Underlying mechanisms and treatment of acetaminophen-induced liver injury (Review)

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Abstract. Acetaminophen (APAP) is safe at therapeutic doses; however, when ingested in excess, it accumulates in the liver and leads to severe hepatotoxicity, which in turn may trigger acute liver failure (ALF). This is known as APAP poisoning and is a major type of drug-related liver injury. In the United States, APAP poisoning accounts for ≥50% of the total number of ALF cases, making it one of the most common triggers of ALF. According to the American Association for the Study of Liver Diseases, the incidence of APAP-associated hepatotoxicity has increased over the past few decades; however, the mechanism underlying liver injury due to APAP poisoning has remained inconclusive. The present study aims to comprehensively review and summarize the latest research progress on the mechanism of APAP-induced liver injury, and to provide scientific and effective guidance for the clinical treatment of APAP poisoning through in-depth analysis of the metabolic pathways, toxicity-producing mechanisms and possible protective mechanisms of APAP in the liver.

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1. Introduction

Drug-induced liver injury (DILI) is a common adverse drug reaction, with an estimated worldwide incidence of 14-19 cases per 100,000 people, 30% of which are associated with jaundice (1). A 2019 retrospective study involving 308 medical centers in the major cities of mainland China noted that the incidence of DILI was 23.80 per 100,000 individuals, which was higher than that in Western countries. It also reported that DILI cases were rising every year, and that traditional Chinese medicines and antituberculosis medicines, among others, were the main drug classes that caused DILI (2). Notably, the incidence of DILI is higher in hospitalized populations (3). DILI refers to an injury to the liver or biliary system caused by the ingestion of hepatotoxic drugs. Mechanistically, DILI can be attributed to either endogenous toxicity (dose-related) or specific toxicity (dose-independent) and indirect injury, a recently proposed type that refers to injury to the liver or biliary system caused by a drug that alters a patient's original liver disease or immune status (4). Endogenous toxicity is predictable and can occur hours or days after an individual is exposed to a drug. By contrast, specific toxicity is often unpredictable and is determined by the interaction of environmental and host factors with the drug (5). Sex is a significant risk factor for DILI; young women are at a higher risk of drug overdose events, which may be due to differences in psychosocial factors, physiological differences and behavioral patterns (6,7). According to the results of the Third National Health and Nutrition Examination Survey in the United States, it was found that women take acetaminophen (APAP) more often than men (8). In addition, it has been shown that the clearance of APAP from men's bodies is 22% higher than that from

women's bodies, because men have higher glucuronidation activity, which is a key enzyme in APAP metabolism (9).

The primary cause of endogenous DILI is considered to be the supratherapeutic use of APAP, which is one of the most widely used and popular over-the-counter analgesic and antipyretic medications across the world (10). APAP is included in the World Health Organization's list of essential medicines recommended as a first-line treatment for the majority of pain and fever cases, and is considered safe at therapeutic doses; however, when overdosed, it can cause life-threatening hepatotoxicity (11). The United States has the highest proportion of DILI cases caused by APAP (~30% of cases), followed by Pakistan (~16% of cases) (12). The first reports of APAP poisoning in humans appeared in the 1960s when Davidson and Eastham (13) reported two patients who developed hepatotoxicity after overdose; both patients succumbed on day 3 after overdosing, and in both cases, the pathologic features of the liver sections were suggestive of marked acute lobulocentric fulminant hepatic necrosis (14). Notably, toxic effects are highly likely with APAP at a dose of 150 mg/kg (15); this dose has remained virtually unchanged over the past 20 years although *N*-acetylcysteine (NAC) has been introduced as a clinical antidote to APAP poisoning. Therefore, understanding the pathogenesis and risk factors of APAP-induced liver injury (AILI), and proposing more effective means of prevention and treatment are imperative.

2. Clinical manifestations and biomarkers

APAP toxicity is a time-dependent event, with the liver being the first organ to come into contact with absorbed APAP. This toxicity can occur from administration errors, accidental ingestion and intentional self-administered overdose (16). APAP hepatotoxicity can generally be divided into four stages of presentation, with the first stage characterized by nausea, vomiting, right upper abdominal pain and other nonspecific symptoms that begin within minutes to hours of ingestion. Some patients may initially be asymptomatic, with hepatic function markers, such as serum aspartate transferase (AST), prothrombin time (PT) and serum total bilirubin (TBIL), at baseline levels (17). During the second stage, abnormalities in liver function, such as an increase in AST activity, PT and TBIL levels, occur and the patient may present with nausea, pain in the right upper abdomen and jaundice (18). The third stage is often the peak of hepatic injury, with marked necrosis of hepatocytes. AST levels can peak at this stage, but some patients do not show typical symptoms. In patients with more severe liver injury, fulminant liver failure, including hepatic encephalopathy and hyperbilirubinemia, can occur (19). The fourth stage is often the recovery stage, when the indicators of various liver functions may return to the baseline. Nevertheless, a small number of patients experience liver and other organ failure, which is life-threatening. Various factors can increase the risk for APAP toxicity, which is mainly mediated by the metabolic pathway of APAP (12). Plasma biomarkers detected through liquid biopsies are currently considered the gold standard for understanding the mechanisms of *in vivo* injury in patients (20). Protein adducts in plasma can serve as specific biomarkers of APAP toxicity. High levels of APAP protein adducts (APAP-AD) in serum samples from patients with acute liver failure (ALF) induced by APAP overdose and lower levels of adducts in other types of liver injury, such as pyrrole protein adducts, can be detected by high-sensitivity high-pressure liquid chromatography combined with electrochemical detection (21). A major advantage of measuring these protein adducts is that their half-life in serum is considerably long; therefore, they can be used to accurately diagnose AILI long after APAP ingestion (22).

