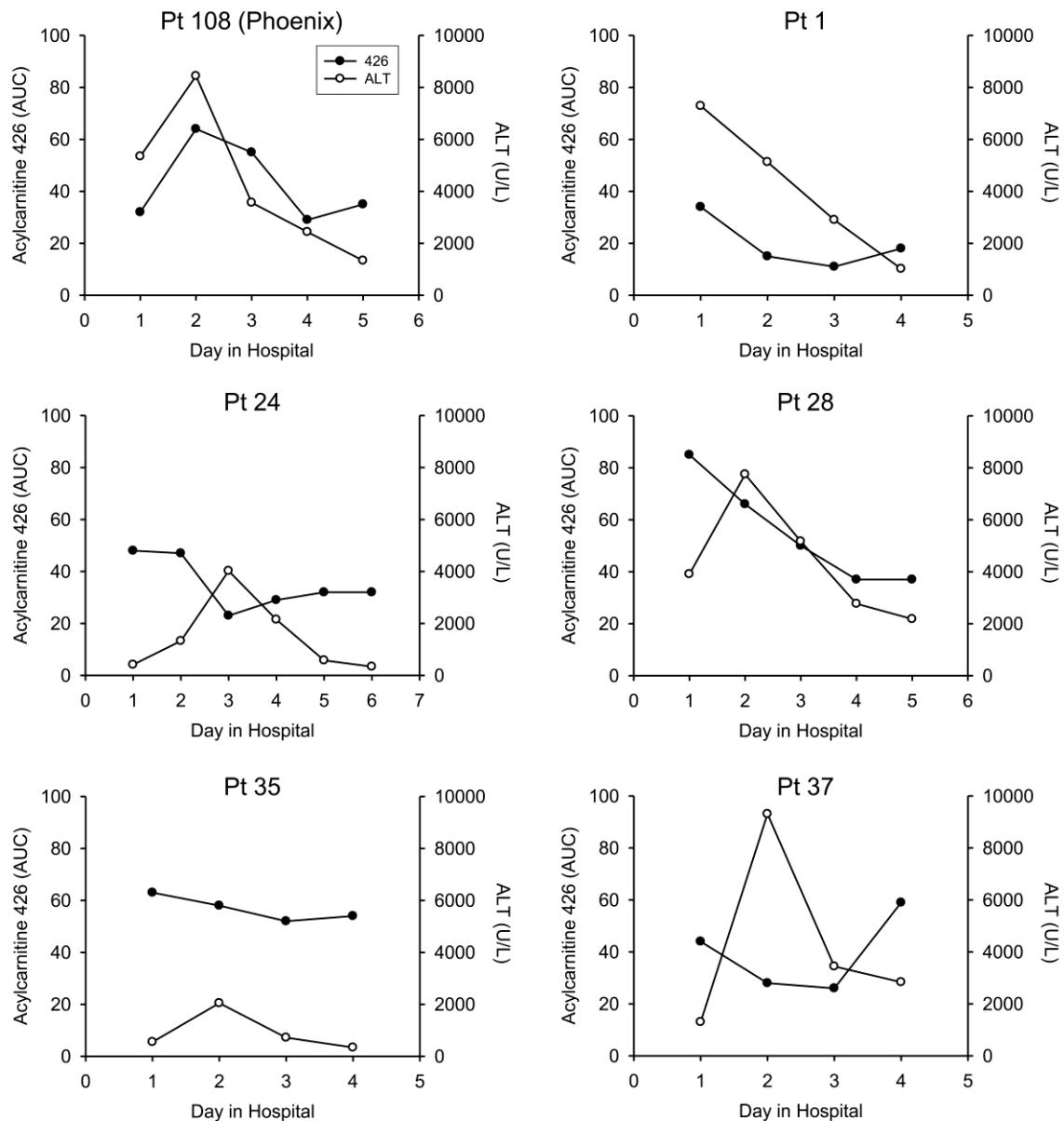


Figure 4.3.11. Plasma acylcarnitines timecourses during APAP hepatotoxicity. Individual timecourses showing ALT (open circles) and the 426 acylcarnitine (filled circles) values in plasma from APAP overdose patients with liver injury. The 424 and 400 acylcarnitines showed similar results.

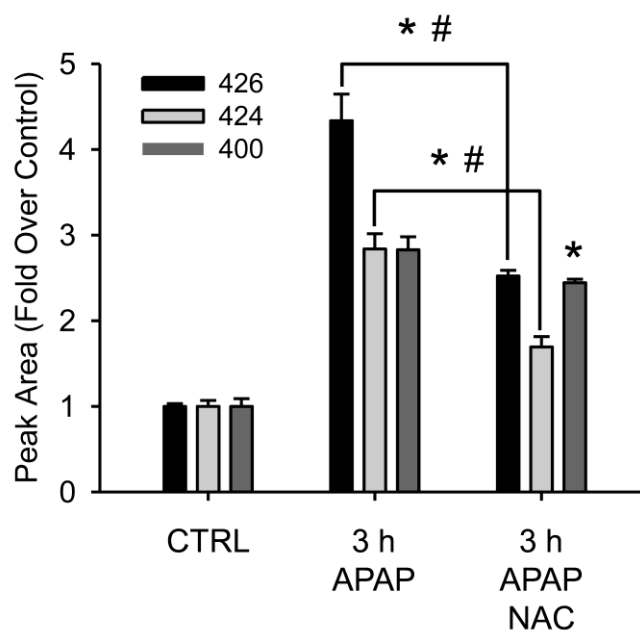


Figure 4.3.12. Plasma acylcarnitines timecourses after post-APAP NAC treatment. Mice were untreated or treated with 300 mg/kg APAP for 3 h. Some animals were treated with 140 mg/kg NAC 1.5 h post-APAP and sacrificed 1.5 h later. Data are expressed as mean \pm SEM of $n = 3$. * $P < 0.05$ vs. control. # $P < 0.05$ vs. 3 h APAP-only.

4.4 DISCUSSION

The objective of this investigation was to gain further insight into the intracellular mechanisms of APAP hepatotoxicity in humans and to assess the mode of cell death. Because there is no diagnostic benefit that would justify the risk of a liver biopsy during the early injury phase after APAP overdose, our approach was to use plasma biomarkers, validated in a mouse model, to obtain reliable mechanistic information. We studied 3 groups of patients. In addition to an age and gender-matched control group, APAP overdose patients were divided into 2 groups: one with evidence of liver injury (peak plasma ALT activities $\geq 1,000$ U/L and peak PT ≥ 18 s) and one with no or very limited liver injury (PT < 18 s and ALT $< 1,000$ U/L; highest: 17.5 s and 158 U/L, respectively). The low injury in this group was likely due, in part, to early admission and treatment, prior to development of toxicity. This would be consistent with the higher plasma APAP levels on admission in the group with normal LTs. Additionally, some of these patients may have taken lower overdoses.

Mitochondrial dysfunction and APAP hepatotoxicity. Mitochondrial dysfunction after toxic doses of APAP has been recognized in rodents since the 1980s, when inhibition of mitochondrial respiration and depletion of ATP were first described (Placke et al., 1987; Meyers et al., 1988; Andersson et al., 1990). More recent studies have shown the development of oxidative and nitrosative stress within mitochondria and occurrence of the mitochondrial membrane permeability transition after APAP treatment (Kon et al., 2004; Masubuchi et al., 2005; Ramachandran et al., 2011a). Using a human cell line

with high expression levels of cytochrome P450 enzymes (HepaRG), mitochondrial dysfunction has also been shown to occur in cultured human hepatocytes (McGill et al., 2011). We reasoned that, if mitochondrial membrane integrity is compromised and mitochondrial contents leak into the cytosol in patients, they must be released into the circulation when the hepatocytes become necrotic. Our data showing the presence of the specific mitochondrial components GDH and mtDNA in plasma of patients with highly elevated ALT levels provide evidence that mitochondrial dysfunction occurs in humans after APAP overdose. The high activity of GDH in the plasma from these patients is consistent with the higher GDH expression in zone 3 of the liver (Racine-Samson, et al., 1996), where APAP causes the greatest tissue injury. Importantly, similar results for GDH and mtDNA in plasma and serum samples from acute liver failure patients (and particularly APAP overdose patients) have been obtained by other groups, working independently, since the publication of our work reported here (Marques et al., 2012; Antoine et al., 2013).

The assumption behind these mechanistic conclusions is that GDH and mtDNA release occurs only when mitochondrial damage is involved, not just cell injury. This hypothesis was confirmed by demonstrating that liver cell damage caused by furosemide, a hepatotoxicant not believed to affect mitochondria (Wong et al., 2000), resulted in ALT release but not a statistically significant increase in either mtDNA or GDH in mouse plasma. In contrast, APAP overdose triggers release of both ALT and large amounts of GDH and mtDNA into the plasma of mice. Thus, it is justified to conclude that APAP-induced liver injury in humans may involve mitochondrial damage. The time course of

the release of GDH and mtDNA correlated well with the appearance of ALT in plasma of all patients, suggesting that mitochondrial damage may be closely related to cell necrosis. In experimental animals, interventions that restored the scavenging capacity for ROS and peroxynitrite in mitochondria (Knight et al., 2002; James et al., 2003b; Bajt et al., 2003) or prevented the MPT (Kon et al., 2004; Masubuchi et al., 2005; Ramachandran et al., 2011a) protected against APAP-induced liver injury. In addition, selective impairment of mitochondrial antioxidant defenses (partial MnSOD deficiency) aggravated APAP hepatotoxicity (Ramachandran et al., 2011 [MnSOD]). Moreover, mitochondrial dysfunction and oxidant stress preceded cell necrosis by several hours in murine hepatocytes and in human HepaRG cells (McGill et al., 2011; Bajt et al., 2004). Thus, mitochondrial damage is central to APAP-induced cell death in murine models and in a human hepatocyte cell line (Kon et al., 2004; Knight et al., 2004; Ramachandran et al., 2011a, McGill et al., 2011; Jones et al., 2010). Based on the close correlation between release of biomarkers of mitochondrial damage and cell necrosis, it is likely that mitochondrial dysfunction is a main determinant of liver cell damage in APAP overdose patients. However, despite these close correlations, our data do not establish that mitochondrial damage is the cause of cell death in humans.

Nuclear DNA damage and APAP hepatotoxicity. In addition to the biomarkers of mitochondrial damage, nuclear DNA fragments were detectable in plasma of patients with severe APAP-induced liver injury. The assay for DNA fragments is based on detection of nuclear histones, which are not present in mitochondrial DNA (Wallace, 1992). Thus, the anti-histone ELISA measures specifically nuclear DNA fragments. No

plasma DNA fragments were detectable in healthy volunteers or in patients without severe liver injury. In contrast, the time course of plasma DNA fragment levels in patients with liver injury closely followed the release of ALT, i.e. cell necrosis. A comparison of plasma DNA fragments and nuclear DNA damage in mouse liver after APAP overdose revealed that both parameters correlate with ALT release, suggesting that nuclear DNA damage occurs along with cell death.

