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Current issues with acetaminophen hepatotoxicity—a clinically relevant model to test the efficacy of natural products

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Abstract

There is a significant need to evaluate the therapeutic potential of natural products and other compounds purported to be hepatoprotective. Acetaminophen-induced liver injury, especially in mice, is an attractive and widely used model for this purpose because it is both clinically relevant and experimentally convenient. However, the pathophysiology of liver injury after acetaminophen overdose is complex. This review describes the multiple steps and signaling pathways involved in acetaminophen-mediated cell death. The toxicity is initiated by formation of a reactive metabolite, which depletes glutathione and binds to cellular proteins, especially in mitochondria. The resulting mitochondrial oxidant stress and peroxynitrite formation, in part through amplification by c-jun-N-terminal kinase activation, leads to mitochondrial DNA damage and opening of the mitochondrial permeability transition pore. Endonucleases from the mitochondrial intermembrane space and lysosomes are responsible for nuclear DNA fragmentation. Despite the oxidant stress, lipid peroxidation is not a relevant mechanism of injury. The mitochondrial dysfunction and nuclear DNA damage ultimately cause oncotic necrotic cell death with release of damage-associated molecular patterns that trigger a sterile inflammatory response. Current evidence supports the hypothesis that innate immune cells do not contribute to injury but are involved in cell debris removal and regeneration. This review discusses the latest mechanistic aspects of acetaminophen hepatotoxicity and demonstrates ways to assess the mechanisms of drug action and design experiments needed to avoid pitfalls and incorrect conclusions. This review should assist investigators in the optimal use of this model to test the efficacy of natural compounds and obtain reliable mechanistic information.

Keywords

Drug-induced liver injury; Sterile inflammation; Oncotic necrosis and apoptosis; Oxidant stress and lipid peroxidation; Mitochondrial dysfunction

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Conflict of interest statement

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Introduction

The search for new drugs and novel therapeutic intervention strategies increasingly includes testing plant extracts and other natural products. In addition, more products of Traditional Eastern Medicine are being evaluated. Independent of whether extracts are considered or if individual ingredients of a mixture are tested, the pharmacological efficacy of these chemicals needs to be investigated. For compounds that are assumed to have hepatoprotective effects, the model of acetaminophen (APAP) overdose in rodents, especially mice, is one of the most popular experimental *in vivo* systems used today (Campos et al. 1989; Chen et al. 2009; Gao and Zhou 2005; Hau et al. 2009; Hsu et al. 2008; Küpeli et al. 2006; Wang et al. 2010; Wu et al. 2008, 2010; Yuan et al., 2010). The advantage of this model is that APAP is a dose-dependent hepatotoxicant, the experiments are technically easy to perform and, most importantly, it is a clinically relevant model. However, after more than 35 years of research there is substantial information in the literature on mechanisms of APAP hepatotoxicity (Hinson et al. 2004; Jaeschke et al. 2003; Jaeschke and Bajt 2006; Jaeschke and Bajt 2010; Nelson, 1990; Nelson and Bruschi, 2001). Some of these mechanisms are well established and others are more or less controversial; some are correct and some are skewed by experimental conditions making them not clinically applicable. In addition, many aspects of APAP-induced cell death and liver injury are still unknown. Thus, the mechanisms of APAP hepatotoxicity are extremely complex and the interpretation of *in vivo* data is difficult. Unfortunately, the model is being used as a tool by investigators who are not necessarily experts in APAP toxicity leading to frequent misinterpretation of data (Jaeschke et al. 2010b). Therefore, the purpose of this review on APAP hepatotoxicity is to discuss some of the established and controversial mechanisms and the potential pitfalls one should be aware of when using this model.