3. Pathophysiological mechanisms of AILI

Continuous improvements in research equipment and methods in the fields of modern medicine and microbiology have enabled a deeper understanding of the mechanisms underlying AILI. Production of toxic metabolites, generation of protein adducts leading to mitochondrial dysfunction and peroxynitrite formation, and cell death due to DNA strand breaks are believed to be the primary mechanisms of APAP-induced hepatotoxicity (Fig. 1). Recent studies have reported the close involvement of several events in the onset of APAP toxicity, which have been described in the following sub-sections.

Toxic metabolites. The liver is a major target for drug toxicity because it serves a crucial role in the metabolism of drugs, toxins and other exogenous substances, and their removal from circulation. The liver is not only responsible for the biotransformation of drugs into more easily excretable forms, but it also protects the body from the potentially harmful effects of drugs through its detoxification system. Approximately 90% of APAP taken at therapeutic doses is metabolized by phase II-coupled enzymes, mainly glucuronosyltransferase (UGT) and sulfonyl transferases (SULTs), which convert it into non-toxic compounds that are then excreted in the urine via the biliary tract and renal system (23). A small portion (~5%) is excreted in the urine without biotransformation, whereas the remaining portion (~5%) is bio-transformed by the cytochrome P450 (CYP450) system, specifically via CYP2E1 and CYP1A2 metabolism, to produce the reactive intermediate molecule *N*-acetyl-*p*-benzoquinone imine (NAPQI), which can be readily detoxified to the water-soluble and innocuous mercapturic acid by glutathione (GSH) (24). Microsomal GSH transferase (GST) catalyzes the binding of NAPQI to GSH, thus reducing its toxicity. Notably, the formation of NAPQI, the active metabolite of APAP, is an important step in the development of hepatotoxicity (25). Exceeding the therapeutic dose of APAP leads to saturation of the phase II-coupled enzymes; therefore, excess APAP is diverted to another metabolic pathway, the CYP450 system, which results in the production of a large amount of NAPQI in hepatocytes (26). This leads to GSH overconsumption, which lowers GSH levels. Excess NAPQI binds to cellular biomolecules, such as proteins, nucleic acids and lipids, a process that occurs predominantly in the centrocytes and hepatocytes of hepatic lobules. The degree of binding and the relative amount of covalent binding have been reported to be associated with the development of toxicity; the formation of protein adducts causes oxidative stress and dysfunction, which further leads to hepatocyte necrosis (27). Notably, therapeutic doses of APAP do not cause liver injury if UGT and SULTs are active and the hepatic GSH stores are

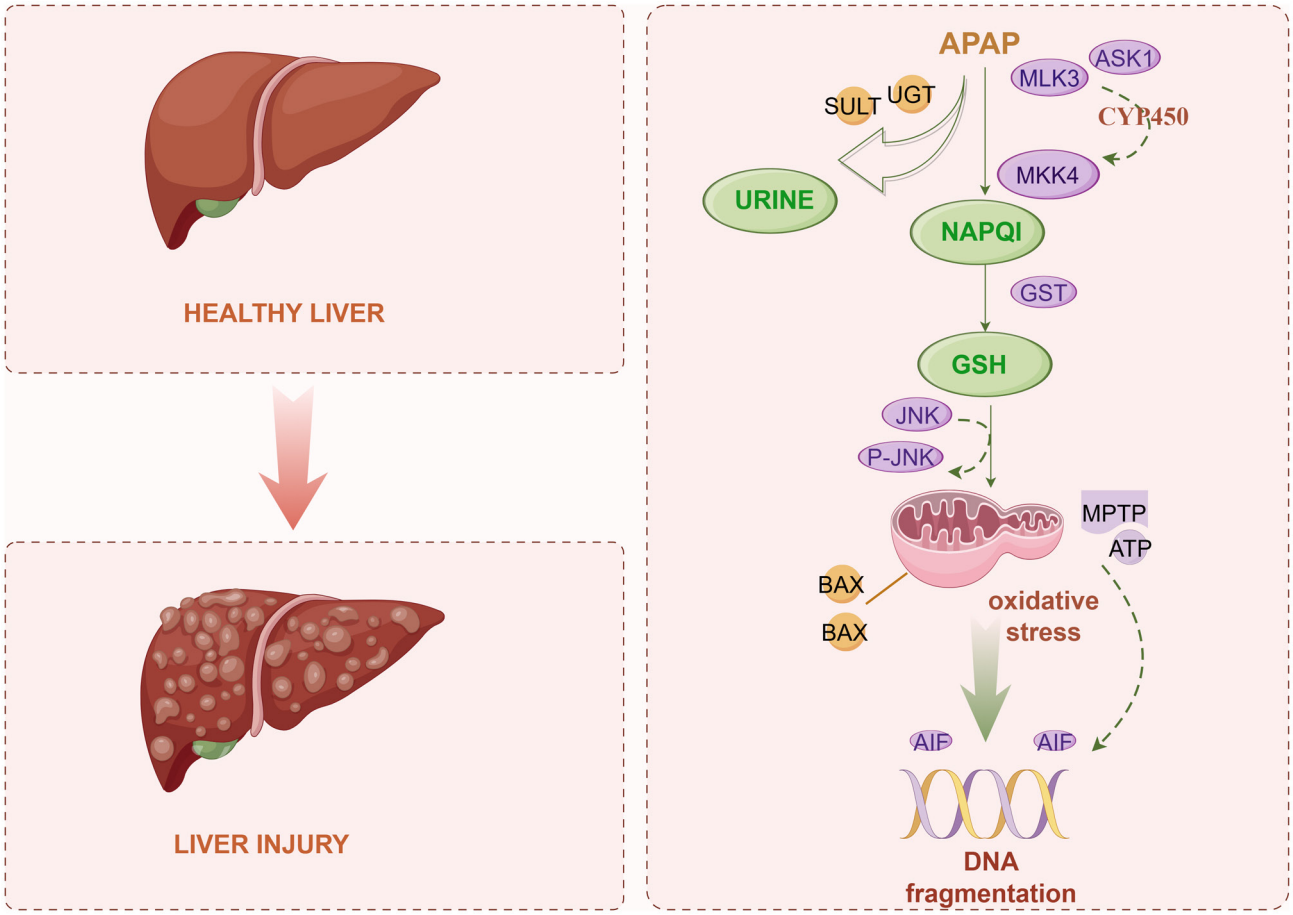

Figure 1. Underlying mechanism of APAP-induced liver injury. APAP, acetaminophen; UGT, glucuronosyltransferase; SULT, sulfonyl transferase; ASK1, apoptosis signal-regulating kinase 1; MLK3, mixed lineage kinase 3; MKK4, mitogen-activated protein kinase kinase 4; NAPQI, *N*-acetyl-*p*-benzoquinone imine; GST, glutathione transferase; GSH, glutathione; MPTP, mitochondrial membrane permeability transition pore; AIF, apoptosis-inducing factor.

adequately maintained, because all of the NAPQI produced is efficiently removed.