Previous studies in rodents documented that DNA fragments after APAP are indistinguishable from apoptotic DNA fragments (Ray et al., 1990; Cover et al., 2005). In addition, no nitrotyrosine residues were detectable in the nucleus (Cover et al., 2005). This indicated that DNA damage was not caused by oxidant stress or peroxynitrite formation but involved endonucleases. Because of the lack of relevant caspase activation during APAP hepatotoxicity (Gujral et al., 2002; Lawson et al., 1999), the traditional caspase-activated DNase can be excluded. Instead, endonuclease G and apoptosis-inducing factor (AIF) are released from the mitochondrial intermembrane space and translocate to the nucleus during APAP-induced cell death (Bajt et al., 2006). Protection against mitochondrial oxidant stress or prevention of the MPT eliminated nuclear DNA fragmentation (Bajt et al., 2004; Cover et al., 2005; Ramachandran et al., 2011a). In a later study, the Bcl-2 family member Bax, which forms pores in the outer mitochondrial membrane during the early phase after APAP exposure, was found to facilitate release of endonuclease G and AIF from mitochondria and nuclear translocation, which triggered the initial DNA damage (Bajt et al., 2008). Partial AIF deficiency reduced the mitochondrial oxidant stress, nuclear translocation of AIF, and

DNA fragmentation (Bajt et al., 2011). There is also evidence that a general endonuclease inhibitor attenuated APAP-induced cell death (Shen et al., 1992). Together, these findings indicate that nuclear DNA damage is dependent on mitochondrial dysfunction and the release of endonucleases from the intermembrane space. Thus, nuclear DNA damage is closely related and even contributes to liver cell death in the murine model. Given the similar appearance of nuclear DNA in patients with APAP-induced liver injury, it is likely that the same mechanisms of DNA damage apply in human liver.

DNA as damage-associated molecular patterns (DAMPs). Our data indicate that nuclear DNA fragments and mtDNA are released into the plasma of patients and in mice after APAP overdose. These molecules can act as DAMPs through activation of toll-like receptors (TLRs), especially TLR9, to induce cytokine formation after APAP (Imaeda et al., 2009). In the mouse model, there is extensive formation of pro-inflammatory mediators and recruitment of neutrophils into the liver in response to APAP overdose (Lawson et al., 2000). However, the preponderance of the experimental evidence argues against a relevant contribution of neutrophils to the injury process (Jaeschke et al., 2012c). Consistent with these findings, neutrophils isolated from the injured liver or from the blood are not activated during the main injury phase of APAP hepatotoxicity in mice (Williams et al., 2010a). Preliminary data for neutrophil activation in APAP overdose patients appear to confirm the lack of neutrophil activation during the injury phase but show a progressive activation at later time points (Williams and Jaeschke, unpublished observations). Thus, it is likely that in patients the release of DAMPs such

as nuclear DNA fragments and mtDNA during the injury phase contributes to activation of innate immune cells, which are involved in the removal of necrotic cell debris and thus contribute to the recovery as observed in mice (Jaeschke et al., 2012c).

Mode of APAP-induced cell death in patients. It is generally accepted that the mode of APAP-induced liver cell death in mice is oncotic necrosis. This is based on morphological evidence (cell swelling, vacuolization, karyorrhexis and karyolysis), the massive release of cell contents (ALT), and the resulting inflammation (Gujral et al., 2002). However, there is limited evidence of apoptosis. Hallmarks of apoptotic cell death include several morphological features such as cell shrinkage, chromatin condensation and formation of apoptotic bodies, as well as extensive caspase activation (Jaeschke and Lemasters, 2003). In general, only very few apoptotic cells are detectable after APAP overdose (Gujral et al., 2002) and there is no relevant caspase activation in mouse livers (Lawson et al., 1999; Adams et al., 2001; Gujral et al., 2002). Furthermore, pancaspase inhibitor did not protect against APAP hepatotoxicity (Lawson et al., 1999; Gujral et al., 2002; Jaeschke et al., 2006; Antoine et al., 2009; Williams et al., 2010b; McGill et al., 2011). However, most of these experiments were done with overnight fasted animals. Recently, it was suggested that in fed mice with higher cellular ATP content, APAP causes limited caspase activation and some apoptotic cell death (Antoine et al., 2009). This conclusion was also based on the detection of a cytokeratin-18 (K18) cleavage product, which is thought to be specific for caspase-3 activity (Cummings et al., 2007; Antoine et al., 2009). Using this assay, there are conflicting data in the clinical literature. A case report showed no significant increase of the

cleavage product over the time course of injury and recovery of an APAP overdose patient (Bechmann et al., 2008). In contrast, two studies found significant increases of this caspase cleavage product in APAP overdose patients (Rutherford et al., 2007; Volkmann et al., 2008). However, as noted by Volkman, et al. (2008), necrotic full-length K18 was the dominant form, suggesting that apoptosis plays a relatively minor part in the mechanism of cell death after APAP overdose in humans. We obtained similar results for K18. Importantly, our data directly analyzing caspase-3 activity or the active fragment of caspase-3 by western blotting did not reveal any evidence for caspase-3 activation in these patients. However, in the overdose patients with extensive liver injury, pro-caspase-3 protein was present in plasma, reflecting the release of cell contents of necrotic cells. These clinical data are similar to our observations in APAP-treated mice, which had an increase in plasma pro-caspase-3 levels but no active fragments and no increase in enzyme activity. In contrast, in the galactosamine/endotoxin model, which is a positive control for caspase-dependent apoptosis in hepatocytes (Jaeschke et al., 1998), extensive caspase-3 activity and the active fragment of caspase-3 were clearly detectable. In a similar experiment, caspase-3 activity was readily detectable in plasma after galactosamine-induced apoptosis in rat liver despite the fact that only 5-6% of hepatocytes were apoptotic (Gujral et al., 2003). Based on these experiments, it can be concluded that the absence of active caspase-3 fragments and of detectable caspase-3 activity in plasma of APAP overdose patients with severe liver injury suggests that apoptotic cell death is not relevant for the overall pathophysiology. The discrepancy between the detection of minor levels of caspase-

dependent K18 cleavage product in this study and others and our lack of direct plasma caspase-3 activity measurement in APAP patients requires further study. This could be related to differences in assay sensitivity or potentially even specificity, i.e. cytokeratin-18 could be cleaved by other proteases. However, there is principle agreement between all studies that necrotic cell death is dominant in these patients.

Currently, much emphasis is placed upon the development of novel biomarkers to aid in both the diagnosis and prognosis of various liver diseases. It is possible that mitochondrial markers such as GDH and mtDNA can also predict outcome. In this study, only one patient did not survive and none received a liver transplant. With these numbers we are unable to assess the prognostic value of these biomarkers. Larger cohorts will be needed to determine whether or not these biomarkers are predictive of patient outcome.

Summary and conclusions. Our data demonstrated the release of biomarkers reflecting mitochondrial damage and nuclear DNA fragmentation in patients with severe APAP-induced liver injury. These events lead to predominantly necrotic cell death. The use of these biomarkers and the mechanistic conclusions were extensively validated by parallel studies in mice where tissue injury and the release of these markers were measured. Our findings provide strong support for the hypothesis that mitochondrial dysfunction and DNA damage are critical events in the mechanism of cell necrosis after APAP overdose in patients. In addition, these data confirm that the in vivo mouse model, primary murine hepatocytes and human HepaRG cells are appropriate

experimental systems to study cell death mechanisms that appear to be relevant for APAP overdose patients.

5. Plasma and Liver Acetaminophen-Protein Adduct Levels in Mice after Acetaminophen Treatment: Dose-Response, Mechanisms, and Clinical Implications

Portions of this section are adapted from McGill et al. (2013), *Toxicol Appl Pharmacol*, in press, with permission from the publisher.

5.1 INTRODUCTION

Acetaminophen (APAP) is one of the most commonly used drugs in the U.S. (Kaufman et al., 2002). At low doses it is a safe and effective analgesic, but high doses can cause severe liver injury. In fact, APAP overdose is the chief cause of acute liver failure throughout the West (Bernal, 2003; Gow et al., 2004; Larson et al., 2005; Canbay et al., 2009). Etiology may be a critical consideration in the treatment of patients with liver injury, particularly with regard to the need for liver transplant. APAP overdose patients are likely to recover without a new organ (Simpson et al., 2009), and it is advisable to avoid transplant whenever possible because of the lifelong immunosuppression and associated costs that follow. Moreover, diagnosis of intentional overdose can help clinicians to ensure that a patient receives proper psychiatric care, as non-fatal drug intoxication is a predictor of later suicide (Qin et al., 2009). Currently, diagnosis of APAP hepatotoxicity is made on the basis of patient-reported history and serum APAP concentration. The former can be unreliable, while the latter is limited by the short serum half-life of the parent drug. Thus, a better diagnostic marker would be clinically useful.

Forty years ago a series of critical papers established that the mechanism of APAP-induced liver injury begins with the P450-catalyzed conversion of the drug to an electrophile that can react with glutathione (GSH) and bind to proteins (Mitchell et al., 1973a, 1973b; Jollow et al., 1973; Potter et al., 1973). This reactive metabolite is generally believed to be *N*-acetyl-*p*-benzoquinone imine (NAPQI) (Dahlin et al., 1984). It

is now thought that binding to proteins, mitochondrial proteins in particular, causes oxidative stress and mitochondrial damage (Jaeschke et al., 2012a) resulting in necrotic cell death (Gujral et al., 2002). Dose-response studies comparing liver APAP-protein adducts and GSH led to the idea that depletion of approximately 70% of total liver GSH is necessary for protein binding to begin (Mitchell et al., 1973b). It was assumed then that this could only occur at toxic doses. Later experiments using antibody-based assays to measure the cysteine adduct of APAP (APAP-CYS) on proteins showed that these adducts also appear in serum, but only after toxic doses or after the onset of injury in time course studies (Pumford et al., 1990; Roberts et al., 1991). This suggested that their presence in serum was the result of liver injury and cell contents release. New, more sensitive and specific techniques have been developed to measure APAP-CYS from proteins (Muldrew et al., 2002; McGill et al., 2011) and it was recently proposed that serum protein adducts could replace serum APAP as the primary diagnostic marker of APAP overdose (Davern et al., 2006). A major advantage is the much longer half-life of these adducts, in the range of 1-2 days (James et al., 2009). While this is a promising approach, comprehensive dose-response studies have yet to be done and there is evidence that APAP-CYS is present in serum even after therapeutic doses (Heard et al., 2011). Furthermore, the effects of polypharmacy and co-morbidities on this parameter are unknown, and the mechanism by which adducts appear in serum without cell death has not been investigated.

To address these issues, we performed detailed dose-response and time course studies to explore the relationships between liver GSH and protein binding, and serum

APAP-protein adducts and toxicity. To determine whether or not co-incidental liver injury could artificially increase the serum concentration of APAP adducts after subtoxic doses, we treated mice with APAP and induced necrosis through ischemia-reperfusion. Finally, primary mouse hepatocytes were used to study the appearance of adducts in extracellular fluid without toxicity. Overall, our data support the clinical use of this parameter, but urge consideration of potential confounding factors.

