Review Article

Acetaminophen-induced Liver Injury: from Animal Models to Humans

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Abstract

Drug-induced liver injury is an important clinical problem and a challenge for drug development. Whereas progress in understanding rare and unpredictable (idiosyncratic) drug hepatotoxicity is severely hampered by the lack of relevant animal models, enormous insight has been gained in the area of predictable hepatotoxins, in particular acetaminophen-induced liver injury, from a broad range of experimental models. Importantly, mechanisms of toxicity obtained with certain experimental systems, such as *in vivo* mouse models, primary mouse hepatocytes, and metabolically competent cell lines, are being confirmed in translational studies in patients and in primary human hepatocytes. Despite this progress, suboptimal models are still being used and experimental data can be confusing, leading to controversial conclusions. Therefore, this review attempts to discuss mechanisms of drug hepatotoxicity using the most studied drug acetaminophen as an example. We compare the various experimental models that are used to investigate mechanisms of acetaminophen hepatotoxicity, discuss controversial topics in the mechanisms, and assess how these experimental findings can be translated to the clinic. The success with acetaminophen in demonstrating the clinical relevance of experimental findings could serve as an example for the study of other drug toxicities.

Keywords: Drug hepatotoxicity; Translational studies; Necrosis, Sterile inflammation.

Abbreviations: AIF, apoptosis inducing factor; ALT, alanine aminotransferase; APAP, acetaminophen; ASK1, apoptosis signal-regulating kinase 1; AST, aspartate aminotransferase; Bax, Bcl2-associated X protein; CAD, caspase-activated DNase; DAMPs, damage-associated molecular patterns; DILI, drug-induced liver injury; EGF, epidermal growth factor; DMSO, dimethyl sulfoxide; GSH, glutathione; GSK-3β, glycogen synthase kinase-3beta; HGF, hepatocyte growth factor; HMGB1, high mobility group box 1 protein; IL, interleukins; JNK, c-jun N-terminal kinase; MAP, mitogen-activated protein; Mkp-1, MAP kinase phosphatase 1; MPT, mitochondrial membrane permeability transition; mtDNA, mitochondrial DNA; NAC, N-acetylcysteine; NAD, nicotinamide-adenine dinucleotide; NAPQI, N-acetyl-p-benzoquinone imine; Nrf-2, nuclear factor-like 2; PARP, poly (ADP-ribose) polymerase; PGC, proliferator-activated receptor gamma coactivator; PHH, primary human hepatocytes; RIP, receptor interacting protein kinase; SECs, sinusoidal endothelial cells; TLR, toll like receptors; TNF, tumor necrosis factor; TUNEL, terminal-deoxynucleoitidyl transferased UTP nick end labeling; VEGF, vascular endothelial growth factors.

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Introduction

Drug-induced liver injury (DILI) is a significant clinical problem worldwide. Fundamentally, DILI can be divided into two categories: idiosyncratic and predictive DILI. Idiosyncratic DILI is mainly caused by therapeutic doses of drugs in susceptible patients after days or months of treatment.[1] It generally affects only <1 in 10,000 patients taking the drug and the symptoms can range from mild, transient liver injury and dysfunction to acute liver failure.[2] The epidemiology and disease patterns of many idiosyncratic hepatotoxic drugs have been described; although the involvement of innate and adaptive immune mechanisms is thought to be critical in most cases, the actual mechanisms of liver injury remain largely unclear.[3] The limited progress in the understanding of the pathogenesis of idiosyncratic DILI comes from the lack of relevant animal models and the rarity of the disease, which makes it difficult to study it in the first place. In addition, genome-wide association studies in a large cohort of DILI cases have failed to identify general risk factors for idiosyncratic DILI.[4] These findings suggest that genetic determinants of DILI risk may be drug specific.[4]