Oxidative stress in mitochondria. Mitochondria are vital organelles for cellular energy production, which also have an important role in cell signaling. Toxic doses of APAP can alter mitochondrial morphology and function. Owing to the reactive nature of NAPQI, one of the early events following its formation and the depletion of hepatic GSH stores is the generation of NAPQI adducts on cellular proteins (28). Mitochondria are a key early target for NAPQI adduct formation. The levels of such adducts markedly increase in mitochondria after APAP overdose into mice, accounting for ~65% of the whole cytoplasm; mitochondrial protein adducts, such as mitochondrial aldehyde dehydrogenase, α-subunit of ATP synthetase and GSH peroxidase (GPX), have been found in mice (29). Large amounts of NAPQI can cause GSH depletion, which leads to increased release of H_2O_2 in mitochondria, which is an important oxidative stress signaling molecule that initiates the auto-activation of apoptosis signal-regulating kinase 1 (ASK1), further triggering the activation of mitogen-activated protein kinase kinase 4. Ultimately, this leads to the cytoplasmic activation of the MAPK, JNK, which belongs to a subgroup of MAPKs that are mainly activated by cytokines and environmental stress (30). JNK activation and attenuation of liver injury have been observed in ASK1-deficient mice, thus demonstrating that ASK1 is upstream of JNK; notably, this activation is more pronounced at a late stage (i.e., ≥3 h after APAP administration), with little effect at an earlier time point (1.5 h) (31).

Another possible mechanism of JNK activation that has recently been proposed is that it can be released from GSTπ (32), an important detoxifying enzyme that is a member of the GST family that binds to JNK. Oxidative stress or direct binding of NAPQI can trigger the release of JNK from GSTπ. Phosphorylated JNK can bind to the mitochondrial outer membrane scaffolding protein and inhibit mitochondrial electron transport through an Src-mediated process. Protein adducts in mitochondria trigger initial electron leakage at the level of complex III of the electron transport chain, causing the formation of superoxide radicals outside the inner mitochondrial membrane (IMM), which in turn triggers mild oxidative stress in the cytoplasm (33). In addition, activated JNK translocates from the cytoplasm to the mitochondria, increasing the mitochondrial permeability transition pore (MPTP) activity, which then elevates mitochondrial oxidative stress; this strongly amplifies mitochondrial superoxide radical formation, which can induce mitochondrial dysfunction and lead to hepatocellular necrosis. NAPQI-induced formation of mitochondrial adducts and the ensuing mitochondrial oxidative stress have been well-studied in recent decades (34,35), but the amplification of JNK is a more recent discovery (36).

Peroxynitrite formation. Although mitochondria exhibit strong antioxidant defense activity, they can experience oxidative stress after ectopic JNK expression, mainly due to the active disruption of electron transfer by JNK-mediated signaling (37). Mitochondrial stress-generated superoxide radicals can react rapidly with nitric oxide to form a potent oxidant, peroxynitrite, which is a highly reactive and nitrifying compound that causes APAP toxicity (38). During APAP hepatotoxicity, peroxynitrite is mainly produced in hepatocytes and liver sinusoidal endothelial cells (LSECs); the formation of nitrotyrosine protein adducts, which are mainly found in mitochondria, indirectly proves that peroxynitrite is formed in the mitochondria of hepatocytes (39). Time course studies of peroxynitrite formation have confirmed its occurrence only in mitochondria after JNK translocation (40). This amplified oxidative and nitrosative stress in the mitochondria, accompanied by inhibition of the antioxidant system and nitrification of mitochondrial components, can induce changes in mitochondrial membrane potential and morphology, such as the opening of the MPTP, which leads to a shift in MPT. This alteration, although reversible in the early stages, eventually leads to an irreversible collapse of the IMM potential and cessation of ATP synthesis. MPT induction may be dependent on the magnitude of the upstream signal from APAP; mice have been shown to exhibit transient MPT induction when the APAP dose is low and irreversible induction at higher doses (41). The result is matrix swelling and outer mitochondrial membrane rupture, which releases intermembrane proteins such as endonuclease G and apoptosis-inducing factor (AIF) into the cytoplasm; these proteins can later translocate into the nucleus, leading to mitochondrial DNA breaks, as well as massive mitochondrial dysfunction and swelling along with DNA damage, which trigger programmed cellular necrosis (42). This event can directly cause the cessation of ATP synthesis.

In addition to the unregulated release of intermembrane proteins via the MPTP, they can be prematurely released through the Bax pore. Bax, a member of the Bcl-2 family of proteins, is located mainly in the cytoplasm; upon stimulation, it can translocate to the outer mitochondrial membrane, form a pore, and trigger the release of intermembrane proteins (43). In addition, JNK and phosphorylated JNK are recruited to the mitochondria via the SH3 homology-associated BTK-binding protein, a mitochondrial outer membrane protein that can further enhance mitochondrial damage and necrosis (44). GSH supplements not only provide excess cysteine as an energy substrate but also serve an important role in scavenging free radicals and peroxynitrite (45). Selective clearance of mitochondrial peroxynitrite increases upon accelerated restoration of mitochondrial GSH levels, further demonstrating the critical role of peroxynitrite in pathophysiology (46).