Models of acetaminophen-induced liver injury

To study mechanisms of APAP toxicity, the mouse *in vivo* or primary mouse hepatocytes are most frequently used. Various strains of mice (outbred or inbred strains) are susceptible to APAP toxicity but some strain differences exist (Harrill et al. 2009). Mice are fasted overnight to reduce hepatic glutathione levels and are treated *i.p.* with doses of 200–400 mg/kg APAP dissolved in warm saline. Liver injury develops between 3–5 h and peaks at 12 h after APAP administration. Fasting allows lower doses of APAP to be used and results in less variation of the injury. Fed mice can also be used but this requires higher doses (500–600 mg/kg) and in general causes more variable results. APAP can also be administered by gavage, which results in more prolonged absorbance and metabolism but yields the same injury. The rat model is also frequently used but rats are generally less susceptible. A dose of 1 g/kg APAP or higher is necessary to get moderate injury. These doses are well above relevant human overdoses, which range mainly between 150–500 mg/kg. In addition, the rat model is rarely used for mechanistic studies. Thus, most of the discussion in this review focuses on data obtained with the mouse model, which has very close similarities to mechanisms of APAP toxicity in a human hepatocyte cell line (HepaRG cells) (McGill et al. 2011b) and to human overdose patients (McGill et al. 2011a).

Metabolic activation of acetaminophen

One of the earliest works on mechanisms of APAP hepatotoxicity demonstrated that a small fraction of the dose is metabolized by the cytochrome P450 (cyp) system to form a reactive metabolite (Jollow et al. 1973; Mitchell et al. 1973a,b). The metabolite was identified as *N*-acetyl-*p*-benzoquinone imine (NAPQI) (Dahlin et al. 1984). The primary enzyme involved is cyp2E1, but others appear to have a role (Thummel et al. 1993; Wolf et al. 2007; Zaher et al. 1998). Glutathione (GSH) initially traps NAPQI and the GSH adduct is excreted.

However, after GSH is depleted, NAPQI reacts with cellular proteins and forms an APAP adduct (Corcoran et al. 1985; Jollow et al. 1973; Mitchell et al. 1973b). Although the original hypothesis that the general protein binding of NAPQI causes toxicity has been questioned, it remains undisputed that metabolic activation of APAP is the critical initiating event of cell death (Smith et al. 1985; Nelson 1990; Nelson and Bruschi 2001) (Figure 1). Thus, any compound that can induce P450 enzymes can aggravate APAP-induced liver injury, e.g. ethanol (Wolf et al. 2007). However, more important for most natural product testing is that any chemical or its solvent that inhibits cytochrome P450 enzymes will substantially protect or even completely eliminate APAP toxicity (Mitchell et al. 1973a). It is important to realize that if a tested drug or mixture inhibits metabolic activation (Chen et al. 2009; Hau et al. 2009), which is the most proximal event in the toxicity mechanism, all subsequent events will be eliminated. It is then impossible to assess if the drug may have additional effects, e.g. may also act as an antioxidant. Thus, one of the most critical initial experiments that must be done is to evaluate if the drug or mixture under consideration affects the metabolic activation of APAP. Protein adducts can be identified by western blotting or immunohistochemistry using an anti-APAP adduct antibody (Bartolone et al. 1989; Roberts et al. 1991) or the cysteine-APAP adducts can be directly quantified using high-performance liquid chromatography with electrochemical detection (Muldrew et al. 2002) or mass spectrometry (McGill et al., 2011b). In addition, one of the most accurate assessments of early NAPQI formation is the depletion kinetics of hepatic glutathione (Jaeschke 1990; Saito et al. 2010b). However, it needs to be kept in mind that this is only accurate during the first 30 minutes (Figure 2). Measurement of GSH at a later time point may not detect delayed metabolism or may be affected by GSH loss due to cell injury (Jaeschke 1990) (Figure 2).

Protein adducts of acetaminophen and oxidant stress

The early “protein binding” hypothesis was repeatedly questioned because the overall fraction of the administered dose that ends up covalently bound to cellular proteins is small and certain interventions appear to be able to separate protein binding from cell injury (Jorgensen et al. 1988). As a result, a competing hypothesis was introduced. Wendel and coworkers hypothesized that reactive oxygen generated during the metabolism of APAP can cause lipid peroxidation (LPO), which may be the actual mechanism of cell death independent of protein binding (Wendel et al. 1979; Wendel and Feuerstein 1981). Although there was convincing evidence for extensive LPO in these animals, what is not very well recognized is the fact that these mice were fed a vitamin E-deficient and polyunsaturated fatty acid-rich diet making them particularly susceptible to LPO. In retrospect, these experiments are an example of unrealistic experimental conditions favoring a mechanism of injury that is of limited relevance when animals are fed a regular diet (Jaeschke et al. 2003). Under normal conditions, i.e. in the absence of massive impairment of antioxidant defense systems, protein binding appears to be the most critical event in the initiation of APAP-induced cell death.