In contrast, predictive DILI occurs mainly after intentional or accidental overdose of a drug. The most clinically relevant drug in this category is acetaminophen (APAP, paracetamol). In the US, APAP overdose is responsible for 78,000 emergency room visits and about 500 deaths per year.[5] In addition, APAP hepatotoxicity is the most frequent cause of acute liver failure of any etiology, accounting for approximately 50% of all cases.[6] Unlike idiosyncratic DILI, APAP hepatotoxicity can be modeled in rodents, primary hepatocytes and in certain cell lines. An increasing number of translational studies demonstrate that these animal models are valuable tools to investigate the mechanisms of toxicity and to identify potential therapeutic targets.[7] However, despite the unique situation of having relevant *in vivo* and *in vitro* experimental systems available, there are still many controversies that hamper progress in understanding the mechanisms of APAP hepatotoxicity and, consequently, the reliable identification of clinically relevant therapeutic targets. This review will address these controversial topics, including intracellular signaling mechanisms of toxicity, mode of cell death, and the role of sterile inflammation from animal models to the most recent clinical findings.

Acetaminophen: Intracellular mechanisms of toxicity

Many drugs cause hepatotoxicity by forming reactive metabolites, which either initiate cell toxicity mechanisms directly or, through formation of protein adducts (haptens), can

trigger immune-mediated toxicity.[8,9] For APAP, it is well established that the cell death mechanisms are initiated by the formation of the presumed reactive metabolite N-acetyl-p-benzoquinone imine (NAPQI), which is generated mainly by the cytochrome P450 enzymes Cyp2e1 and 1a2 in mice and humans.[10,11] Although NAPQI can be readily detoxified by conjugation with glutathione (GSH), the availability of GSH is limited in case of an overdose.[12] The resulting depletion of GSH enables reactions of NAPQI with protein sulfhydryl groups of cysteine, causing the binding of APAP to cellular proteins.[12] Originally, it was thought that the protein adducts formed could directly trigger cell death, but no critical protein target could be identified.[13] In contrast, it was recognized that the binding to mitochondrial proteins after APAP overdose correlated with toxicity,[14] suggesting that mitochondria could be a critical target. This observation was in agreement with the impaired mitochondrial respiration and increased mitochondrial oxidant stress observed after APAP overdose in mice (Fig. 1).[15,16] The enhanced superoxide formation leads to generation of the potent oxidant peroxynitrite in mitochondria.[17] This oxidant stress, together with the uptake of lysosomal iron,[18] causes the formation of the mitochondrial membrane permeability transition (MPT) pore, which is responsible for the collapse of the membrane potential and cessation of ATP synthesis.[19–21] The critical role of this oxidant stress for mitochondrial dysfunction and cell necrosis has been shown by the protective effects of delayed treatment with GSH or N-acetylcysteine (NAC)[22–24] and the aggravation of injury in mice with reduced MnSod (Sod2) activity in mitochondria.[25,26]

Although the importance of the mitochondrial oxidant stress is well established,[27] there is a discrepancy between early GSH depletion and mitochondrial dysfunction with some oxidant stress and the delayed necrosis.[28] This led to the hypothesis that the initial oxidant stress is insufficient to trigger the MPT and a "second hit" is needed to amplify this oxidant stress. This second hit appears to be the mitogen-activated protein (MAP) kinase c-jun-N-terminal kinase (JNK) (Fig. 1), which is activated (phosphorylated) in the cytosol very early during APAP toxicity in mice.[29] P-JNK then translocates to the mitochondria and triggers the MPT by amplifying the mitochondrial oxidant stress.[29,30] The effect of P-JNK on the mitochondria is mediated by interaction with the mitochondrial protein Sab (SH3 domain-binding protein that preferentially associates with Btk), which is located in the outer membrane.[31] Knock-down of Sab attenuated JNK activation and prevented APAP-induced liver injury, suggesting that Sab is a critical link between JNK activation and mitochondrial dysfunction.[31] The critical role of JNK in the pathophysiology of APAP-induced liver injury has been documented by the protective effect of a JNK inhibitor and by gene knock-down experiments.[32] However, JNK does not seem to be directly activated by the early events of APAP toxicity. Instead, a number of upstream kinases have been identified, all of which can be activated directly or indirectly by the early oxidant stress generated during APAP-induced mitochondrial dysfunction. These kinases include apoptosis signal-regulating kinase 1 (ASK1),[33] glycogen synthase kinase-3beta (GSK-3β),[34] mixed-lineage kinase 3 (MLK 3),[35] receptor interacting protein kinase (RIP) 1[36] and RIP3.[37] However, several phosphatases that counteract JNK activation have been shown to protect against APAP toxicity, including MAP kinase phosphatase 1 (Mkp-1) and protein tyrosine phosphatase 1B.[38,39] Thus, the amplification of the