Microcirculatory dysfunction. The hepatic microcirculatory milieu, which consists mainly of LSECs, hepatic stellate cells and hepatic macrophages, serves an important role in maintaining intrahepatic homeostasis and hepatocyte function, regulating vascular tone and controlling inflammation (47). Although hepatocytes are the primary target of the cytotoxic effects of APAP overdose, morphological alterations in LSECs are also associated with APAP-induced liver disease. Drugs are transported through blood sinusoids by various mechanisms, including organic anion and cation transport proteins of the basolateral membrane, other transport proteins such as Na^+-taurine bile acid co-transporting polypeptides or prostaglandin transport proteins, and passive diffusion, before metabolism and clearance within hepatocytes (48). APAP-induced LSEC injury precedes liver injury; thus, LSEC injury is considered an early event in liver injury (49). Intra-endothelial perturbations, including swelling of LSECs, gap formation and estradiol incorporation, occur as early as 2 h after APAP overdose, suggesting that LSECs can metabolize APAP (49). In a previous study, gavage administration of APAP to male C57Bl/6 mice revealed that a large number of SECs swelled at 0.5 and 1 h, whereas the hepatocyte morphology remained intact, and serum AST and alanine amiotransferase (ALT) levels, which are indicators of hepatocyte injury, were not significantly increased. These results suggested that LSECs are damaged before hepatocytes (50). Furthermore, scanning electron microscopy after APAP intoxication has revealed the induction of large pores in LSECs, accompanied by the accumulation of erythrocytes, enlargement of the Disse space and collapse of sinusoidal lumen in a mouse model (51). Massive centrilobular congestion is an important feature of APAP-induced hepatotoxicity in mice, which occurs before the appearance of necrosis, and is induced by hepatocytes and their relationship with LSECs. Vascular congestion, interstitial congestion and sinusoidal collapse may impair hepatic circulation, thereby exacerbating toxicity by causing additional hypoxic injury (52). Furthermore, serum hyaluronic acid (HA) levels have been shown to be elevated in patients with APAP-induced acute hepatic injury (53); HA is mainly produced by mesenchymal cells and is found at low levels in normal human plasma, as it is rapidly cleared by LSECs. Notably, clearance of HA is impaired when LSECs dysfunction, which may be related to the HA receptor and DNA breaks on defective LSECs (54). Hence, HA levels may be used to determine the extent of sinusoidal vascular endothelial cell damage (55). Formaldehyde-treated serum albumin (FSA) is a commonly used model ligand for the LSEC scavenger receptor that has been widely used to study the scavenger receptor function of LSECs, particularly when assessing the ability of LSECs to scavenge blood-borne waste macromolecules. FSA expression in the hemorrhagic centrilobular region has been reported to diminish 2 h after APAP gavage in a mouse model, suggesting impaired LSEC scavenger receptor function or a reduced number of cell surface receptors (50).

Endoplasmic reticulum (ER) stress. The ER serves a crucial role in a number of cellular processes, including protein folding, assembly, transport, post-regulation, secretion and quality control of membrane proteins, lipid synthesis and regulation of intracellular calcium homeostasis. Disturbance of ER function by stimuli such as altered cellular redox status, oxidative stress, calcium homeostasis imbalance, hypoxia or energy deprivation can lead to the accumulation of unfolded proteins in the ER, leading to ER stress (56). The ER can respond to stress through a complex series of events known as the unfolded protein response (UPR). The UPR comprises a series of signaling events, such as the activation of ER transmembrane proteins, including activating transcription factor

6 (ATF6), which induces various transcription factors such as pro-apoptotic proteins (e.g., CHOP) (57).

In contrast to other mechanisms of toxicity, such as mitochondrial dysfunction and oxidative stress, ER stress has not been comprehensively evaluated in the context of drug-induced side effects. Some data from mouse experiments have suggested that ER stress is a relatively late event in APAP toxicity, with significant stress occurring only 12 h after APAP administration (58). By contrast, hepatic mitochondrial alterations, JNK activation and oxidative stress occur much earlier in mice after the same dose of APAP (59). Sublethal doses of APAP can result in a redox shift in ER compartments; notably, a shift of ER oxidoreductase from the redox state to the oxidized state in hepatic microsomes suggests that the ER is in a state of redox imbalance. This state impairs ER oxidoreductase function and may initiate ER-associated signaling pathways, including ATF6 and CHOP activation, which inhibits the expression of anti-apoptotic genes and activates pro-apoptotic genes (60). By contrast, CHOP deletion has been reported to protect mice from liver injury after APAP poisoning (61). Moreover, caspase-12, an ER-resident caspase, is transiently activated by ER stress-related factors such as calcium ion imbalance, misfolded protein accumulation, and induction of ER stress inducers. It can be considered that the activated state of caspase-12 does not last long, but occurs for a short time and then disappears. By contrast, the mode of cell death in APAP hepatotoxicity is caspase-independent apoptosis, triggered instead by activation of poly(ADP-ribose) polymerase 1 (62). The mode of cell death in APAP hepatotoxicity is thought to be necrosis rather than apoptosis because there is no caspase activation following APAP overdose, despite mitochondrial rupture and release of intermembrane proteins. Moreover, caspase inhibitors are ineffective in protecting the liver from APAP toxicity (63).

NAPQI can covalently bind to a variety of microsomal proteins, such as GST, protein disulfide isomerase (PDI) and calreticulin. Since PDI and calreticulin have major roles in protein folding and calcium chelation within the ER, covalent binding of NAPQI to these proteins can induce ER stress (64). Furthermore, ER stress may be a secondary consequence of mitochondrial dysfunction. The ER and mitochondria are closely linked, and the physical binding of these two organelles creates a structure called the mitochondria-associated ER membrane; in a physiological context, this connection allows for continuous calcium transfer. During ER stress, calcium can be transferred to the mitochondria in large amounts through these microstructural domains or as a result of increased cytoplasmic calcium levels; thus, the induction of ER stress can increase mitochondrial calcium overload, which in turn favors mitochondrial membrane permeability and the release of pro-apoptotic factors, such as cytochrome *c* and AIF (65). Notably, ER stress can also stimulate the expression of the mitochondrial stress factor heat shock protein 60 (HSP60) in mouse hepatocytes, and may impair mitochondrial respiration and decrease mitochondrial membrane potential. This finding not only reveals the complex interaction between the ER and mitochondria, but also demonstrates that ER stress can induce mitochondrial stress (66).