After the initial concerns, the protein binding hypothesis was modified. Rather than general binding to proteins, adducts of very specific vital proteins in the cell were proposed to be critical. However, despite the fact that a substantial number of APAP-adducted proteins were subsequently identified, no obvious target was found that could explain the rapid cell death (Cohen et al. 1997; Qiu et al. 1998). With the recognition of a mitochondrial oxidant stress (Jaeschke 1990) and the fact that formation of mitochondrial protein adducts correlated with liver injury (Tirmenstein and Nelson 1989; Qiu et al. 2001), the current concept emerged (Jaeschke et al. 2003). In this hypothesis, reactive metabolite formation and protein binding, especially to mitochondrial proteins, is an important initiating event that by itself is not sufficient to cause cell death. In contrast, this protein binding induces

mitochondrial oxidant stress and peroxynitrite formation, which amplifies the original stress eventually leading to necrotic cell death. The pathophysiological importance of this mitochondrial oxidant stress has been documented by delayed treatment with antioxidants, which scavenged reactive oxygen and reactive nitrogen species without relevant effect on protein binding (Bajt et al. 2003; James et al. 2003b; Knight et al. 2002; Saito et al. 2010c; Salminen et al. 1998). Thus, testing natural products for antioxidant effects in the APAP model has to rule out any effect on metabolic activation and protein binding when used as a pretreatment. Alternatively, animals can be treated with the drugs only after the metabolic phase is over in order to avoid interference with metabolic activation.

Sources of reactive oxygen and reactive nitrogen species

APAP overdose causes an oxidant stress (Jaeschke 1990) and peroxynitrite formation (Hinson et al. 1998). The increase in tissue glutathione disulfide (GSSG) levels *in vivo*, as a specific marker for hydrogen peroxide, is caused by a selective accumulation of GSSG in mitochondria (Jaeschke 1990; Knight et al. 2001). This suggests that enhanced amounts of superoxide are being generated by the electron transport chain and released into the mitochondrial matrix (Figure 3). This conclusion is confirmed by the increased MitoSox_{red} fluorescence observed after APAP treatment in cultured hepatocytes (Yan et al. 2010). The most likely source of the superoxide is complex I of the mitochondrial electron transport chain (Murphy 2009). Because superoxide is an anion which cannot escape the mitochondria, the reaction with nitric oxide (NO) to form peroxynitrite must also occur in mitochondria (Cover et al. 2005). These reactive oxygen and reactive nitrogen species directly cause mitochondrial DNA damage (Cover et al. 2005) and early activation of c-jun-N-terminal kinase (JNK) (Hanawa et al. 2008). The subsequent translocation of activated JNK (P-JNK) to the mitochondria (Hanawa et al. 2008) in turn further promotes the mitochondrial oxidant stress (Saito et al. 2010a). Ultimately, the mitochondrial oxidant stress and peroxynitrite formation triggers the mitochondrial permeability transition (MPT) pore formation resulting in collapse of the mitochondrial membrane potential, cessation of ATP synthesis and ultimately necrotic cell death (Kon et al. 2004; Masubuchi et al. 2005; Ramachandran et al. 2011). Direct evidence for the pathophysiological importance of ROS and in particular peroxynitrite has been provided by scavenging of these reactive intermediates through accelerated recovery of cellular and mitochondrial GSH (Knight et al. 2002; Saito et al. 2010c). Thus, useful measurements to assess the effects of natural product interventions on mitochondrial oxidant stress and peroxynitrite include GSSG (tissue or mitochondria), nitrotyrosine protein adducts (immunohistochemistry or western blotting), loss of mitochondrial DNA, protein carbonyls in mitochondria, mitochondrial release of cytochrome c, endonuclease G and apoptosis-inducing factor (AIF) (western blotting), and nuclear DNA damage (TUNEL assay). A moderate increase of thiobarbituric acid-reactive substances (TBARS) and/or a moderate decrease of antioxidant enzyme activities are frequently reported (Campos et al. 1989; Gao and Zhou 2005; Hsu et al. 2008; Küpeli et al. 2006; Wang et al. 2010; Wu et al. 2008, 2010; Yuan et al. 2010), but are of limited pathophysiological relevance and do not provide relevant mechanistic information. However, as mentioned, all of these measurements on oxidant stress should only be done if an effect on upstream events (i.e. metabolic activation and protein binding) has been ruled out.