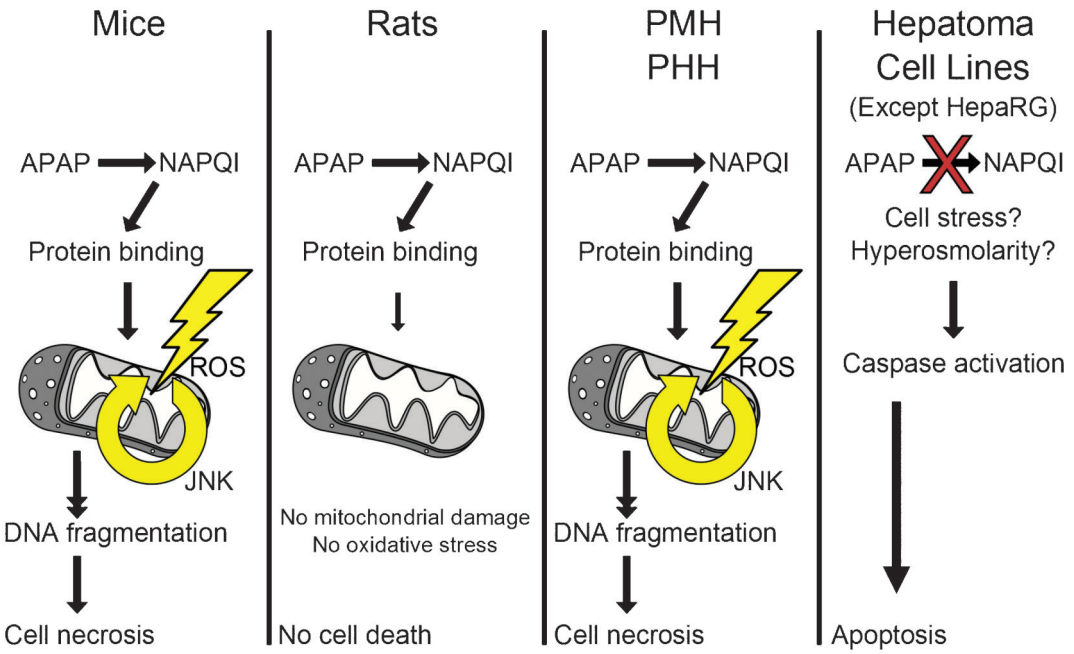

Fig. 1. Experimental models to study acetaminophen (APAP) hepatotoxicity. The most common models used to study APAP hepatotoxicity are mice, rats, primary mouse and human hepatocytes (PMH and PHH, respectively), and hepatoma cell lines. However, the mechanisms of injury and mode of cell death differ. In mouse models and in humans, APAP-induced liver injury involves mitochondrial damage, oxidative stress, c-jun N-terminal kinase (JNK) activation, and nuclear DNA fragmentation. The mode of cell death in these models is oncotic necrosis. However, rats develop little or no oxidative stress and thus no injury, while hepatoma cells may develop injury but through different mechanisms than mouse or human hepatocytes. In the latter case, the mode of cell death is almost always apoptosis. The results suggest that mice and PHH are the best available models for the study of APAP toxicity.

mitochondrial oxidant stress involves a complex and redundant network of kinases and phosphatases. However, the JNK-dependent and independent kinase network may not only be involved in pro-death signaling pathways but can also promote survival mechanisms such as promoting autophagy, as discussed later.[40]