5.2 MATERIALS AND METHODS

Animals. All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Kansas Medical Center. Male C57Bl/6 mice were purchased from Jackson Laboratories (Bar Harbor, ME). The animals were kept in a temperature-controlled room with a 12 h light/dark cycle and *ad libitum* access to food and water. Food was removed 12-16 h prior to treatment with the indicated doses of acetaminophen or before hepatocyte isolation for cell culture. For *in vivo* studies, APAP was dissolved in warm saline and injected i.p. The concentration of each APAP solution was adjusted so that all mice received approximately the same volume. At the indicated time points, the mice were sacrificed by cervical dislocation. Blood was drawn from the caudal vena cava into heparinized syringes and plasma was obtained by centrifugation at 12,000 g for 3 min. A section was taken from the left lobe of each mouse liver and fixed in 10% phosphate-buffered formalin for histology. Remaining portions of the livers were collected and flash frozen in liquid nitrogen for later biochemical analysis.

Ischemia-reperfusion surgery. Liver injury was induced by ischemia-reperfusion as described (Hasegawa et al., 2007). Briefly, each mouse was treated with 75 mg APAP / kg body weight. Under ketamine/xylazine/acepromazine anesthesia (i.m. injection), midline laparotomy was performed and the blood vessels supplying the left and median lobes were occluded with an atraumatic clamp at approximately 1 h post-APAP. After forty-five minutes, the clamp was removed to restore blood flow and the abdomen was closed using suture thread and wound clips. Body temperature was monitored with a rectal probe and maintained at 37.0 – 37.5 °C using a heat lamp. The animals were sacrificed while still under anesthesia after 1.5 h of reperfusion.

Primary hepatocyte culture. Primary mouse hepatocytes were isolated as described (Bajt et al., 2004). Briefly, the caudal vena cava was cannulated and the liver was perfused at 8 mL/min with warmed and oxygenated Hank's buffer salt solution (HBSS) containing penicillin/streptomycin at pH 7.4 for 10 min. This was followed by perfusion with a solution of the same composition plus 1 mM Ca^{2+} and Mg^{2+} and 0.04% collagenase D (Roche Molecular Biochemicals, Mannheim, Germany). After perfusion, livers were minced and strained through a series of wire mesh filters. The cells were then pelleted by gentle centrifugation and washed three times before being re-suspended in cell culture medium and seeded on sterile collagen-coated dishes. The cells were maintained in Williams' E medium supplemented with fetal bovine serum, insulin, and penicillin/streptomycin. Protein adducts in cells and supernatant were assessed after exposure to 5 mM APAP for 3 h. For some experiments, cultures were washed 4-5 times with 1x PBS and changed to serum- and insulin-free medium

immediately before use. To monitor protein secretion in the absence of serum, the protein in the culture medium was concentrated using Amicon Ultra centrifugal filters with 3,000 Da MWCO (Millipore, Billerica, MA), separated by gel electrophoresis, and stained with Coomassie blue.

Bilateral renal ischemia. Under ketamine/xylazine/acepromazine cocktail anesthesia, mice were subjected to quarter-inch bilateral incisions made approximately 35 – 45 mm behind the ears to provide access to the kidney on each side. The kidneys were exteriorized and the renal arteries and veins were ligated near the renal pedicles using silk suture thread. The ligated kidneys were returned to the body and the incisions were closed using suture thread and wound clips. Sham controls were subjected to bilateral incision but not renal ligation. Within 15 min of closing the wounds, the mice were injected i.p. with 300 mg/kg APAP followed 15 min later by 80 μ mol / kg GSH or an equal volume of saline. Body temperature was monitored with a rectal probe and maintained at 37.0 – 37.5 °C using a heat lamp. The animals were sacrificed while still under anesthesia 1.5 h post-APAP.

Biochemistry. Alanine aminotransferase (ALT) activity was measured using a kit from Pointe Scientific (Canton, MI). Glutamate dehydrogenase (GDH) was measured as described (McGill et al., 2012a).

APAP-protein adduct measurement. To remove low molecular weight compounds with the potential to interfere with detection and to isolate proteins, plasma samples, liver homogenates, cell lysates, and cell culture medium were filtered through Bio-Spin 6

columns (Bio-Rad, Hercules, CA) that were pre-washed with 10 mM sodium acetate buffer (pH 6.5). The filtered samples were digested overnight with proteases to free APAP-CYS. After digestion, remaining protein in the cell culture medium, cell lysate, and in some plasma samples was precipitated using cold isopropanol/methanol (McGill et al., 2011) and pelleted by centrifugation. The supernatants were evaporated at 55 °C and 16 psi and the protein-derived APAP-CYS-containing residues were re-suspended in small volumes of 10 mM sodium acetate. APAP-CYS in liver homogenates was prepared as described (Ni et al., 2012b). For time course and dose-response experiments, APAP-CYS was measured by LC-MS/MS (McGill et al., 2011). For ischemia-reperfusion and cell culture experiments, APAP-CYS was measured using HPLC with electrochemical detection (Muldrew et al., 2002; Ni et al., 2012b).

TUNEL staining. For the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay, sections of liver were stained with the In Situ Cell Death Detection Kit, AP (Roche Diagnostics, Indianapolis, IN) as described in the manufacturer's instructions

Statistical methods. The Shapiro-Wilk test was used to assess normality. For normally distributed data, one-way analysis of variance (ANOVA) was performed to test for significance, with Student-Newman-Keuls post-hoc comparison between groups. For non-normally distributed data, ANOVA was performed on ranks, followed by Dunn's multiple comparisons. For all tests, $p < 0.05$ was considered significant.

5.3 RESULTS

Dose-response of liver injury. Mice were treated with various doses of APAP (15, 75, 150, 300, or 600 mg/kg) and sacrificed at the indicated times. As expected, APAP caused a dose-dependent increase in plasma levels of ALT and the mitochondrial enzyme GDH at 24 h (Fig. 5.3.1A,B). Neither enzyme was elevated after treatment with doses lower than 150 mg/kg, which produced injury in approximately half of the animals tested (Fig. 5.3.1A,B). The 300 and 600 mg/kg doses caused dramatic increases in plasma ALT activities (Fig. 5.3.1A), with the highest values measured 12 – 24 h after treatment. Unfortunately, hemorrhage limited the volume of plasma available for enzyme measurements after treatment with 600 mg/kg, so we could not collect a complete set of data for GDH at the highest dose. However, histology could be obtained and we observed increased necrosis and TUNEL staining with increasing doses (Fig. 5.3.1C,D), consistent with increasing plasma ALT and GDH activities.

Time course and dose-response of liver GSH and APAP-protein adducts. To explore the relationship between liver GSH and covalent binding of the reactive metabolite of APAP to proteins, we measured GSH and APAP-protein binding in the livers from these animals. Upon reaction with a free sulphydryl group, the structure of NAPQI reverts to that of APAP. The cysteine adduct is the major protein adduct formed by NAPQI (Streeter et al., 1984) and is often referred to as APAP-CYS (Muldrew et al., 2002). Protein-derived APAP-CYS was measured after purification of proteins using size exclusion chromatography and digestion with proteases to liberate the APAP-CYS

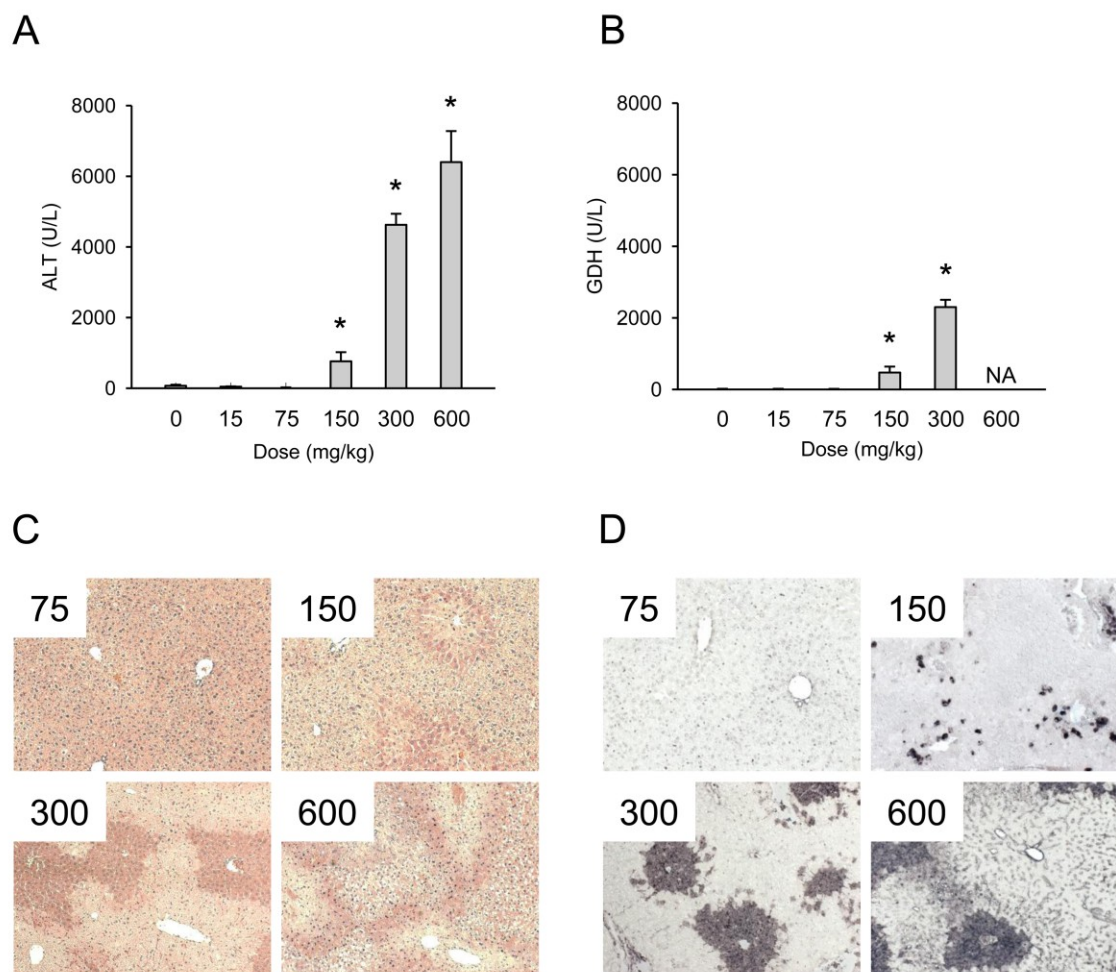


Figure 5.3.1. Dose-response of liver injury. Mice were treated with 0, 15, 75, 150, 300, or 600 mg APAP / kg bodyweight and sacrificed 24 h later. (A) Dose-response of plasma alanine aminotransferase (ALT) activities. (B) Dose-response of plasma glutamate dehydrogenase (GDH) activities. (C) Hematoxylin and eosin staining of liver sections. (D) TUNEL staining of liver sections. Data are expressed as mean \pm SEM of $n = 6$ animals per time point. * $P < 0.05$ (compared to $t=0$); NA = Not Available.