Lipid peroxidation

Due to the involvement of oxidant stress, LPO is a popular hypothesis to explain massive cell death after APAP overdose. Antioxidant function and protection against LPO are probably the most invoked mechanisms of protection by natural products (Campos et al. 1989; Gao and Zhou 2005; Hsu et al. 2008; Küpeli et al. 2006; Wang et al. 2010; Wu et al.

2008, 2010; Yuan et al. 2010). LPO is a multistep process requiring initiation of a radical chain reaction and propagation through iron-dependent Fenton-like chemistry (Jaeschke 2000). This process can lead to complete destruction of cell membranes and thus rapid cell death. However, because of the severe injury potential of LPO, cells have very effective defense systems at multiple levels to prevent this process (Jaeschke 2010). A number of enzymes effectively metabolize and detoxify ROS including superoxide dismutases, glutathione peroxidase, catalase, thioredoxins, and peroxiredoxins. GSH can be used as a cofactor in these reactions, or it can react directly with ROS itself (Jaeschke 2010). If the radical chain is initiated, chain-breaking antioxidants in the lipid membrane such as vitamin E can effectively interrupt this process. In addition, the effective chelation of iron by ferritin and transferrin further prevents the propagation of the radical chain reaction (Jaeschke 2010). Therefore, because of these highly effective defense systems, LPO is rarely a direct mechanism of cell death in vivo (Jaeschke 2000). This conclusion is based on quantitative consideration of LPO. There is very little evidence for LPO after APAP treatment and loading liver cells with vitamin E does not protect (Knight et al. 2003). In contrast, if animals are fed a diet high in polyunsaturated fatty acids (substrate of LPO) and low in vitamin E (essential for defense against LPO), the animals become highly susceptible and LPO is the dominant mechanism of cell injury after APAP (Wendel and Feuerstein 1981; Wendel et al. 1979, 1982) and other chemicals such as allyl alcohol (Jaeschke et al. 1992b). Under these conditions, pretreatment with vitamin E or iron chelation effectively protects against both chemicals (Jaeschke et al. 1992b; Knight et al. 2003; Werner and Wendel 1990). However, in these animals indicators of LPO are increased 30- to 50-fold (3,000–5,000 %) above baseline (Jaeschke et al. 1992b; Mathews et al. 1994; Wendel et al. 1979, 1982). If this extensive LPO on a vitamin E-deficient diet is compared to APAP toxicity in animals on a regular diet, LPO is either undetectable (Knight et al. 2003) or at best 100% above baseline (Campos et al. 1989; Gao and Zhou 2005; Hsu et al. 2008; Küpeli et al. 2006; Wang et al. 2010; Wu et al. 2008, 2010; Yuan et al., 2010). Quantitatively, this minor LPO can not account for the massive cell injury observed after APAP on a regular diet. For LPO to be relevant, very large increases of specific parameters of LPO such as ethane and pentane exhalation, hydroxy fatty acids, hydroxy-nonanal, diene conjugation, etc. should be measured. In addition, these parameters should be drastically reduced (along with reduced injury) after treatment with a lipid soluble antioxidant like vitamin E (Jaeschke et al. 1992b; Knight et al. 2003; Mathews et al. 1994; Werner and Wendel 1990), which is not the case after APAP treatment in mice on a normal diet (Knight et al. 2003). Moreover, it takes only 2–4 hours after APAP to completely destroy a liver by LPO in mice fed a vitamin E-deficient diet (Wendel and Feuerstein 1981; Wendel et al. 1979, 1982) or allyl alcohol (Jaeschke et al. 1992b), while this is not seen under normal circumstances. Thus, the minor LPO and small reduction of antioxidant enzyme activity frequently reported after APAP (Campos et al. 1989; Gao and Zhou 2005; Hsu et al. 2008; Küpeli et al. 2006; Wang et al. 2010; Wu et al. 2008, 2010; Yuan et al., 2010) are at best general and not very specific indicators of oxidant stress. The selective generation of ROS and peroxynitrite within the mitochondria triggers the MPT and the mitochondrial release of endonucleases that cause nuclear DNA fragmentation are the critical events in the induction of necrotic cell death (Jaeschke and Bajt 2006).