A consequence of the mitochondrial dysfunction is nuclear DNA damage (Fig. 1). Genomic DNA fragmentation as a hallmark of APAP toxicity in mice and mouse hepatocytes has been recognized for some time.[41,42] DNA fragments can be internucleosomal fragments of approximately 180 base pairs and multiples thereof, as detected by DNA ladder.[41] This implicates a role for endonucleases in this process rather than oxidant stress.[17] The nuclear translocation of endonuclease G and apoptosis inducing factor (AIF) from the mitochondria correlates with the nuclear DNA fragmentation.[43,44] Endonuclease G and AIF, which are mitochondrial intermembrane proteins, are released initially by mitochondrial outer membrane permeabilization through a Bcl2-associated X protein (bax) pore and later, after the MPT and mitochondrial matrix swelling, by rupture of the outer membrane.[45] The DNA damage can activate DNA repair processes including poly(ADP-ribose) polymerase (PARP), which may accelerate cell death by excessive consumption of nicotinamide-adenine dinucleotide (NAD). Although APAP-induced DNA damage activates PARP and depletes NAD, the absence of the enzyme actually enhanced the injury, indicating that activation of DNA repair is beneficial.[46] Thus, DNA damage caused by mitochondria-derived endonuclease and others contribute to cell necrosis, and attempts to repair the damage limit the injury and support recovery.

Damaged mitochondria are critical for the pathophysiology of APAP-induced cell death. Endogenous defense systems (autophagy) can remove damaged cell organelles and modified protein by enveloping them with membranes (autophagosomes) and fusing these structures with lysosomes.[47] Autophagy is activated after APAP overdose.[48] Inhibition of autophagy attenuates liver injury, suggesting that autophagy is an adaptive mechanism to stress and limits injury.[48] In addition to classical autophagy mechanisms, newly recognized mitochondrial spheroids can also contribute to the removal of damaged mitochondria.[49] However, these processes are only effective in the outer area of risk.[47] Additional effects, such as drp1 translocation to mitochondria that promotes mitochondrial fission, appear to enhance cell death.[37] The role of mitochondria biogenesis remains unclear because the protection against APAP-induced liver damage by peroxisome proliferator-activated receptor gamma coactivator (PGC)-1alpha,was caused by the activation of nuclear factor-like 2 (Nrf-2)-dependent antioxidant genes.[50] Thus, adaptive mechanisms to drug-induced cellular stress are clearly affecting liver injury and could be potential drug targets.

The vast majority of APAP-induced liver injury studies focus on hepatocytes. However, non-parenchymal cells may also play a role. In addition to resident and newly recruited inflammatory cells, which will be discussed later, sinusoidal endothelial cells (SECs) can be affected by an APAP overdose.[51] Direct morphological evidence of SEC damage and indirect evidence of SEC dysfunction (hemorrhage) have been reported.[52–54] Nitrotyrosine staining in SECs precedes staining of hepatocytes, suggesting that SEC damage may be independent of parenchymal cell injury.[55] Isolated SECs exposed to APAP from specific mouse strains show substantial GSH depletion[56] and lactoferrin protects SECs from APAP-induced injury in specific mouse strains.[57] The strain-dependent SEC injury correlates with Cyp2E1 levels in these SECs.[57] In addition, severe SEC injury in susceptible strains leads to extensive congestion and microvascular dysfunction and also impacts parenchymal cell injury.[52,56,57]

All of the discussed mechanisms of APAP-induced liver injury were investigated using a number of different *in vivo* and *in vitro* models. Selecting the most appropriate experimental model, which closely resembles the human pathophysiology, is critical for the clinical relevance of the mechanisms derived from these models.

In vivo models of drug hepatotoxicity

The most frequently used preclinical species for drug hepatotoxicity are rats and mice. Testing new drugs in rats is still required for standard safety evaluations by regulatory agencies. In the case of APAP-induced DILI, the rat is clearly a poor model.[12] Rats can metabolize APAP to form a reactive metabolite and cause GSH depletion and protein adducts formation, even in mitochondria.[58] However, they do not develop mitochondrial oxidant stress or JNK activation and consequently develop very little if any liver injury (Fig. 1).[58] It remains unclear at this point if the lack of progression in rat liver is due to the delay in protein adducts formation, or if as of yet unidentified specific protein targets are not hit by NAPQI. Although there is the potential to learn more about the initiating events, the mechanisms of injury in rats and the severity of injury do not reflect the human pathophysiology. Nevertheless, large numbers of studies are still being published that test the hepatoprotective potential of natural products using the rat model (reviewed[59]). In addition to using an insensitive animal model for their drug hepatotoxicity studies, another caveat of most of these investigations is that the plant extracts are largely uncharacterized and the potential effect as a P450 inhibitor of one or more of the chemicals in the extract is rarely tested.