Aseptic inflammation. Inflammatory liver diseases that are not caused by pathogens, such as AILI, non-alcoholic steatohepatitis and alcoholic liver disease, are considered 'sterile' (67). While hepatocellular necrosis is the initial underlying mechanism of APAP-induced hepatotoxicity, the second step in liver injury is a sterile inflammatory response to necrotic hepatocytes. The total number of leukocytes in the livers of mice has been shown to increase ~3-fold a total of 18 h after APAP injection, suggesting a notable inflammatory response during AILI (68). Hepatocytes release intracellular components that can act as damage-associated molecular patterns (DAMPs), such as high mobility group box 1 protein, DNA fragments and HSPs, which act as ligands for Toll-like receptors on macrophages (69), thus leading to transcriptional activation of cytokines and chemokines. Most of these inflammatory factors can be released directly into circulation, but a few cytokines require caspase-1 activation to trigger their activity. Activated caspase-1 induces the production of the NLRP3 inflammasome complex, which comprises NLRP3, caspase-activated deoxyribonuclease and procaspase-1 (70); this complex mediates a pro-inflammatory response through neutrophil and monocyte activation, and recruitment to the liver (71). When neutrophils are activated following an APAP overdose, those in the peripheral circulation tend to move toward the site of injury in the early stages, with the majority migrating to areas of necrosis, after which the injury begins to worsen, peaking at 24 h. Subsequently, the neutrophil levels begin to decrease during the recovery phase of liver injury. Monocytes participate in inflammation via phagocytosis, regulates connective tissue remodeling and fibrosis processes and the formation of extracellular traps (72). Monocytes also serve an important role during the aseptic response. On the one hand, activated monocytes can control neutrophil activation and recruitment through chemokines; on the other hand, when monocytes reach the necrotic area, they can be transformed into monocyte-derived macrophages, which leads to a rapid increase in the number of hepatic macrophages. CCL2 is a mediator of monocyte entry from bone marrow into circulation (73). Although the aseptic response removes necrotic cell debris and promotes tissue repair, leukocytes contribute to tissue damage. Given the dual role of inflammation in APAP hepatotoxicity, it could be hypothesized that a moderate inflammatory response might contribute to tissue repair and hepatocyte regeneration, whereas an excessive response could exacerbate liver injury (74).

4. Treatments

The primary treatment for APAP poisoning is the use of the specific detoxifying drug NAC, which can minimize APAP damage to the liver. In addition, liver transplantation is an option to consider for patients with ALF. However, NAC may cause some adverse effects in clinical application and there is a problem of drug resistance. Therefore, there is an urgent need to develop new drugs to improve the therapeutic efficacy. The present study explores the possible therapeutic targets for APAP poisoning and reviews the current major therapeutic approaches to inform future treatment strategies.

Inhibition of toxic metabolite production. Historical progress of research on NAC is shown in Table I. The first demonstration of the detoxifying role of GSH in DILI was made by Mitchell *et al* (75) at the National Institutes of Health in 1973,

Table I. Historical progression of NAC for the treatment of APAP hepatotoxicity.

First author, year	Research progress	(Refs.)
Peterson, 1977	The first report of NAC treatment of APAP overdose was presented.	(120)
Akakpo, 1985	Oral NAC was approved by the FDA for APAP overdose.	(33)
Corcoran, 1985	After 1 h of APAP treatment, NAC effectively promoted detoxification of NAPQI in mice, leading to attenuated protein adduct formation.	(121)
Smilkstein, 1988	Administration of NAC within the first 8-10 h after APAP ingestion was revealed to be effective in preventing liver injury and liver failure.	(122)
Rumack, 2002	The IV NAC dosing regimen of 300 mg/kg infused over 21 h was approved by the FDA.	(123)
Yarema, 2009	When NAC was initiated within 12 h, the relative risk of hepatotoxicity was lower in the IV group vs. the oral group.	(124)
Yang, 2009	Long-term NAC treatment was revealed to impair APAP-induced liver regeneration in patients with ALI, at least in part by inhibiting the NF-κB activation pathway.	(125)
Blieden, 2010	Common side effects, such as nausea and vomiting, were reported to occur after NAC treatment.	(126)
Saito, 2010	The most effective protective mechanism of NAC was revealed to be the enhancement of hepatocyte clearance of NAPQI, which could prevent covalent modification of cellular proteins, thereby blocking the initiation of APAP toxicity.	(77)
Waring, 2012	The side effects of NAC were reported to be similar to the clinical manifestations of allergic reactions, including rash, pruritus, angioedema and bronchospasm.	(127)
de Andrade, 2015	NAC was revealed to attenuate hepatic injury inflammation during APAP-induced hepatotoxicity and to exert antioxidant effects.	(128)
Khayyat, 2016	After NAC treatment, GSH was significantly elevated in an APAP-treated group; the protective effect of NAC may occur by its promotion of GSH biosynthesis.	(129)
Downs, 2021	A standard dose of NAC was sufficient to prevent 91% of hepatotoxicity in a large number of patients with APAP overdose treated with NAC within 8 h.	(130)

ALI, acute liver injury; APAP, acetaminophen; FDA, Food and Drug Administration; GSH, glutathione; IV, intravenous; NAC, *N*-acetylcysteine; NAPQI, *N*-acetyl-*p*-benzoquinone imine.