Necrosis and apoptosis

APAP-induced liver injury is characterized by extensive cell contents release (liver enzymes), cell swelling, nuclear degradation (karyorrhexis and karyolysis) and an inflammatory response (Gujral et al. 2002). These are typical characteristics of oncotic necrosis (Jaeschke and Lemasters 2003). Thus, it is generally concluded that APAP-induced cell death in vivo (Gujral et al. 2002) and in vitro (Bajt et al. 2004; Kon et al. 2004) is caused by oncotic necrosis (Figure 4). However, cell death induced by APAP involves some

signaling mechanisms which are thought to be more characteristic of apoptosis such as mitochondrial translocation of Bax and Bid (Adams et al. 2001; Bajt et al. 2008; El-Hassan et al. 2003; Jaeschke and Bajt 2006), mitochondrial release of cytochrome c (Adams et al. 2001; El-Hassan et al. 2003; Knight and Jaeschke 2002), activation of JNK (Gunawan et al. 2006; Henderson et al. 2006), DNA fragmentation as indicated by a DNA ladder (Cover et al. 2005; Ray et al. 1990; Shen et al. 1991), DNA fragments in the cytosol (Lawson et al. 1999) and cells staining positive for the TUNEL assay (Gujral et al. 2002; Lawson et al. 1999). Therefore, occasionally the hypothesis is brought forward that apoptosis is an important component of APAP-induced liver injury (Chen et al. 2009; Ray et al. 1996) or that the injury process begins with apoptosis and quickly deteriorates into secondary necrosis (El-Hassan et al. 2003). However, there is really no reliable evidence that apoptotic cell death is involved in the pathogenesis of APAP hepatotoxicity. First, there is no morphological evidence of apoptosis, i.e. cell shrinkage, nuclear condensation and chromatin margination (Figure 4), and second, all other parameters (TUNEL assay, DNA ladder, Bax translocation, cytochrome c release, etc) are not specific for apoptosis. Most importantly, there is no evidence for relevant caspase activation (Adams et al. 2001; El-Hassan et al. 2003; Gujral et al. 2002; Jaeschke et al. 2006; Lawson et al. 1999) and caspase inhibitors do not protect (Jaeschke et al. 2006; Lawson et al. 1999). The critical issue is not whether there is a minor increase of caspase activity or evidence for a minor processing of a caspase, the issue is if the caspase activity increase can quantitatively explain the massive cell injury observed after APAP overdose. To answer this question, it is useful to compare APAP-induced liver injury with TNF- or Fas receptor-mediated apoptosis. In these models, apoptotic cell death of 20–30% of hepatocytes correlates with >100-fold increase of caspase-3 activity (Bajt et al. 2000; Gujral et al. 2004; Jaeschke et al. 2000). Furthermore, pancaspase inhibitors completely prevent TNF- and Fas receptor-induced apoptosis (Bajt et al. 2000; Jaeschke et al. 2000) but have absolutely no effect on APAP toxicity (Jaeschke et al. 2006; Lawson et al. 1999; Williams et al. 2010b). The two manuscripts claiming protection with caspase inhibitors in vivo used a pretreatment regimen (El-Hassan et al. 2003; Hu and Colletti 2010). The problem is that most caspase inhibitors are only soluble in dimethyl sulfoxide (DMSO), which is a potent inhibitor of cytochrome P450 enzymes (Park et al. 1988). Even very low doses of DMSO as used with these inhibitors are sufficient to eliminate metabolic activation of APAP and completely protect when used as pretreatment (Jaeschke et al. 2006; Saito et al. 2010a). Thus, in both of these manuscripts that reported caspase inhibitor protection (El-Hassan et al. 2003; Hu and Colletti 2010), no proper solvent controls were included and protection was caused by DMSO, not the caspase inhibitor (Jaeschke et al. 2006; Jaeschke et al. 2010a).