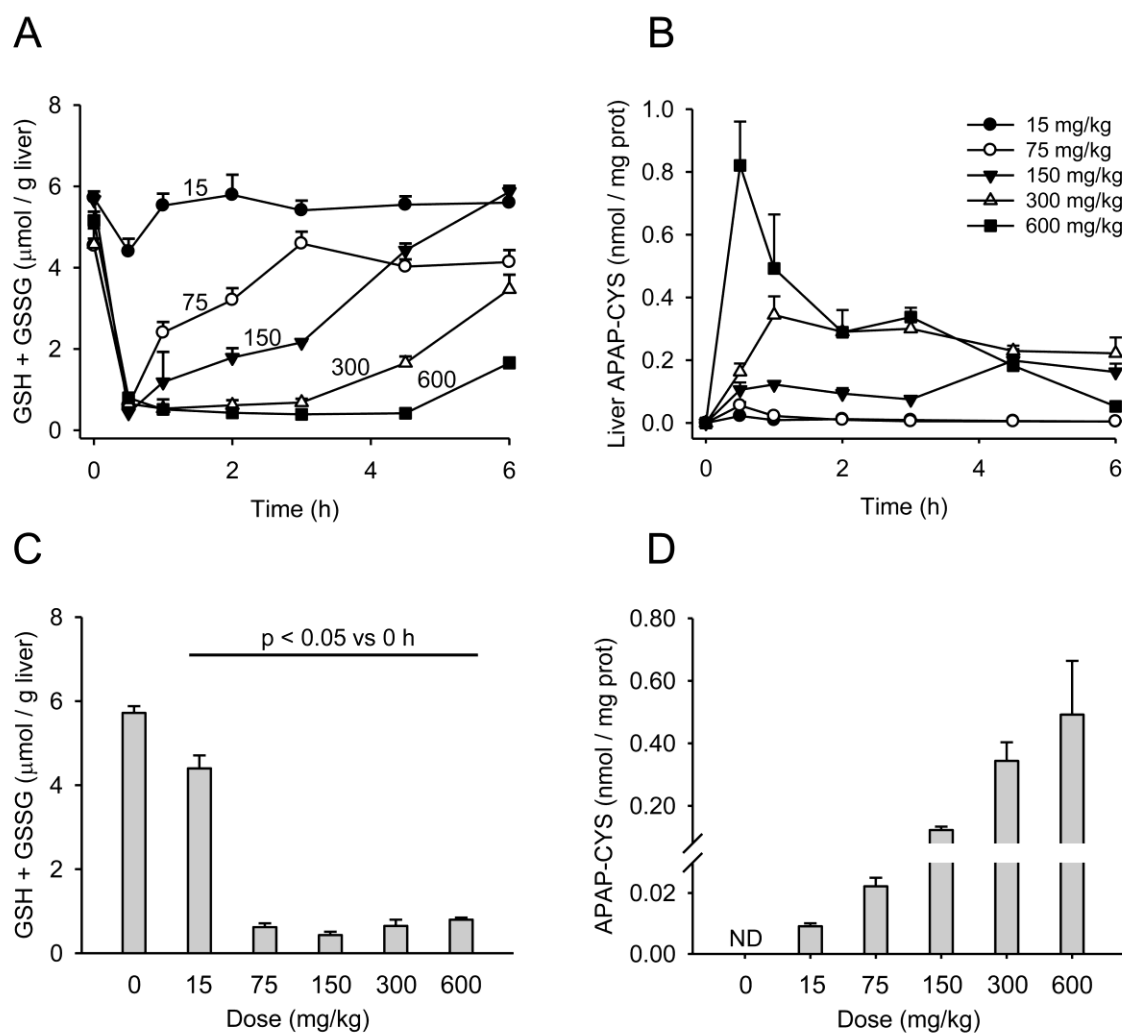


Figure 5.3.2. Dose-response and time course of total liver GSH (GSH + GSSG) and liver APAP-protein adducts. Mice were treated with 0, 15, 75, 150, 300, or 600 mg APAP / kg bodyweight and sacrificed at the indicated time points. (A) Total liver GSH over time. (B) Liver protein-derived APAP-CYS over time. (C) Total liver GSH 0.5 h after APAP treatment. (D) Liver protein-derived APAP-CYS concentration, 1 h after APAP treatment. Data are expressed as mean \pm SEM of $n = 6$ animals per time point.

residues. Thus, our assay specifically measures APAP-CYS from proteins, not from APAP-GSH or free cysteine. Except for the lowest dose, GSH was depleted to the same extent within 0.5 h of each treatment (Fig. 5.3.2A,C). Significant differences in GSH levels were observed only during the time of GSH recovery (Fig. 5.3.2A). Importantly, protein-derived APAP-CYS could be detected in liver samples even after the 15 mg/kg dose, albeit at low levels (Fig. 5.3.2B,D). These data show that extensive GSH depletion is not necessary for protein binding to occur. Nevertheless, it is clear that GSH is an important scavenger of NAPQI.

Time course and dose-response of liver injury and plasma APAP-protein adducts. We next studied the dose-response of plasma APAP-protein adducts and compared these values with ALT. APAP-CYS concentration was below our lower limit of quantitation in all plasma samples from mice treated with the lowest dose (15 mg/kg). However, adducts could be measured in plasma after treatment with the 75 mg/kg subtoxic dose, and increased dose-dependently thereafter (Fig. 5.3.3A,B). Comparing adducts with ALT revealed that protein-derived APAP-CYS is detectable in plasma without increased ALT after a subtoxic dose (Fig. 5.3.4A) and appears in plasma as early as 0.5 – 1 h after treatment with a toxic dose, well before the onset of injury and ALT release (Figs. 5.3.4 and 5.3.5). These data contradict the earlier view that necrosis is required for release of adducted proteins, and are consistent with more recent work showing that adducts can be detected in serum from humans after therapeutic doses (Heard et al., 2011). Interestingly, we also found that APAP-protein adducts were detectable in serum from rats, which are not susceptible to APAP overdose (Fig. 5.3.6). Protein binding is

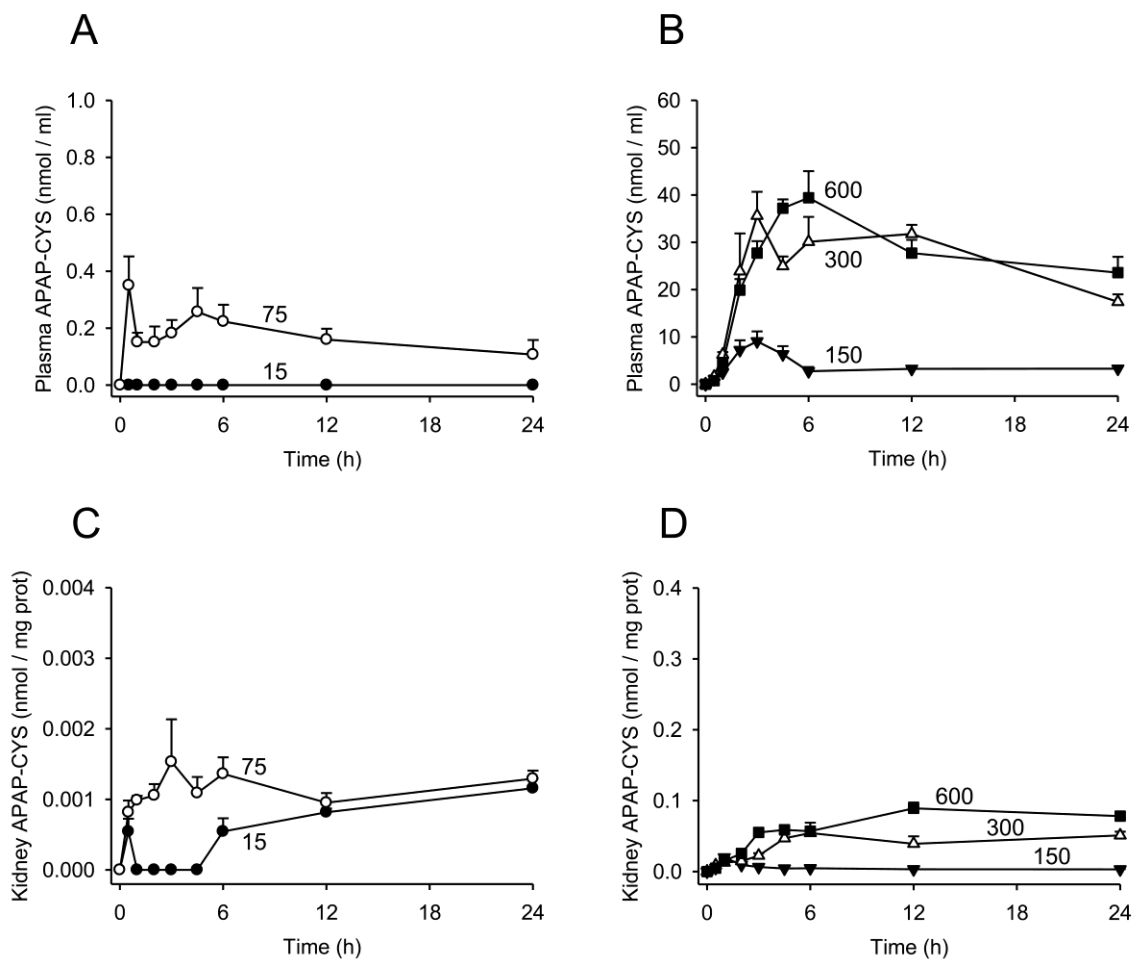


Figure 5.3.3. Dose-response and time course of plasma and kidney APAP-protein adducts. Mice were treated with 0, 15, 75, 150, 300, or 600 mg APAP / kg bodyweight and sacrificed at the indicated time points. Protein-derived APAP-CYS was measured in plasma (A and B) and kidneys (C and D) from these animals. Data are expressed as mean \pm SEM of $n = 6$ animals per time point.

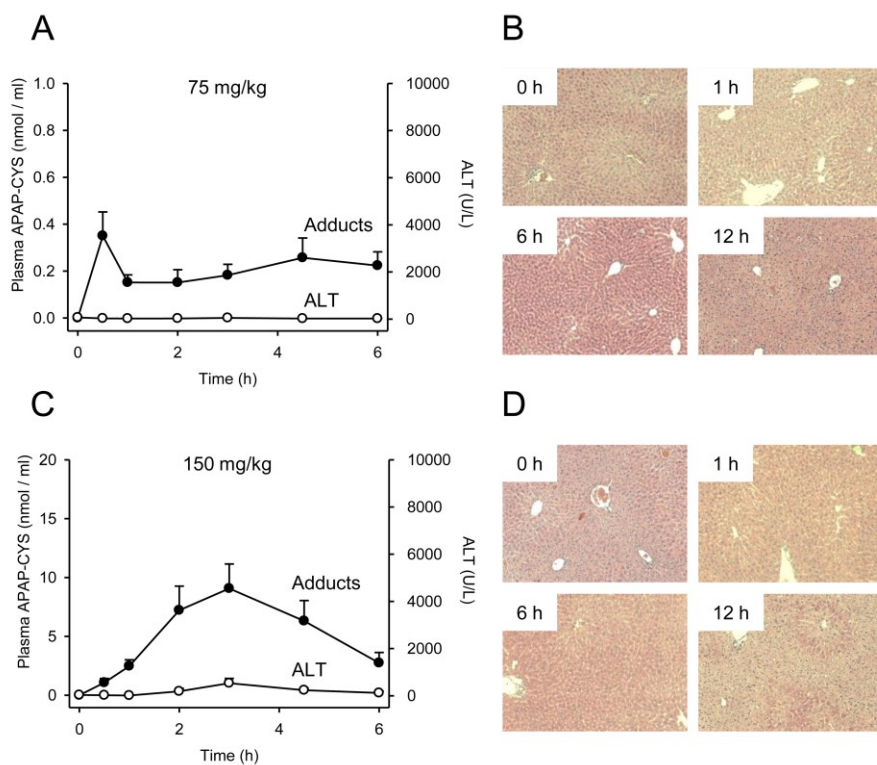


Figure 5.3.4. Time course of plasma APAP-protein adducts and liver injury after 75 mg/kg or 150 mg/kg dose. Mice were treated with 75 or 150 mg/kg and sacrificed at the indicated time points. (A,C) ALT and protein-derived APAP-CYS in plasma. (B,D) Liver histology time course. Data are expressed as mean \pm SEM of $n = 6$ animals per time point.