The mouse model of APAP hepatotoxicity was established in the 1970s.[12] Based on the early mechanistic understanding, NAC was developed as an antidote against APAP overdose.[60] More recently, the use of gene knock-out or transgenic mice has helped to further popularize the mouse model for investigation of DILI. Most of the mechanistic insight into the pathophysiology has been gained from experiments with the murine system. Fortunately, many fundamental mechanisms of APAP hepatotoxicity in the mouse have been confirmed in patients with APAP overdose[61] and in human hepatocytes.[62,63] In addition, the severity of the overall liver injury is very similar between mice and humans.[61] However, the injury process progresses much faster in mice than in humans, with peak alanine aminotransferase (ALT) values, as indicator of liver cell death, between 12 and 24 h in the mouse[64] and 36–48 h in humans after overdose.[65] The reason for this delay in humans does not appear to be reduced drug metabolism and reactive metabolite formation but may be related to delayed mitochondrial protein binding and delayed JNK activation.[63] Thus, the mouse model of APAP hepatotoxicity is superior to other animal models and most closely resembles the human pathophysiology in terms of liver injury and recovery. In the future, large animal models may be needed to better mimic the acute liver failure observed in humans.[66]

In vitro models of APAP-induced hepatocyte cell death

In vitro models are indispensable tools to identify drug toxicity and assess molecular mechanisms. Many of the mechanistic details described in previous paragraphs were investigated in freshly isolated mouse hepatocytes. However, in addition to the obvious advantages of an isolated cell preparation, there are limitations that need to be considered.[67] Among others, primary hepatocytes lack nonparenchymal cells, they are exposed to artificial media during isolation and culture, and they are generally cultured under hyperoxic conditions (room air oxygen levels).[67] This may lead to numerous gene expression changes, enhanced oxidant stress, and many other potential modifications.[67,68] Loss of cytochrome P450 enzyme activity over time in primary cells in culture is one of the most critical gene expression changes in studies of drug toxicity.[69] This will affect the sensitivity to drugs such as APAP. Recent advances in sandwich culture techniques and 3D culture ameliorate some of these problems,[67] but this improvement comes with a price. These additional manipulations are more cumbersome and time consuming and likely increase the cost of the experiments. Nevertheless, studies with primary cultured hepatocytes can provide new mechanistic understanding if the limitations are considered and the data are verified *in vivo*.

In contrast to primary hepatocytes, many established hepatocyte cell lines are readily available. An advantage of these cells is that they proliferate easily, can be cryopreserved and thawed out without much loss of functionality, are easy to work with, and are available in large quantities. However, most of these immortalized cell lines, including the most popular hepatoma cell lines (e.g. HepG2, Hep3B, Huh7) have a drastically modified gene expression profile and lack, among other critical proteins, essential drug metabolizing enzymes and transporters (Fig. 1).[70,71] The caveat of working with these types of cells is that they still respond to cellular stress such as APAP exposure with changes in gene or protein expression.[72] However, the relevance to human pathophysiology is questionable because the nature of the stress is different than a cell that has the capacity to generate a reactive metabolite. This discrepancy is most clearly demonstrated by the fact that APAP causes apoptotic cell death in hepatoma cell lines[73] but necrosis in primary hepatocytes.[19,28,42,63] If these hepatoma cells are transfected with a specific cytochrome P450, e.g. CYP2E1, some of the sensitivity can be restored.[74]