The hypothesis that APAP-induced cell death is apoptosis deteriorating into secondary necrosis is also not supported by any data. Secondary necrosis always involves a previous phase of apoptosis with massive caspase activation, which can still be observed during the secondary necrosis phase (Bajt et al. 2000). Because secondary necrosis depends exclusively on the preceding apoptosis, secondary necrosis is also completely preventable by caspase inhibitors (Bajt et al. 2000). Based on these data, APAP-induced cell death clearly does not involve relevant apoptosis or secondary necrosis.

Because there is no caspase activation after APAP overdose, the traditional caspase-activated DNase associated with apoptosis (Nagata et al. 2003), cannot be activated. Nevertheless, APAP-induced DNA fragmentation as assessed by DNA laddering is indistinguishable from actual apoptosis (Ray et al. 1990; Cover et al. 2005). This indicates that nuclear DNA fragmentation after APAP overdose is an endonuclease-mediated process. The endonucleases involved are endonuclease G and apoptosis-inducing factor derived from the mitochondria (Bajt et al. 2006) and DNase1 from lysosomes (Napirei et al. 2006).

Innate immune response

The cell contents released after APAP-induced necrotic cell death initiates an inflammatory response with activation of Kupffer cells and recruitment of neutrophils and monocytes into the liver (Laskin 2009; Laskin and Pilaro 1986; Lawson et al. 2000) (Figure 5). It was recently recognized that some of the compounds generally released by dying hepatocytes can stimulate toll-like receptors on macrophages and other non-parenchymal cells and promote cytokine formation initiating an inflammatory response in the absence of pathogens (sterile inflammation) (Bianchi 2007). The same compounds i.e. high mobility group box 1 (HMGB1) protein, heat shock proteins and DNA fragments, are also released during APAP toxicity (Antoine et al. 2009; Jahr et al. 2001; Martin-Murphy et al. 2010) and are responsible for hepatic neutrophil accumulation (Scaffidi et al. 2002). It is now well accepted that sterile inflammation in response to hepatic ischemia-induced cell death substantially aggravates liver injury during reperfusion through Kupffer cell and neutrophil cytotoxicity (Jaeschke 2006). However, antibodies against β_2 integrins (CD18) on neutrophils (Lawson et al. 2000), neutropenia-inducing antibodies (Cover et al. 2006), or deficiency of intercellular adhesion molecule-1 (ICAM-1) (Cover et al. 2006) or CD18 (Williams et al. 2010a) did not protect against APAP-induced liver injury. In addition, chemical inhibitors against NADPH oxidase, the enzyme most critical for reactive oxygen-mediated cell killing by phagocytes (Gujral et al. 2004), did not protect against APAP toxicity (Cover et al. 2006). In support of these results, animals deficient in gp91^{phox}, a component of NADPH oxidase, were not protected against the oxidant stress or cell death induced by APAP overdose (James et al. 2003a). Interestingly, neutrophils are not even activated at the time of the initial recruitment into the liver during APAP toxicity (Williams et al. 2010a). This is in contrast to models where neutrophils contribute to liver injury; under these conditions neutrophils are primed or even fully activated during their accumulation in the liver (Jaeschke et al., 1992a; Gujral et al. 2003). Taken together, there is extensive experimental evidence to support the hypothesis that neutrophils do not actively contribute to APAP-induced liver injury. However, there are individual reports that may suggest a role of inflammatory cells in the pathogenesis (Liu and Kaplowitz 2006). Unfortunately, most of these data can be explained with initially unrecognized experimental problems leading to incorrect conclusions (Jaeschke, 2008). For example, an involvement of natural killer (NK) cells and NK T cells in the pathogenesis of APAP hepatotoxicity originally reported by Liu et al. (2004) was triggered by the use of the solvent DMSO, which can activate and recruit NK cells into the liver (Masson et al. 2008). The use of DMSO was not disclosed in the original paper by Liu et al (2004). Thus, when APAP is dissolved in saline, NK or NKT cells do not contribute to the toxicity (Masson et al. 2008). In addition, the report by Liu et al. (2006) that neutrophils are critical for APAP-induced liver injury could not be reproduced when neutropenia was induced after APAP treatment (Cover et al. 2006). The reason for these discrepancies is the fact that only 24 h pretreatment with a neutropenia-inducing antibody causes Kupffer cell activation and a preconditioning effect with upregulation of hepatoprotective genes, which questions the conclusions about the importance of neutrophils in the pathophysiology (Jaeschke and Liu 2007).