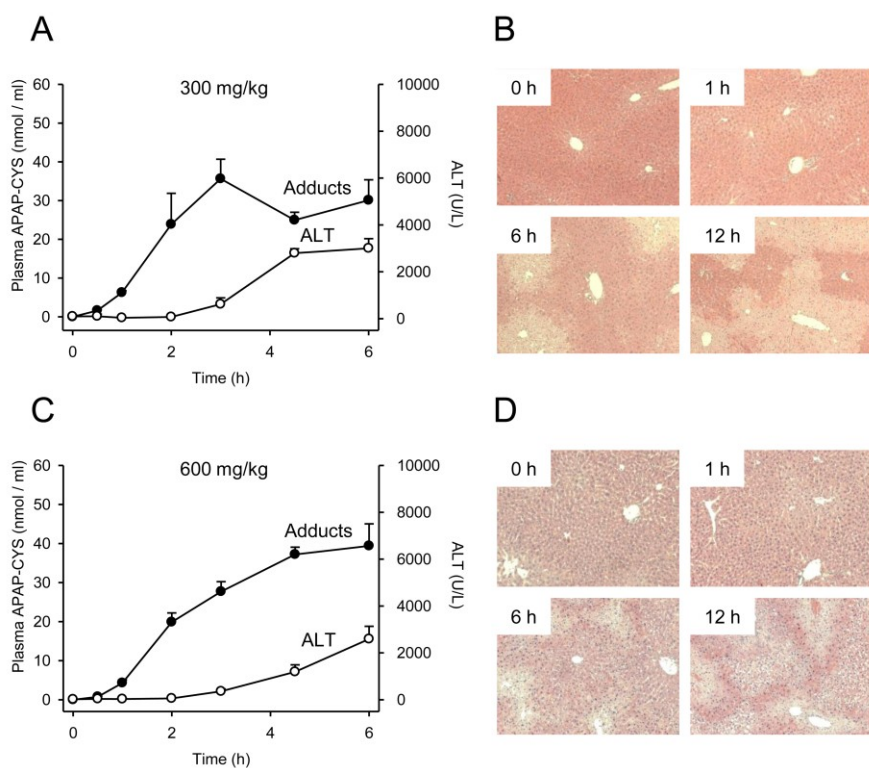


Figure 5.3.5. Time course of plasma APAP-protein adducts and liver injury after 300 mg/kg or 600 mg/kg dose. Mice were treated with 300 or 600 mg/kg and sacrificed at the indicated time points. (A,C) ALT and protein-derived APAP-CYS in plasma. (B,D) Liver histology time course. Data are expressed as mean \pm SEM of $n = 6$ animals per time point.

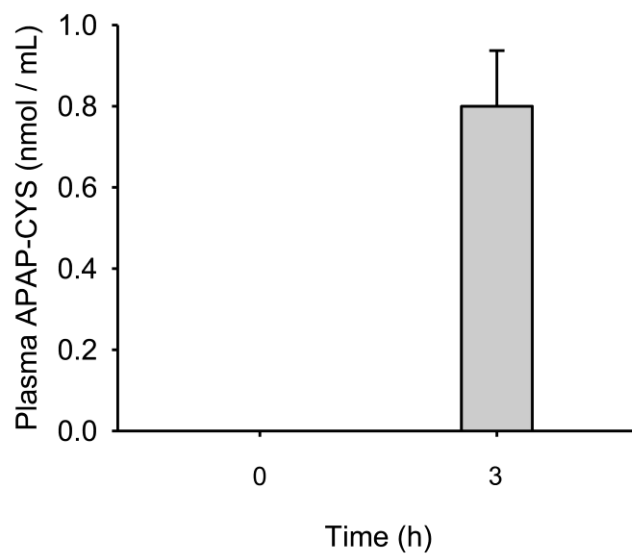


Figure 5.3.6. APAP-protein adducts in rat plasma. Rats were treated with 1 g/kg APAP and sacrificed 3 h later.

also known to occur in the kidney after treatment with APAP (Mudge et al., 1978; McMurtry et al., 1978). However, our data show that the concentration of APAP-protein adducts in the kidney is very low, even after high doses of APAP (Fig. 5.3.3C,D). Thus, most of the plasma adducts in our study were probably liver-derived and the contribution of the kidneys is negligible.

Mechanisms of the appearance of APAP-protein adducts in plasma. We hypothesized that the appearance of APAP-protein adducts in plasma occurs in one of two ways. Protein binding may take place exclusively within hepatocytes, followed by secretion or exocytosis of some of the adducted proteins into plasma. Alternatively, NAPQI could diffuse out of the hepatocyte and bind to plasma proteins in situ. We decided to take an in vitro approach to test these hypotheses. When cells were kept in serum-free medium (without APAP), we observed a clear increase in extracellular protein within 3 h (Fig. 5.3.7A), without an increase in cell death or enzyme release when compared with serum-containing cultures (Fig. 5.3.7B). These data suggest that primary mouse hepatocytes can actively secrete proteins into the culture medium. We then treated primary mouse hepatocytes with 5 mM APAP in the presence or absence of extracellular protein from FBS and measured protein-derived APAP-CYS in the culture medium. Importantly, we were able to measure APAP-protein adducts in medium from cultures without serum (Fig. 5.3.7C). This is consistent with the hypothesis that adducts are formed within hepatocytes and secreted. However, the concentration of adducts in the medium from this group was approximately half of that in medium from the group with serum. This could not be explained by a reduction in metabolism of APAP. In fact,

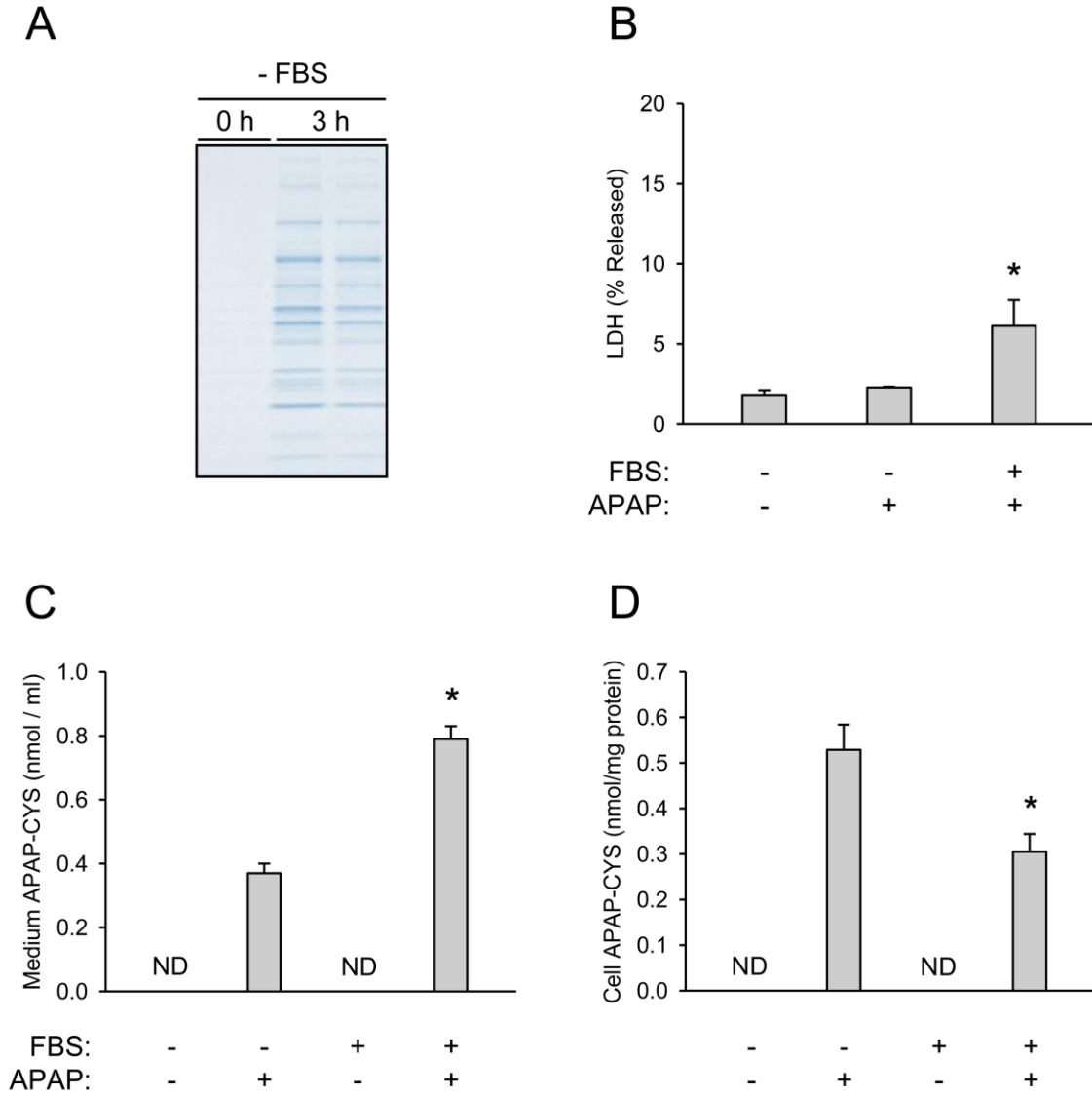


Figure 5.3.7. Mechanisms of the appearance of extracellular APAP-protein adducts in vitro. (A) Primary mouse hepatocytes were washed and cultured in serum-free medium for 3 h in the presence of 5 mM APAP. Extracellular protein was measured using SDS-PAGE with Coomassie blue. (B) LDH release was measured in cultures of primary mouse hepatocytes treated with APAP in the presence or absence of serum for 3 h. (C) Protein-derived APAP-CYS was measured in the culture medium of hepatocytes treated with APAP in the presence or absence of serum for 3 h. (D) Protein-derived APAP-CYS was measured in hepatocytes treated with APAP in the presence or absence of serum for 3 h. Data are expressed as mean \pm SEM of 3-6 experiments. *P < 0.05 vs. cultures with serum-free medium. ND = Not Detectable.

protein binding within cells was increased in the absence of extracellular protein (Fig. 5.3.7D). One possible reason for the difference in medium adducts is that both adduct secretion and NAPQI diffusion are involved. Secretion of adducted proteins would explain the appearance of adducts in serum-free medium, while diffused NAPQI returning to the cell to bind to cellular proteins would explain the increase in intracellular adducts. To test the latter possibility, mice were treated with APAP and GSH following bilateral renal ischemia to prevent the breakdown of GSH in the kidney (Fig. 5.3.8). We hypothesized that the increased plasma GSH would scavenge any NAPQI that diffused out of the hepatocytes and would thereby reduce serum APAP-protein adduct concentration. Unfortunately, we could not detect a difference in serum adducts. Interestingly, bilateral ischemia alone appeared to be sufficient to increase plasma GSH above the expected 5 – 10 μ M range, likely by reducing serum GSH turnover. This may be why no difference was found between the groups receiving or not receiving GSH. The extreme variation in the results was likely also a factor, making the data difficult to interpret. Additional work is needed to fully understand the mechanism by which APAP-protein adducts appear in plasma.