and their first report on oral NAC treatment for APAP overdose was published in May 1977. In 1985, NAC treatment was found to be effective in detoxifying NAPQI in mice 1 h after APAP administration (76), and it was suggested that this detoxification may be related to enhancement in APAP-GSH metabolite formation and attenuation in protein adduct synthesis. Notably, NAC is a precursor of GSH; NAC is deacetylated to cysteine and conjugated to glutamate, which is further converted to glutamylcysteine by glutamate-cysteine ligase (GCL) in hepatocytes. Glutamylcysteine binds to glycine via GSH synthetase to produce GSH, which effectively prevents hepatotoxicity due to GSH depletion (77). NAC also reduces the covalent bonding of APAP, thereby attenuating hepatic necrosis, and improves mitochondrial energy metabolism through ATP maintenance in addition to scavenging reactive oxygen species (ROS) and peroxynitrite (78). This energy generation is accomplished primarily through the Krebs cycle, also known as the tricarboxylic acid cycle or aerobic respiration, which is part of the cellular respiration process. The Krebs cycle maintains cell survival and function by breaking down glucose into carbon dioxide and water to produce energy (ATP) (77). Furthermore, NAC exhibits anti-inflammatory and antioxidant properties (79). Approximately 40 years after its introduction, NAC remains the only Food and Drug Administration-approved antidote for APAP overdose. The standard oral or intravenous dosing regimen of NAC is highly effective in patients who develop a moderate overdose within 8 h of APAP ingestion (80). NAC was initially administered orally; however, intravenous infusion is currently more common in clinical practice. Mainly due to the fact that the patient is in a coma and cannot be administered orally, at the same time, intravenous injection can ensure 100% of the drug enters the blood circulation, avoiding uncertainty in the oral absorption process.

In the United States, the approved dose of oral NAC is 140 mg/kg body weight followed by 70 mg/kg body weight every 4 h for a total of 17 doses (81); however, the efficacy of NAC reduces in patients with advanced disease or after a large overdose because the drug has often been metabolized in the body for some time before the patient is admitted to the clinic (39). A series of adverse reactions can occur during NAC treatment; some examples are intravenous NAC side effects such as allergic reactions, nausea, vomiting, diarrhea or constipation, fever, headache, drowsiness and low blood pressure (82). These reactions mainly occur in the first hour after NAC infusion, corresponding to the peak of NAC concentration (83); therefore, a drug that can prolong the therapeutic

window period of NAC is urgently awaited. Based on NAC trials, scientists have shown through animal experiments that co-treatment of fomepizole (4MP) with APAP can effectively prevent AILI (84,85); 4MP can strongly attenuate hepatic GSH depletion, and can almost eliminate APAP-AD and all oxidative metabolites of APAP, suggesting that it effectively inhibits CYP2E1 and largely prevents the formation of NAPQI. 4MP may be considered a good candidate for NAC-assisted treatment of patients with selective APAP overdose (33).

Activation of autophagy. Autophagy is the conserved process by which substrates in the cytoplasm are translocated to lysosomes through intermediate double-membrane-bound vesicles called autophagosomes (86). It is a tightly regulated cellular process that is essential for the maintenance of cellular homeostasis through lysosomal degradation to remove unwanted cytoplasmic contents, including misfolded or aggregated proteins, accumulated lipids, excessive or damaged organelles, and harmful pathogens (87), in order to achieve cell renewal (88). It has been demonstrated that APAP treatment induces the formation of autophagosomes in mouse livers and that autophagy defects enhance hepatocyte apoptosis and necrotic cell death resulting from APAP treatment, a process that relies on MPTP opening, depolarization of mitochondrial membranes, swelling and loss of proteins such as cytochrome *c* (89).

Mitochondrial damage is a key event in APAP hepatotoxicity. Autophagy selectively removes damaged organelles, especially mitochondria, thereby preventing oxidative stress and necrotic cell death caused by mitochondrial damage (90). This process is largely dependent on a mitochondrial serine/threonine kinase (PINK1). In healthy mitochondria, the mitochondrial transmembrane potential drives PINK1 into the IMM via outer mitochondrial membrane translocase; mitochondrial damage induces the accumulation of PINK1, which recruits Parkin to initiate mitosis. Activated Parkin mediates ubiquitination of mitochondrial outer membrane proteins, which acts as a signal to recruit autophagy aptamers, such as OPTN, NDP52 and p62. As a result, the autophagy machinery is recruited to degrade damaged mitochondria (91). Excess APAP can activate autophagy in mouse livers and primary hepatocytes for protective purposes, which can counteract the mitochondrial oxidative stress induced by APAP and JNK activation, among others (92). Mechanistically, this is likely a compensatory response to excess ROS after APAP intoxication, and in this regard, mitochondria are the main source of ROS required for autophagy-inducing signaling; ROS accumulation can induce autophagy by directly affecting core autophagy mechanisms and indirectly affecting components of autophagy-regulated signaling pathways (93). Considering that the accumulation of APAP-AD is an early and critical step in mitochondrial damage, the formation of such adducts poses a potential threat to mitochondria. Therefore, timely and effective removal of APAP-AD may be an important strategy to alleviate the toxic effects of APAP on mitochondria, thus maintaining their normal function, ensuring smooth intracellular energy metabolism, and providing sufficient ATP for cells. Autophagy can selectively remove deleterious APAP-AD, thereby protecting the liver from AILI to a certain extent (89). Furthermore, activated autophagy can remove damaged mitochondria, which are the sites of ROS production; elevated ROS levels can induce autophagy. Removal of damaged mitochondria can reduce ROS production and eliminate inflammatory vesicles (e.g., NLRP3 inflammatory vesicles), potentially inhibiting inflammatory responses and maintaining the normal turnover and regulatory functions of the ER (94).