Kupffer cells are activated during APAP toxicity (Laskin 2009; Laskin and Pilaro 1986; Holt et al. 2008). Although functional inactivation of Kupffer cells by gadolinium chloride modestly protects, complete elimination of the resident hepatic macrophages actually aggravates APAP-induced liver injury (Ju et al. 2002). The loss of Kupffer cells prevents interleukin-10 formation, which reduces induction of inducible nitric oxide synthase (iNOS) and therefore limits generation of peroxynitrite and liver injury in hepatocytes (Bourdi et al. 2002). In addition to the effects of Kupffer cell-derived mediators on oxidant stress in hepatocytes, these resident macrophages have the capability to directly generate reactive oxygen through NADPH oxidase and cause hepatocellular injury (Bilzer et al. 1999).

However, a direct cytotoxic effect of Kupffer cells is unlikely because mice with gp91^{phox}-deficiency, which eliminates NADPH oxidase activity in Kupffer cells, did not affect the oxidant stress, peroxynitrite formation and liver injury after APAP overdose (James et al. 2003a). In addition, those Kupffer cells most active in cytotoxicity are located in the periportal area (Jaeschke et al. 1992a) away from the centrilobular region of injury. Furthermore, the elimination of Kupffer cells did not protect against APAP-induced liver injury (Ju et al. 2002). Together these data support the conclusion that Kupffer cells do not directly cause liver injury in this model.

In addition to neutrophils, monocytes are also recruited into the liver, although at later time points (Laskin 2009). Formation of monocyte chemoattractant protein 1 (MCP-1) by macrophages and hepatocytes in the area of injury promotes the infiltration of these blood cells (Dambach et al. 2002; Holt et al. 2008). In addition, when monocyte recruitment is attenuated in mice deficient of CC chemokine receptor 2, the receptor for MCP-1, the injury is not affected but repair of the tissue damage is substantially impaired (Dambach et al. 2002; Holt 2008). This suggests that the newly recruited monocyte-derived tissue macrophages are involved in cell debris removal during the later phase of APAP hepatotoxicity as a prerequisite for regeneration and replacement of necrotic cells by viable ones (Chiu et al. 2003; Dambach et al. 2002; Holt et al. 2008).

Taken together, there is a substantial sterile inflammatory response after APAP hepatotoxicity (Figure 5). However, the vast majority of data suggest that innate immune cells do not actively contribute to the injury process in this model. In contrast, macrophages and potentially also neutrophils appear to be mainly involved in removal of necrotic cells and therefore promote tissue repair. However, because of the extensive use of immunological reagents and of gene knock-out mice with sometimes unrecognized characteristics, many contradictory results are being published. It is therefore critical, especially in experiments where immunological mechanisms are investigated, to confirm the validity of any new findings through independent approaches in order to avoid the very common problem of unrecognized methodological pitfalls.

Conclusions

APAP-induced liver injury, especially in mice, is a clinically relevant model that is suitable to test the efficacy of hepatoprotective natural products and other compounds in vivo. Given the extensive knowledge of the mechanisms of APAP-induced liver injury, the model can also be used to investigate mechanisms of therapeutic action. Importantly, though many studies demonstrate an antioxidant effect or protection against LPO with natural products after APAP treatment, this is highly unlikely because LPO is not a relevant mechanism of APAP-induced liver injury. This conclusion is often due to a weakness in study design or an incomplete understanding of the mechanisms of toxicity. Only when the full knowledge of APAP-induced pathomechanisms is applied to the testing of new compounds can we expect to obtain a realistic picture of the therapeutic potential of natural products and their mechanisms of action. This information is critical when considering a potential clinical application of new drug entities.