Effect of co-incidental liver injury on plasma APAP-protein adducts. Although necrosis is not necessary for adduct release, it is possible that co-incidental liver injury could lead to artificially elevated plasma concentrations after a subtoxic dose. To test this, we treated mice with the subtoxic 75 mg/kg dose of APAP for 1 h and induced necrosis in the left lateral and median lobes with forty-five minutes of ischemia followed by 1.5 h of

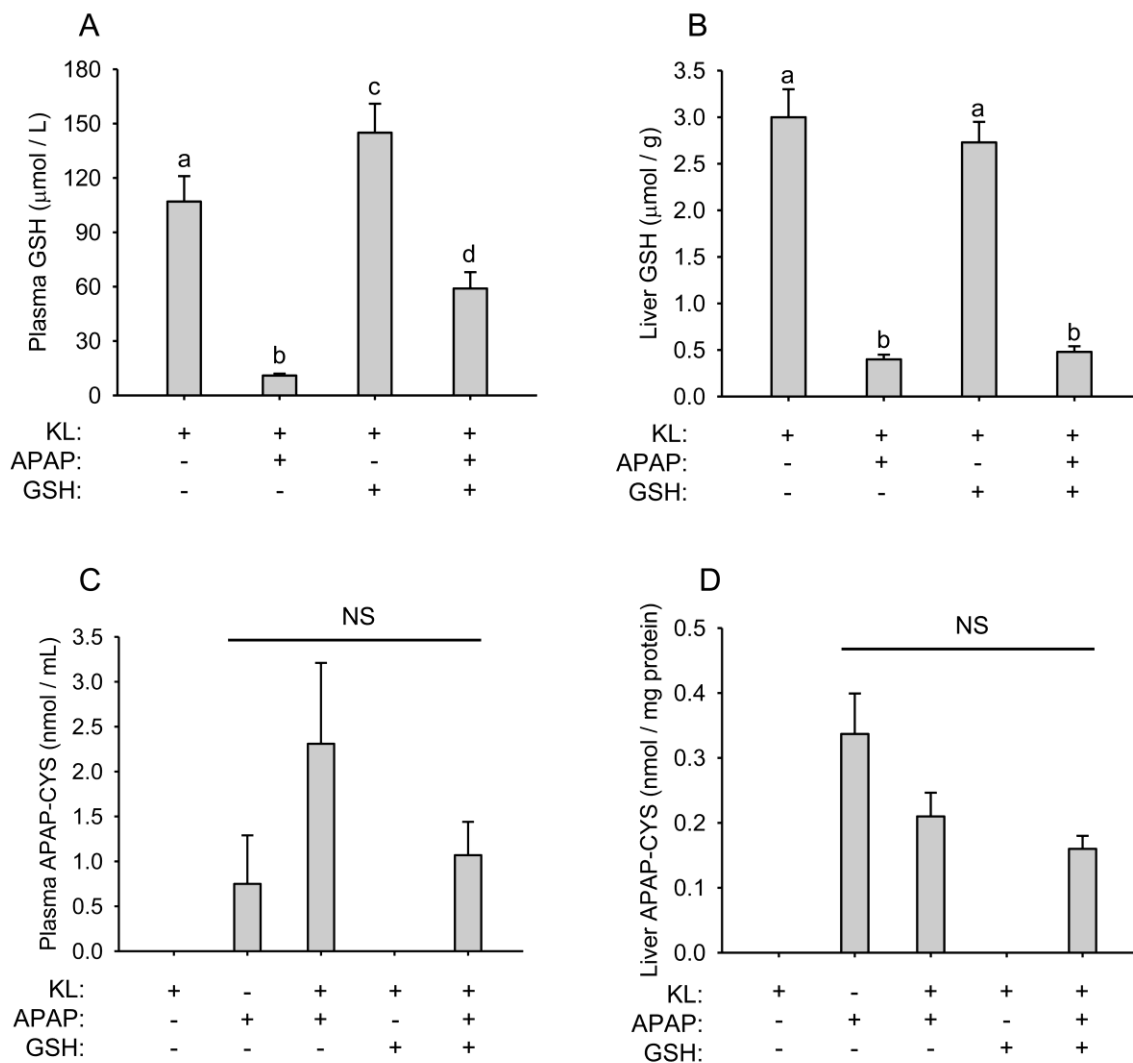


Figure 5.3.8. Increased plasma GSH does not affect plasma APAP-protein adduct levels. Mice were subjected to bilateral renal ischemia or sham surgery and treated with 300 mg/kg APAP followed by saline or 80 $\mu\text{mol/kg}$ GSH. (A) Plasma GSH values. (B) Liver GSH. (C) Protein-derived APAP-CYS in plasma. (D) Protein-derived APAP-CYS in liver. Data are expressed as mean \pm SEM of $n = 3-9$ animals per treatment group. Different letters indicate significant differences between groups ($P < 0.05$). NS = not significant.

reperfusion. Sham controls were anesthetized and underwent midline laparotomy, but were not subjected to ischemia. All animals were sacrificed at the same time point after APAP treatment. Ischemia-reperfusion resulted in significant liver injury and cell contents release, as indicated by plasma ALT (Fig. 5.3.9A). Only a very minor increase in ALT over control was also observed in the sham-treated animals. APAP-protein adducts could be detected in plasma from sham-operated mice, but was significantly increased in samples from the ischemia-reperfusion group (Fig. 5.3.9B). No difference in the metabolic activation of APAP could be discerned between the two groups, based on APAP adducts and GSH levels in the left lateral lobe (Fig. 5.3.9C,D). This is consistent with our time course data showing that NAPQI and liver adduct formation plateau by 1 h (Fig. 5.3.9B), before the mice were subjected to hepatic ischemia. These data show that plasma APAP-protein adduct concentrations can be increased by non-APAP-related liver injury after exposure to subtoxic doses.

5.4 DISCUSSION

Recent work in the field of APAP hepatotoxicity has focused on late events in the mechanism of injury. We now know that high doses of APAP can inhibit mitochondrial respiration (Meyers et al., 1988) and cause mitochondrial oxidative stress in both mice (Jaeschke, 1990; Cover et al., 2005; Fujimoto et al., 2009; Ramachandran et al., 2011b; Bajt et al., 2011) and human HepaRG cells (McGill et al., 2011). In mice, an initial oxidative stress leads to activation of the c-Jun N-terminal kinase (JNK) (Gunawan et

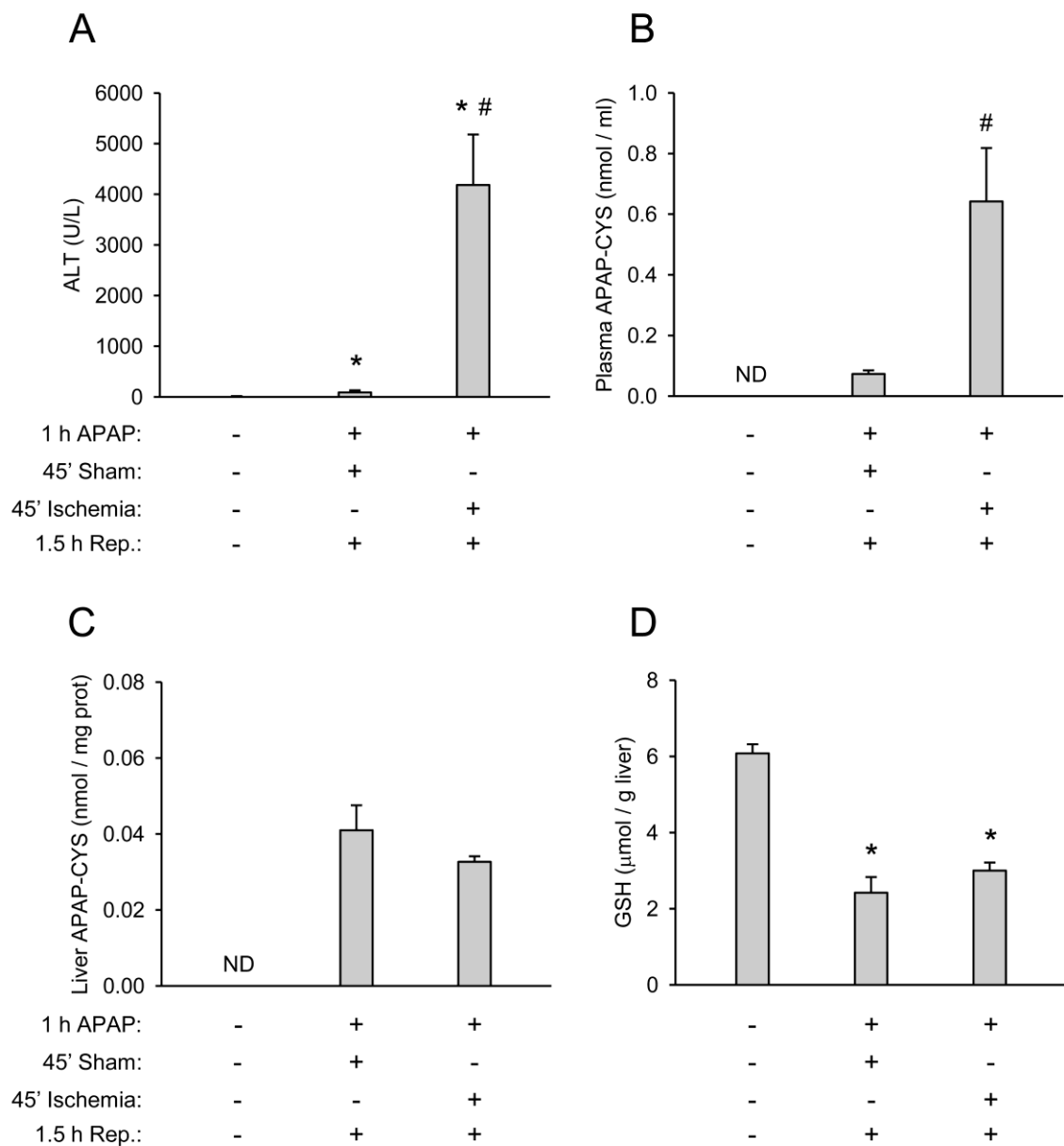


Figure 5.3.9. Ischemia-reperfusion liver injury increases plasma APAP-protein adduct levels. Mice were pretreated for 1 h with APAP at 75 mg/kg and subjected to ischemia-reperfusion of the liver. (A) Plasma ALT values. (B) Protein-derived APAP-CYS in plasma. (C) Protein-derived APAP-CYS in livers. (D) Total GSH (GSH + GSSG) in livers. Data are expressed as mean \pm SEM of $n = 4-6$ animals per treatment group. * $P < 0.05$ (compared to untreated controls). # $P < 0.05$ (compared to sham). ND = Not Detectable.