HepaRG cells are hepatoma cells isolated and differentiated from a patient with hepatocellular carcinoma that are metabolically competent, i.e. express a large number of drug metabolizing enzymes, including P450 enzymes, and transporter similar to primary hepatocytes.[70] Although there are some limitations of these cells, such as being derived from a single donor and differentiating into both hepatocytes and biliary epithelial cells, the fact that they are metabolically competent hepatoma cells with unlimited availability is a major advantage over most other hepatoma cell lines.[70] Consequently, upon exposure to APAP, HepaRG cells develop cell necrosis with many mechanistic characteristics similar to mouse hepatocytes but with a time line closely resembling that of human overdose patients.[62]

Primary human hepatocytes (PHH) are the most relevant *in vitro* model for studying human pathophysiology. However, availability is limited and it can be prohibitively expensive. Cryopreservation of cells can make them more easily available, but in general, cryopreserved cells are of lower quality.While these cryopreserved cells may be suitable for drug metabolism studies, they are not useful for many other types of experiments. This is especially important for drug toxicity studies. Freshly isolated cultured human cells are clearly superior as they reflect closely the gene expression pattern of the liver *in vivo*. Recent studies with APAP hepatotoxicity documented the very close correlation between the time line of toxicity (onset and peak of cell death) in PHH and onset and peak of ALT elevation as an indicator of liver injury in overdose patients.[63,65] However, the overall sequence of events leading to cell death after APAP in PHH is very similar to mouse hepatocytes, including GSH depletion, cytosolic and mitochondrial protein adducts formation, JNK activation and translocation to mitochondria, mitochondrial dysfunction, and collapse of the mitochondrial membrane potential preceding cell death (Fig. 1).[63] Importantly, APAP induces necrotic cell death in PHH similar to mouse hepatocytes.[63] Overall, like the translational work with overdose patient, these studies with PHH confirm that the mouse model of APAP hepatotoxicity is the most relevant animal model for studying these mechanisms.

Despite using clinically relevant experimental models, some topics remain controversial. This is almost always an issue of experimental design and interpretation of experimental data.

Mechanisms of drug-induced cell death: Apoptosis versus necrosis

The mode of cell death during APAP hepatotoxicity is controversial. Although the vast majority of animal studies have concluded that the injury is caused by necrosis, an increasing number of reports suggest that apoptotic cell death plays a significant role. The key problem is that there are very few specific parameters for apoptosis, and even those can be misinterpreted. For example, frequently used parameters such as mitochondrial cytochrome c release, increased bax protein expression and bax translocation to the mitochondria, BH3 interacting domain death agonist (bid) cleavage, DNA strand breaks (terminal deoxynucleotidyltransferase dUTP nick end labeling (TUNEL) assay), and DNA laddering (internucleosomal DNA cleavage) are all observed during apoptosis and necrosis, not allowing any distinction between these forms of cell death.[75]

One of the more specific features of apoptosis is the activation of caspases, including the initiator caspase-8 and the executioner caspase-3.[76] An extensive increase in caspase activity in combination with processing of procaspases to the active fragments can be easily detected during hepatic apoptosis.[77] Consequently, caspase inhibitors are highly effective in preventing apoptosis in the liver.[77] In contrast, during APAP-induced liver injury, no increase in caspase-3 activity is detectable, and caspase inhibitors offer no protection.[78] A caveat of the use of pancaspase inhibitors is the fact that they generally require the solvent dimethyl sulfoxide (DMSO), a potent inhibitor of P450 enzymes,[79] and higher doses of caspase inhibitors may inhibit other proteases through off-target effects.[80]

DNA fragmentation is observed during apoptotic and necrotic cell death. Predominantly small fragments are formed during apoptosis, and larger fragments are generated during APAP-induced necrosis.[81] The characteristic smaller

DNA fragments during apoptosis (multiples of 180–185 base-pairs) are formed by caspase-activated DNase (CAD).[76] In contrast, DNA fragmentation during APAP-induced cell death is caused by mitochondria-derived endonuclease G and AIF, as discussed above.[43,44] One of the most frequently used assays to visualize DNA strand breaks is the TUNEL assay. Because the assay detects DNA strand breaks, apoptotic and necrotic cells will stain positive. However, the staining patterns are different, with mainly nuclear staining in apoptotic cells and both nuclear and cytosolic staining in necrotic cells.[75] The cytosolic staining is likely caused by the release of large DNA fragments due to karyorrhexis and karyolysis, which are still recognized by the terminal deoxynucleotidyltransferase.