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Figure 1. Metabolic activation of acetaminophen (APAP)

More than 80-90% of an administered dose of APAP is conjugated with glucuronic acid or sulfate and excreted. A small fraction is metabolized by cytochrome P450 enzymes to form the reactive metabolite N-acetyl-p-benzoquinone imine (NAPQI), which can be conjugated and detoxified by glutathione (GSH). After an overdose of APAP, excess NAPQI is formed leading to cellular GSH depletion and consequently covalent binding of NAPQI to cellular proteins, which is the initiating step of toxicity. Any therapeutic intervention (natural products, solvents) administered before APAP has the potential to inhibit cytochrome P450 enzymes and effectively protect against APAP toxicity.

Figure 2. Glutathione depletion kinetics in rodent liver after a high dose of APAP

A hypothetical graph based on Knight et al. 2001 and Saito et al. 2010b,^c. Glutathione (GSH) levels fall quickly after 200–600 mg/kg APAP in the absence of a metabolism inhibitor during the first 20–30 min leading to hepatic GSH depletion of about 90% of baseline values (solid line). In the presence of dimethyl sulfoxide (DMSO) or another inhibitor of APAP metabolism, NAPQI formation and consequently the kinetics of GSH depletion are delayed (dashed line). The length of the delay and the extent of inhibition will depend upon the clearance rate of the inhibitor and the dose of APAP (among other considerations). At doses of 200–300 mg/kg APAP, a small amount of DMSO may sufficiently inhibit metabolism and cause only moderate GSH depletion, which is insufficient to initiate toxicity. In contrast, a high overdose (500–600 mg/kg APAP) may lead to delayed depletion (dashed line) and consequently less toxicity compared to a saline-treated mouse receiving APAP. The critical point is that measuring GSH levels at two hours would not detect this metabolic inhibition.

Figure 3. Mitochondrial oxidant stress and peroxynitrite formation during APAP hepatotoxicity Metabolism of APAP causes formation of the reactive metabolite NAPQI, which binds to mitochondrial proteins and initiates mitochondrial oxidative stress. This results in formation of peroxynitrite within mitochondria as well as activation and translocation of JNK to the mitochondria from the cytosol. The activation of JNK triggers an amplification of mitochondrial oxidative stress and peroxynitrite formation, ultimately resulting in induction of the mitochondrial permeability transition. As a consequence, mitochondrial factors such as cytochrome c, endonuclease G and the apoptosis inducing factor (AIF) are released from the mitochondrial intermembrane space. Endonuclease G and AIF then translocate to the nucleus to initiate nuclear DNA fragmentation.

Figure 4. Mode of cell death

Fundamental differences in cell morphology between apoptotic cell death and oncotic necrosis. Hematoxylin & eosin-stained sections of livers obtained from animals treated with either 700 mg/kg galactosamine/100 µg/kg endotoxin (left panel) or 300 mg/kg acetaminophen (right panel) for 6 hours. Hepatocellular apoptosis is characterized by apoptotic body formation, chromatin condensation and cell shrinkage (arrows in left panel). Oncotic necrosis is characterized by karyorrhexis, karyolysis, cell swelling and loss of membrane integrity (arrows on right panel). X400 (all panels)

Figure 5. Innate immune response during acetaminophen hepatotoxicity

During APAP-induced hepatotoxicity damaged hepatocytes release cellular contents including DAMPs. DAMPs activate innate immune cells resulting in an inflammatory response including cytokine/chemokine production and immune cell recruitment. These innate immune cells are present to maintain host defense against invading pathogens and to remove cellular debris thereby promoting liver regeneration but in this model they are not actively participating in the injury process. Abbreviations: DAMPs, Damage-Associated Molecular Patterns; TLRs, Toll-like Receptors; HMGB1, High mobility group box-1 protein; HSPs, Heat shock proteins; MIP-2, Macrophage inflammatory protein-2 (CXCL2); MCP-1, Monocyte chemoattractant protein-1 (CCL2); IL-6, Interleukin-6; TNF- α , Tumor necrosis factor- α