Activation of the nuclear factor erythroid 2-related factor 2 (Nrf2) signaling pathway and inhibition of ferroptosis. Nrf2 is involved in various aspects of cellular and organismal detoxification against oxidative stress, regulation of cellular metabolism and promotion of cellular proliferation, and serves a crucial role in the pathological mechanisms of several diseases as well as homeostasis (95). The Nrf2 pathway is considered to have a key role in AILI, and activation of Nrf2 signaling is a potential target for ameliorating APAP hepatotoxicity. NAPQI, the intermediate metabolite produced by APAP, contributes to GSH depletion and ROS formation; this triggers oxidative stress, which leads to mitochondrial dysfunction, hepatocyte necrosis and liver injury (70). To counteract oxidative stress induced by ROS, the body maintains cellular redox homeostasis through a series of antioxidant molecules and detoxifying enzymes. The Nrf2/ARE pathway is one of the major nuclear transcription factor-associated mechanisms that respond to this process by activating phase II detoxification enzymes; Nrf2 can detoxify NAPQI by activating antioxidant enzymes (96). GCL, a key enzyme in GSH synthesis, consists of catalytic (GCLC) and modifying subunits; GCLC is one of the downstream targets of Nrf2 activation (97).

Iron-dependent cell death (ferroptosis) is a regulated form of cell death that can be induced when intracellular GPX4 is directly or indirectly inhibited by low GSH levels (98). APAP has been extensively studied and shown to induce hepatic lipid peroxidation and ferroptosis, a cascading effect that ultimately leads to ALF in mice (99). Notably, APAP-induced ALF may be prevented by pharmacological and genetic inhibition of ferroptosis, which acts as an initial event during the course of APAP-induced hepatotoxicity and is not only capable of directly damaging hepatocytes, but also triggers the release of extracellular substances, such as DAMPs, that further exacerbate inflammatory responses and intensify liver injury. Inhibition of the Nrf2 pathway increases sensitivity to ferroptosis and GPX4 is a downstream target of Nrf2 (100). Several natural small-molecule drugs can activate the Nrf2 pathway, and typically exhibit better biocompatibility and lower side effects than conventional chemical drugs; they are of natural origin, easily accessible and cost-effective. For example, curcumin, a plant polyphenol extracted from turmeric, has been shown to upregulate the expression of antioxidant enzymes, such as GPX4, by promoting the release and activation of Nrf2, thereby attenuating ferroptosis and alleviating APAP-induced hepatotoxicity; this finding provides a new strategy and direction for the treatment of APAP-induced ALF (101). Research progress on natural small-molecule drugs for attenuating AILI has been summarized in Table II, which lists the names and mechanisms of action of various natural small-molecule drugs, providing valuable references for researchers and clinicians.

Table II. Advances in the study of natural small molecule drug substances to ameliorate APAP liver injury.

First author, year	Small molecule drug	Mechanism	(Refs.)
Cai, 2022	ASX	ASX ameliorated AILI *in vivo* and *in vitro* by reducing inflammation, inhibiting oxidative stress and ferroptosis via the NF-κB pathway, and increasing autophagy via the Nrf2/HO-1 pathway.	(101)
Li, 2023	KA	KA activated the Nrf2 signaling pathway and inhibited ferroptosis. KA pretreatment improved biochemical indices related to liver function, and attenuated liver necrosis and inflammation in APAP-induced mice.	(97)
Pang, 2016	CA	CA prevented APAP-induced hepatotoxicity by decreasing the expression of Keap1 and inhibiting the binding of Keap1 to Nrf2, which activated Nrf2, leading to increased expression of antioxidant signaling.	(131)
Yang, 2020	Limonin	Limonin attenuated APAP-induced hepatotoxicity by activating the Nrf2 antioxidant pathway and inhibiting the NF-κB inflammatory response through upregulation of Sirt1.	(132)
An, 2023	Abietic acid	Abietic acid inhibited APAP-induced NF-κB activation, and increased Nrf2 and HO-1 expression. *In vitro*, the inhibitory effects of Abietic acid on APAP-induced inflammation and iron death were reversed when Nrf2 was knocked down.	(133)
Lv, 2019	Coriandrin	Coriandrin could counteract APAP-induced ALF by inducing Nrf2 via the AMPK/GSK3β pathway.	(134)
Wang, 2016	EsA	EsA enhanced Nrf2-regulated survival mechanisms through the AMPK/Akt/GSK3β pathway and has been shown to possess protective potential against APAP toxicity.	(135)
Wang, 2019	Fariro	Activation of Nrf2 and induction of autophagy by Fariro through the AMPK/AKT pathway contributed to its hepatoprotective activity *in vitro*, and had protective potential against APAP-induced hepatotoxicity.	(96)
Zhu, 2023	Xanthohumol	Xanthohumol inhibited APAP-induced liver injury by suppressing oxidative stress and mitochondrial dysfunction through activation of Nrf2 by AMPK/Akt/GSK3β.	(136)
Yao, 2022	Rosmarinic acid	Rosmarinic acid was shown to protect against AILI through Nrf2-mediated inhibition of the NEK7-NLRP3 signaling pathway.	(137)
Jiang, 2022	Sarmentosin	Sarmentosin attenuated APAP-induced ALF and protected hepatocytes from APAP injury in mice, and the important role of Nrf2-mediated oxidative stress in the dynamics of this process was demonstrated.	(138)

AILI, APAP-induced liver injury; ALF, acute liver failure; APAP, acetaminophen; ASX, astaxanthin; CA, caffeic acid; HO-1, heme oxygenase-1; KA, kaempferol; Nrf2, nuclear factor erythroid 2-related factor 2.

Liver repair and regeneration. Liver regeneration is a compensatory and beneficial process in response to hepatocyte death and tissue damage; stimulating this process may be a potential therapeutic strategy to control APAP hepatotoxicity (102). The role played by innate immune cells in APAP hepatotoxicity has been controversial, but evidence has emphasized that innate immune cells are critical for liver repair (103). Immune cells, such as neutrophils and macrophages, stay mainly in necrotic areas after APAP overdose; hepatic neutrophil infiltration is a key step of the innate immune response to acute liver injury (104) and is critical for recovery after APAP overdose (105). In addition, platelets serve a key role in the course of AILI and platelet depletion can attenuate hepatic injury to some extent. Platelet-expressed c-type lectin-like receptor (CLEC)-2 is a key mediator of platelet activation, and CLEC-2-mediated elimination of platelet signaling may enhance recovery after acute liver injury via TNF-α-dependent hepatic recruitment of neutrophils (103). In this context, neutrophil infiltration represents an innate immune repair response that can aid in rapid liver repair after acute injury.