In patients, nuclear DNA fragments were detectable in plasma after APAP overdose.[61] However, similar to the mouse model, extensive procaspase-3 protein levels were observed in blood of patients with APAP-induced liver injury but not in patients without liver injury.[61] In support of these findings, no caspase-3 enzyme activity was measured in any APAP overdose patient.[61] In addition, the caspase-cleaved cytokeratin-18 fragment was either not detectable or represented only a minor fraction (<15%) of the total full-length cytokeratin-18 released into the blood in these patients.[82] Together these results in patients and, as discussed in the previous paragraph, in PHH support the hypothesis that, similar to mice and mouse hepatocytes, the primary mode of cell death in APAP hepatotoxicity in humans is necrosis.

As the example of APAP-induced liver injury demonstrates, a clear distinction between apoptotic and necrotic cell death cannot be achieved by assessing a single parameter. Use of a combination of parameters, e.g., caspase activation, cell morphology, and DNA fragmentation, is mandatory for solid conclusions. In addition, the use of positive controls will give insight into how much parameters change with a certain percentage of apoptosis and allow more confident conclusions regarding the mode of cell death. Knowledge of the type of cell death is not only important because of the intracellular signaling pathways involved, but also because it determines the degree of inflammation that occurs in response to the tissue damage.

Pathophysiological implications of sterile inflammation

Extensive cell necrosis causes the release of cell contents, as indicated by the massive increase of liver enzymes in serum. In recent years, it has been recognized that many of these cellular components can activate various pattern recognition receptors, such as toll like receptors (TLRs).[83,84] These damage-associated molecular patterns (DAMPs) released by cells include high mobility group box 1 (HMGB1) protein, nuclear DNA fragments, mitochondrial DNA (mtDNA), heat shock proteins, hyaluronic acid, and many more.[83,84] DAMPs can activate TLRs on macrophages (e.g. DNA binds to TLR9, HMGB1and heat shock proteins work through TLR4) to induce the transcription of pro-inflammatory cytokines and activate the inflammasome, which can trigger the cleavage of pro-forms of certain interleukins (IL) and other cytokines (e.g. pro-IL-1β, and pro-IL-18). Extensive cytokine formation and release recruits neutrophils and monocytes into the liver with the potential to aggravate the initial injury. This general scheme of a sterile inflammatory response has been investigated in the mouse model of APAP-induced liver injury *in vivo*. During APAP hepatotoxicity, HMGB1, DNA fragments, heat shock proteins, and others are detectable in plasma,[61,85,86] as are the formation of cytokines[53] and the recruitment of first neutrophils[53,87] and later monocytes.[88] Thus, there is no doubt that the severe cell necrosis induces an extensive sterile inflammatory response in mice. However, it is controversial whether neutrophils and macrophages actually enhance the injury or contribute to the repair and recovery of the damaged liver by removing cell debris. Although a few studies have suggested direct involvement of neutrophils, most studies do not find evidence for neutrophil cytotoxicity aggravating APAP-induced liver injury (reviewed[84]). Importantly, liver neutrophils are not activated during the injury phase, and deficiency of key neutrophil adhesion molecules has no effect on APAP toxicity.[89] In addition, release of acetylated HMGB1 as indicator of macrophage activation is delayed in mice and occurs at the end of the injury phase.[85] Taken together, these findings suggest that neutrophils, in addition to monocytes, are recruited into the liver in preparation for regeneration.[84,88] Cytokines generated during the sterile inflammatory response may modulate intracellular events and promote injury by inducing inducible nitric oxide synthase and enhancing peroxynitrite formation.[90]

In APAP overdose patients, DAMPs such as nuclear DNA fragments, mtDNA, and HMGB1 are extensively released into the plasma with a time course similar to ALT and aspartate aminotransferase (AST).[61,82] Interestingly, serum levels of these DAMPs are better predictors of poor outcome (liver failure and death) than ALT or AST.[82,91] Certain cytokines, including IL-6, IL-8, and monocyte chemoattractant protein 1, are substantially elevated during APAP toxicity.[92] However, neutrophils are not activated in patients during the peak of liver injury.[93] Neutrophil activation correlates with declining ALT levels, i.e. with the recovery phase during APAP overdose in patients.[93] These findings suggest that no direct neutrophil cytotoxicity is involved in the human pathophysiology. Similarly, hepatic macrophages derived from resident Kupffer cells and recruited monocytes contribute to the tissue repair process during APAP-induced acute liver failure rather than aggravate the injury.[94] In addition to removing necrotic tissue, the lost liver cells need to be replaced by proliferation of surviving cells.