al., 2006; Hanawa et al., 2008; Saito, et al., 2010a) through apoptosis signal-regulating kinase 1 (ASK1) (Nakagawa et al., 2008) and mixed lineage kinase 3 (MLK3) (Sharma et al., 2012). The activated JNK translocates to mitochondria and exacerbates the oxidative stress and injury (Hanawa et al., 2008; Win et al., 2011; Ramachandran et al., 2011b; Jaeschke et al., 2012a). Loss of mitochondrial membrane integrity and translocation of Bax into mitochondria cause release of endonucleases that can cleave nuclear DNA (Bajt et al., 2006, 2008, 2011). Importantly, these mechanisms are not limited to mice or human cell lines. There is evidence that mitochondrial damage and nuclear DNA fragmentation also occur in humans *in vivo* after APAP overdose (McGill et al., 2012a), leading to hepatocellular necrosis (McGill et al., 2012a; Antoine et al., 2012). While these mechanisms are critical in APAP-induced liver injury, it is important to remember that they occur downstream of the metabolic activation of APAP. Inhibiting NAPQI formation prevents the oxidative stress, JNK activation, and mitochondrial damage (Jaeschke et al., 2011). Unfortunately, the relationship between protein binding and liver injury is not fully understood. While a number of specific targets of NAPQI have been identified, the activities of these proteins are in general only modestly affected (Cohen et al., 1997; Qiu et al., 1998). Importantly, early work comparing APAP with the non-hepatotoxic isomer *N*-acetyl-*m*-aminophenol (AMAP) revealed that the reactive metabolite of APAP binds more to mitochondrial proteins (Tirmenstein and Nelson, 1989), suggesting that mitochondrial protein binding is particularly important. Consistent with this, we have shown that rats, which are less susceptible to APAP-induced liver injury, have lower APAP-protein adduct concentrations in mitochondria

than mice (McGill et al., 2012b). However, these data are largely correlative. A recent *in vitro* study showed that AMAP can actually cause toxicity in liver slices from rats and humans and even mice at higher doses (Hadi et al., 2013). Unfortunately, no protein adducts were measured in these experiments (Hadi et al., 2013). Additional work is clearly needed to fully understand the connection between protein adducts and toxicity.

Liver glutathione and protein binding. It has long been believed that extensive GSH depletion is required for protein binding to occur after APAP. Dose-response data supporting this were first published forty years ago (Mitchell et al., 1973b) and it has since become a paradigm in the study of electrophile-mediated hepatotoxicity. However, more recent work has challenged this idea. Protein adducts could be measured in human HepaRG cells as early as 1 h after treatment with APAP, well before any appreciable loss of GSH had occurred (McGill et al., 2011). Moreover, protein-derived APAP-CYS could be detected in serum from humans after only therapeutic doses (Heard et al., 2011). The discrepancy is likely due to the lack of multiple time points in the earlier dose-response data, which were collected 2 h post-APAP (Mitchell et al., 1973b). Our results show that the peak of protein adduct formation in the liver is reached by 0.5 – 1 h after administration of subtoxic doses and that adduct concentration decreases thereafter. Importantly, we were able to detect protein binding after treatment with 15 mg/kg APAP at these earlier time points, with only a minimal loss of liver GSH. Together, it is clear from these studies that some protein binding can occur without extensive GSH depletion and without toxicity.

Interestingly, liver GSH was similar at 0.5 h after APAP for all doses ≥ 75 mg/kg, while recovery of GSH showed a clear dose-response (Fig. 2A). It is worth noting that GSH recovered to near control levels by 12 h after all but the highest dose. Furthermore, our data show that APAP-protein adduct formation in the liver peaks within 1 – 2 h and that adduct concentration decreases beyond this time point, possibly as a result of autophagy of adducted proteins and mitochondria (Ni et al., 2012a). Thus, to make an accurate assessment of metabolic activation of APAP in mice it is necessary to measure GSH and/or protein adducts at an early time point, in the 0.5 – 2 h post-treatment range. Use of a single late time point, as done in most natural product testing experiments, does not give a reliable assessment of APAP metabolism and NAPQI formation (Jaeschke et al., 2011).

Clinical use of plasma APAP-protein adducts. Etiology can be a consideration in determining whether or not an individual with liver injury is in need of a transplant. Patients with APAP hepatotoxicity are more likely to survive without a new organ than patients with liver injury due to other causes (Simpson et al., 2009). Moreover, when intentional overdose can be established, it is important to ensure that the patient receives proper care and counseling to avoid future incidents (Qin et al., 2009). Thus, accurate diagnosis of APAP overdose is critical. Presently, diagnosis is largely based on serum APAP concentration. Unfortunately, APAP has a relatively short serum half-life in humans. The confident use of serum APAP requires a patient to present early after overdose, which is often not the case. Measurement of serum APAP-protein adducts, which have a half-life of 1-2 days (James et al., 2009), is a promising solution.

Initially, this was based on the idea that adducts are released from dying hepatocytes (Davern et al., 2006) and are therefore a strong indication of APAP toxicity. However, data from this study and from other groups have shown that adducts can appear in plasma before ALT after a toxic dose and are detectable after subtoxic and even therapeutic doses (Heard et al., 2011). To deal with this, a threshold of ≥ 1.1 nmol/mL serum APAP-CYS combined with serum ALT $\geq 1,000$ U/L has been proposed for diagnostic use (James et al., 2009). Our data are in general agreement with this. In our study, plasma levels of protein-derived APAP-CYS peaked at 0.35 ± 0.1 nmol/mL after treatment with the 75 mg/kg subtoxic dose, but reached 9.1 ± 2.1 nmol/mL after the lowest toxic dose tested (150 mg/kg). The results from our *in vitro* experiments support a role for secretion of adducted proteins into plasma as an important mechanism by which adducts appear there without liver injury. However, we cannot rule out the possibility that NAPQI formed in hepatocytes diffuses out of the cells and binds to plasma proteins *in situ*. The latter would be consistent with the earlier finding in mice of APAP-hemoglobin adducts in red blood cells, which do not express cytochrome P450 enzymes (Axworthy et al., 1988) and the identification of adducts on serum albumin (Switzar et al., 2013). It is likely that both mechanisms are involved.

While these data support the clinical use of plasma APAP-protein adducts, there may be circumstances that require special consideration. Though data from our study and others show that liver injury is not required for the appearance of APAP-protein adducts in plasma, it is possible that cell death and cell contents release can contribute to the plasma levels. APAP is a very popular drug and a person taking therapeutic doses of

APAP could develop liver injury incidental to their APAP use, possibly resulting in higher concentrations of protein-derived APAP-CYS in plasma. Our data show that ischemia-reperfusion-induced liver injury can significantly increase APAP-protein adducts in plasma after a subtoxic dose of APAP (Fig. 7). Thus, care should be taken in the clinical interpretation of this parameter. Other possible causes of liver injury may need to be ruled out before a diagnosis of APAP overdose is made on the basis of plasma adducts.

Summary and conclusions. APAP is a widely used drug and hepatotoxicity as a result of overdose is a major clinical issue. Protein binding is the critical initiating event in the mechanism of injury. However, contrary to early reports, APAP-protein binding can occur even after subtoxic doses, without extensive GSH depletion. It is likely that either a threshold of protein binding (particularly mitochondrial protein binding) needs to be achieved before the development of toxicity, or that specific binding targets are spared at low doses. Interestingly, while our data support the measurement of protein-derived APAP-CYS in plasma as a promising new diagnostic method, it is important to note that these adducts can appear in plasma without injury. Establishment of a sensitive but still specific threshold concentration is important. Our results show that co-incidental liver injury after a subtoxic dose of APAP can dramatically increase the plasma concentration of adducts. This may have important clinical implications. The effects of other diseases and polypharmacy on plasma APAP-protein adduct levels remain to be investigated.

6. CONCLUSIONS AND DISCUSSION

6.1 FUTURE DIRECTIONS IN ACETAMINOPHEN TOXICITY RESEARCH

Mitochondrial protein binding. Although it is clear that mitochondrial dysfunction and oxidative stress are central in the mechanism of APAP hepatotoxicity in mice, it must be emphasized that it is not known exactly how these things develop. As mentioned previously, correlative data suggest that covalent binding of NAPQI to mitochondrial proteins is a factor. However, none of the available evidence can prove that mitochondrial protein binding is the initiator of the mitochondrial damage or increased ROS production. Our ability to demonstrate this is constrained by methodological and theoretical limitations. Although it has been shown that the reactive metabolites of the non-hepatotoxic isomer AMAP bind less to mitochondrial proteins than that of APAP (Tirmenstein and Nelson, 1989) and that APAP-resistant rats have lower mitochondrial protein binding than mice (McGill et al., 2012b; Chapter 2), in both cases mitochondrial protein binding was still detectable in the animals that did not develop liver injury. There are three possible interpretations of these data. First, there may be a threshold of mitochondrial protein binding that must be achieved in order to cause serious injury. This is supported by our mitochondrial APAP-protein adduct dose-response data (Fig. 2.3.4). Second, it is possible that specific mitochondrial proteins must be adducted. Although a number of specific protein targets of NAPQI have been identified, as yet none appear to be critical in the mechanism of injury (Jaeschke et al., 2012a). Moreover, the techniques used to discover some of these targets are plagued by fundamental flaws. For example, Qiu and Burlingame treated mice with radiolabeled APAP or AMAP and separated proteins from whole liver homogenates using 2D gel

electrophoresis (Qiu and Burlingame, 1998; 2001). They then picked the radiolabeled protein bands from the gel, digested them, and performed mass spectrometry to sequence the resulting peptides. While this was a major improvement over earlier methods that had been used to achieve similar goals, the fact remains that any proteins that co-migrated with the radiolabeled targets could have interfered with the sequencing. This method is likely to identify only the highest abundance proteins at those spots on the gel that were picked. Without additional steps to further purify all of the proteins located in those spots, interference cannot be ruled out. Finally, mitochondrial protein binding may not be important at all. The mitochondrial dysfunction may be the result of something else that occurs downstream of the metabolic activation of APAP but before the occurrence of the MPT.