Hepatocytes have a major role in the process of liver regeneration. Liver recovery after APAP hepatotoxicity involves a combination of numerous factors, such as hepatic microvascular reconstruction and interactions between different cell types. Among these factors, vascular endothelial growth factor (VEGF), a major regulator of tumorigenesis, inflammation and wound healing, can effectively promote liver regeneration (106). Cellular crosstalk

between LSECs and hepatocytes during liver regeneration serves an important role in hepatic sinusoidal homeostasis and physiological angiogenesis (107). A comparison of VEGF receptor 1 (VEGFR1) tyrosine kinase-knockout (VEGFR1 TK2/2) and wild-type (WT) mice revealed that the expression levels of proliferating cell nuclear antigen and growth factors, such as hepatocyte growth factor, CD31 and basic fibroblast growth factor, were lower in the knockout mice than in WT mice. In addition, the VEGFR1 TK2/2 mice showed impaired hepatic microvascular function, which was reduced by ~40% compared with that in WT mice, a phenomenon that was associated with enhanced hepatic MMP-9 expression (108). Selective activation of VEGFR1 could serve as a target to promote tissue repair by facilitating blood sinusoidal cell recovery after acute liver injury; however, several risks are associated with VEGFR1 activation. Its activation on endothelial cells can promote malignant angiogenesis, thereby enhancing the migration and activity of endothelial cells, which may facilitate the invasion of hepatocellular carcinoma cells (109).

Liver transplant. Hepatotoxicity can be completely prevented if NAC is administered within 10 h of overdose. Patients who do not receive NAC in time can develop severe liver injury, which may progress to ALF. Therefore, in cases of severe liver injury caused by APAP, liver transplantation may be the only treatment option to save the life of the patient (110). Moreover, liver transplantation is the only effective strategy for the treatment of APAP-induced liver failure (111). The decision to undergo liver transplantation for APAP overdose-induced ALF is challenging and may be affected by the scarcity of donor liver grafts, acute graft rejection, lifelong immunosuppression and unaffordable costs. Psychosocial issues of patients with APAP overdose are of concern, as >30% of those who meet the criteria for transplantation have severe mental illness, or alcohol and/or drug abuse problems (112).

5. Discussion

The prognosis of AILI is influenced by multiple factors. APAP, a widely used analgesic and antipyretic, has become a major cause of ALF in several countries and AILI is a major public health problem worldwide. The causes of APAP intoxication can be divided into two categories. For the first cause, i.e., self-administered overdose, patients are taken to the emergency room for NAC treatment early and serious injury can be avoided. In the other category, i.e., accidental overdoses, the optimal time for treatment is often missed before an adverse reaction sets in because the symptoms of poisoning are not obvious in the early stages (24). The total ingested dose of APAP is the most important determinant of the development and severity of APAP-induced hepatotoxicity. The Rumack-Matthew nomogram, developed in the 1970s as a tool for assessing the risk of hepatotoxicity after ingestion of APAP, consists of two main lines: The Treatment Line and the Rumack-Matthew Line, which predict the risk of hepatotoxicity from 4 to 24 h after ingestion. However, this chart does not apply to patients tested 24 h after ingestion or with a history of multiple ingestions (113). In addition, patterns of use and certain factors [e.g., chronic alcohol abuse (114), age, concomitant use of certain medications, genetic factors, pre-existing liver disease and nutritional status] can influence susceptibility to APAP hepatotoxicity via multiple mechanisms (115). For example, a study of 1,039 children aged <7 years found no susceptibility to APAP-induced hepatotoxicity after mild to moderate (up to 200 mg/kg) APAP ingestion (116). This observation could be explained by the fact that the sulfate coupling of the drug is more pronounced in children, whereas the glucuronide coupling is more predominant in adults, which means that differences in the binding pathways may result in a reduced risk of APAP-related toxicity in children. In addition, because NAPQI formation is dependent on APAP metabolism via the CYP system and coupling to mercapturic acid metabolites after therapeutic doses, urinary concentrations of these metabolites are much lower in children than in adults. This suggests that CYP serves a less significant role in APAP metabolism in children than in adults (117). Timely medical intervention, such as the clinical application of NAC, can minimize liver injury.

Research on the epidemiology, mechanism underlying toxicity, diagnosis and treatment of AILI has progressed considerably. However, some persistent challenges include the early diagnosis of APAP-induced hepatotoxicity (when it is not clinically apparent) to avoid severe injury later, and the identification of safe doses for different populations in terms of their sensitivities. Since LSEC damage often occurs in the early stages of liver injury, it could be proposed that the detection of LSEC-specific damage may provide a timely and accurate diagnosis of patients when conventional markers, such as ALT and AST, do not show notable changes. For example, changes in their morphology can be observed by electron microscopy; in normal liver samples, the thin and weak cytoplasm of LSECs contains a large number of pores, which is its unique structure (118). However, when the liver is diseased, the size of the pores will gradually change; for example, in liver fibrosis, the pores will disappear progressively until a continuous basement membrane is formed (119). To achieve this goal, future studies are required to explore the specific relationship between LSEC injury and APAP hepatotoxicity, and to develop more sensitive and specific assays.

In conclusion, research on AILI is continuously advancing, and researchers are exploring the mechanisms of toxicity, biomarkers, therapeutic targets and new therapeutic approaches from multiple perspectives. The results of these research studies are expected to provide new ideas and methods for the prevention and treatment of DILI, and to further reduce the risk of liver injury caused by APAP and other drugs.

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Not applicable.

Competing interests

The authors declare that they have no competing interests.

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