Regeneration

Liver regeneration is an intricate and well-orchestrated process. The remnant liver will proliferate to reestablish the original architecture and function after surgical or toxicological insult.[95,96] In some cases, the remaining hepatocytes will proliferate in a hyperplastic manner to restore the functional liver mass, while in others, liver progenitor cells will participate to generate new hepatocytes.[96–98] Generally, the production of tumor necrosis factor (TNF) α and IL-6 by non-parenchymal cells will prime the hepatocytes for proliferation, and cooperative signals from vascular endothelial growth factors (VEGF), epidermal growth factors (EGF), hepatocyte growth factors (HGF), and cytokines will stimulate hepatocytes to overcome cell-cycle checkpoints to proliferate and regain the normal liver size. This is followed by termination of regeneration, which is primarily mediated by TGF-β and renewed quiescence.[95,97]

Considerable progress has been made in our understanding of the central mediators of liver regeneration. TNF

receptor knockout mice exhibit exaggerated injury and impaired regenerative responses after APAP, which can be explained by delayed mitogenic cytokine signaling.[99] In mice, APAP time-dependently induces IL-6 levels in serum and liver.[23,100,101] IL-6 knockout mice are more susceptible to APAP challenge[101] and display a delayed injury resolution due to impaired liver regeneration.[102] In addition, APAP triggers a significant upregulation of VEGF in mice,[103] while pharmacological inhibition of VEGF resulted in similar injury but decreased recovery.[103] Administering human recombinant VEGF mitigates the injury after APAP and facilitates regeneration in mice.[104] Indirectly, scavenging peroxynitrite by glutathione treatment also enhances regeneration in mice after APAP.[100]

More recent data have provided insights about additional players in liver regeneration after APAP. The Wnt/β-catenin pathway is emerging as a central player in the regulation of liver development, growth, and regeneration;[105] and its importance in APAP-induced hepatotoxicity has been explored. Biphasic increases of β-catenin expression are observed in mice, and β-catenin knockout mice have reduced liver regeneration.[106] Interestingly, the evaluation of human liver biopsy samples has indicated that β-catenin activation is strongly associated with patients who undergo spontaneous regeneration without liver transplantation.[106] Similarly, increased serum alpha-fetoprotein, a sign of hepatic regeneration,[107] correlates with a favorable outcome in APAP overdose patients.[108,109] From studies like these, a better understanding of liver regeneration may lead to improved prognostic indicators that can supplement existing criteria to determine candidates for liver transplantation among APAP overdose patients.

Conclusions

APAP-induced liver injury is the most frequently encountered drug hepatotoxicity and cause of acute liver failure in the United States. In addition, APAP is the most used experimental model to generate DILI. During the last decade, there has been substantial progress in understanding the intracellular mechanisms of cell death, sterile inflammation, and recovery after APAP overdose. The most relevant models that have fueled this new knowledge are *in vivo* mouse models, primary mouse and human hepatocytes, and certain metabolically competent hepatoma cell lines (e.g. HepaRG) (Fig. 1). Additional models, such as humanized mice, are in development and could expand the experimental tool box. Importantly, translational studies with patients provide support for the clinical relevance of many experimental models. It can be expected that this increased insight into toxicity mechanisms in experimental models and in humans will lead to improved therapeutic interventions and better strategies to detect potential relevant hepatotoxic drugs in development. Furthermore, the success with APAP from understanding experimental systems to the human pathophysiology could serve as an example for making relevant progress in other areas of drug hepatotoxicity.

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Conflict of interest

None

Author contributions

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