In order to prove that a specific mitochondrial protein (or any specific protein) is a critical target of NAPQI, one would have to show not only that the protein in question is adducted but that preventing binding to that single target also prevents or reduces the liver injury. Currently, there is no way to prevent binding of NAPQI to one specific protein. A potential strategy is to knock down the proposed target in cultured cells (e.g. HepaRG cells) and determine whether or not this prevents the injury caused by APAP. However, if the protein is so vital that covalent binding by NAPQI is lethal to the cell, then knocking it down would likely also be lethal. While this would be a form of evidence in itself, it would not be conclusive. Another possibility is that cells could be transfected with cDNA encoding a form of the suspected target that is not vulnerable to NAPQI in an effort to rescue or preserve the protein's function and prevent toxicity after APAP

treatment. This might be possible with a form of the protein lacking the amino acid targeted by NAPQI. However, if the amino acid in question is so important for the proper function of the protein, substituting the critical residue with another could have an effect similar to NAPQI binding. This is especially true considering that NAPQI primarily binds to cysteine residues (Streeter et al., 1984), which are often necessary for proper protein folding. Furthermore, the latter approach would likely require the generation of mutants of numerous proteins that have been identified as NAPQI targets and many rounds of screening of transfected cells. The task would be laborious to say the least. Even if successful, doubts would likely remain concerning the validity of these in vitro models. Until a practical strategy for the investigation of this issue can be developed, our assessment of the importance of mitochondrial protein binding in the mechanism of APAP-induced liver injury will continue to rest on correlative data.

Mechanisms of APAP toxicity in humans. Although our data represent an important first step toward the development of an improved understanding of the mechanism by which APAP can cause liver injury in humans, they are by no means conclusive. While we have some evidence that elevated GDH, mtDNA, and acylcarnitines in plasma may be specific evidence for mitochondrial dysfunction, it is possible that our results are peculiar to the furosemide model that we used. For example, it has been suggested that the diuretic action of the drug could result in increased elimination of these markers from the circulation (Lemasters, personal communication, 2012). Clearly, additional work is needed to fully explore the role of mitochondrial damage in APAP hepatotoxicity

in humans. Other mechanistic issues that need to be investigated clinically include oxidative stress and the role of the innate immune system.

Clinical biomarkers of outcome during APAP-induced liver injury. As it stands, clinicians rely principally on markers of liver injury and function (e.g. ALT, prothrombin time, bilirubin, etc.) to follow patient progress. While a number of formulae have been devised to apply these data to the prediction of patient outcome (e.g. King's College Criteria [KCC], Model for End-Stage Liver Disease [MELD] scores, etc.), the results are sometimes unsatisfactory. New evidence is emerging that the plasma or serum levels of mechanistic biomarkers such as GDH and keratin 18 correlate with outcome. For example, Antoine et al. (2012) found that full length keratin 18 was higher in patients who either died or received a liver transplant. More recently, the same group reported that admission GDH levels for some patients weakly correlated with severity of peak injury later in the course of hospitalization (Antoine et al., 2013). In a preliminary analysis, we could not find evidence to support the prognostic use of GDH, mtDNA, or nuclear DNA fragments. However, this was tested using very small sample sizes that could not achieve the necessary statistical power to draw a conclusion. It is very possible that additional work will reveal an association between one of these parameters and outcome. One particularly promising prognostic biomarker that can serve as an example is α -fetoprotein (AFP) (Schmidt and Dalhoff, 2005; Schiødt et al., 2006). AFP is expressed and secreted by proliferating hepatocytes and is thus a mechanistic biomarker of liver regeneration. A faster increase in serum AFP levels after liver injury is associated with improved outcome (Schmidt and Dalhoff, 2005; Schiødt et

al., 2006). A major advantage of this marker is that many clinical labs are already equipped to measure it as an aid in the diagnosis of liver cancer. We found that the ratio of serum AFP concentration at the time of peak injury (“Day 1”) and two days later (“Day 3”) was predictive of outcome even in a very small cohort of 23 patients with APAP-induced liver injury (Fig. 6.2.1). Unfortunately, the greatest predictive power is achieved when relatively late AFP levels can be obtained for the second time point, after the peak injury has occurred. If the patient has developed high grade encephalopathy by this time then liver transplant may no longer be an option and the prognostic use of this biomarker becomes moot. This is likely the reason that AFP measurements are rarely ordered for APAP overdose patients.

6.2 FUTURE TREATMENTS FOR ACETAMINOPHEN HEPATOTOXICITY

Although NAC is a very effective, safe, and affordable treatment for APAP hepatotoxicity, the greatest benefit is achieved when administered within 24 h of overdose. A better understanding of the mechanisms of APAP-induced liver injury in humans may lead to new therapies for late-presenting patients. Two drugs already in use that may be useful are the MPT inhibitor cyclosporine A (CsA) (Kon et al., 2004) and the JNK inhibitor leflunomide (Latchoumycandane et al., 2007). Unfortunately, both drugs have immunosuppressive effects and are therefore not recommended for acute liver failure patients who are already immunologically compromised. Additionally, hepatotoxicity is a potential side effect of leflunomide and is therefore a contraindication

for the drug. NIM811 is a relatively new non-immunosuppressive CsA derivative that can also protect against APAP toxicity in some models (Kon et al., 2004). Clinical trials of NIM811 for other conditions including viral hepatitis are currently underway. The antibiotic minocycline is also thought to inhibit the MPT, but considerably less work has been done to study this effect. A more promising approach than interrupting the mechanisms of toxicity may be the promotion or enhancement of liver regeneration and recovery. There is some evidence from rodent studies that vascular endothelial growth factor (VEGF) (Donahower et al., 2006; Donahower et al., 2010), interleukin-6 (IL-6) (James et al., 2003a), stem cell factor (SCF) (Simpson et al., 2003; Hu and Colletti, 2008), and the Wnt/ β -catenin pathway (Apte et al., 2009) are important in liver regeneration after drug-induced liver injury. In particular, treatment of mice with recombinant VEGF, IL-6, and SCF has been shown to enhance liver regeneration and reduce injury after toxic doses of APAP (Donahower et al., 2010; James et al., 2003a; Simpson et al., 2003). It is not yet clear if a similar treatment could work in humans. Even if successful, recombinant protein therapies can be very expensive, while the current standard of care is both cheap and effective. Until it becomes more affordable, it seems unlikely that such a treatment will be adopted clinically.

6.3 STRATEGIES TO PREVENT ACETAMINOPHEN HEPATOTOXICITY

Reducing availability. In 1998, legislation intended to limit the sale of both aspirin and APAP was put into effect in the UK. The maximum number of tablets or capsules of

either drug that could be sold in a single pack at a commercial non-pharmaceutical outlet was lowered from twenty-five to sixteen. For registered pharmacies, the maximum was set at thirty-two (it was previously unlimited), and the total number that could be purchased by a single person at any one time was set at one-hundred. The new legislation also required manufacturers to add a label to all APAP-containing products warning consumers about the danger of APAP overdose. It has also been noted that around this same time there was a shift toward distribution of APAP in blister packs, although this was not a legal requirement. A number of studies have been conducted to assess the impact of these changes on the incidence of APAP-induced liver injury (Prince et al., 2000; Hawton, et al., 2001; 2004; Sheen et al., 2002; Morgan et al., 2005; 2007; Bateman et al., 2006; Hawkins et al., 2007). Interpretation of these data is complicated by differences in inclusion criteria, normalization, and endpoint (e.g. mortality, hospital admission, serum APAP levels, etc.), but overall conclusions can be drawn. Initial reports of decreased post-legislation incidence of severe APAP-induced liver injury in England and Wales were promising (Prince et al., 2000; Hawton et al., 2001; 2004; Morgan et al., 2005). Unfortunately, APAP hepatotoxicity trends in Scotland were not affected by the new regulations (Sheen et al., 2002; Bateman et al., 2006). Moreover, it has been suggested that the decrease in APAP-related deaths and hospital admissions in the southern countries of Great Britain merely reflects an overall decrease in poisoning deaths in recent years in the UK (Hawkins et al., 2007; Morgan et al., 2007). Even stricter regulations on pack size were introduced in the Republic of Ireland in 2001. Although fewer data are available from Irish hospitals and poison

control centers, a comparison of the average size of an APAP overdose taken in non-fatal self-poisonings in Ireland and the UK did not reveal a significant difference (Hawton et al., 2011). Together the data provide little solid evidence for a beneficial effect of pack size restrictions.

Additional insight can be gained from non-legislative changes in other countries. Two consecutive APAP recall periods in Australia in 2000 resulting from extortion and contamination threats against two local pharmaceutical companies provided a unique opportunity to study the effect of reduced APAP availability in a population. Although there was a modest decrease in APAP-related hospital admission rates (Kisely et al., 2003), the number of APAP-related deliberate self-poisoning calls and in-person presentations to poison control centers did not significantly change (Balit et al., 2002). Furthermore, no changes in the incidence of APAP-related hospitalizations were observed after point-of-sale restrictions for many non-prescription drugs including APAP were lifted by the National Association of Pharmacy Regulatory Authorities in Canada (Prior et al., 2004). Overall, the data suggest that changing APAP availability has little or no effect on the frequency of APAP overdose or APAP-induced liver injury. Nevertheless, other countries including the US and Germany have followed the example set by the UK by introducing their own legislation to reduce the potential for APAP overdose.

Acetaminophen / cysteine co-formulations. Another strategy to prevent APAP hepatotoxicity is co-formulation with a GSH precursor. Presently, only one such

preparation exists. Paradote / co-methiamol is a combination of APAP and methionine that is available in the UK. Unfortunately, a comparison of liver injury after overdose of Paradote and other APAP-containing products has not been made. Additionally, there is some concern over the nutritional and possibly toxic effects of long-term high-dose methionine ingestion (Jones et al., 1997). In fact, a previous formulation with a 2.5-fold larger amount of the amino acid was withdrawn from the market due to safety concerns. Because intentional APAP overdose is usually done on impulse with whatever formulation is available in the home, a combination product such as this would likely have to completely replace existing APAP-only formulations in order to be successful. Unfortunately, the unpleasant smell and taste of sulfur-containing amino acids could hinder this. More importantly, the increase in manufacturing costs could prove to be a crippling and unnecessary financial burden in developing countries where APAP is popular but overdose is rare (Saha and Kale, 1998).

6.4 WHY STUDY ACETAMINOPHEN HEPATOTOXICITY?

Currently, National Institutes of Health (NIH)-supported research commands in excess of 30 billion US taxpayer dollars each year (NIH appropriations list, 2012). Although this is less than 1% of the total federal operating budget, it is not an insignificant amount of money. As professionals entrusted with the growth of this investment, it is incumbent upon biomedical scientists to ensure that it is spent responsibly. Additionally, given that the NIH has been forced to operate on a flat or even below-inflation budget for roughly a

decade, the allocation of this money is not trivial. Within this cultural context, I am occasionally questioned regarding the wisdom of investing in research aimed to improve outcomes for victims of a predominantly self-inflicted malady. However facetious the question, it may be worth considering. Although it is true that most APAP overdoses are suicide attempts, it is also true that most overdose patients are relatively young. Poor judgement and a tendency to act on impulse are hallmarks of youth. It is questionable whether such a serious decision when young should be made binding, in effect, and permanent by a diversion of research dollars. It is my opinion that youth warrants compassion. Additionally, not all APAP overdoses are intentional. APAP is an ingredient in numerous over-the-counter and prescription medications. Regular use of multiple APAP-containing products can lead to accidental overdose. These cases should not be overlooked simply because they are the minority. Furthermore, aside from their direct application, results from research of APAP-induced liver injury may be translatable to other forms of liver injury and disease.

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