Minireview

The molecular mechanisms of acetaminophen-induced hepatotoxicity and its potential therapeutic targets

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Impact Statement

With the widespread clinical use of acetaminophen (APAP), APAP-induced hepatotoxicity (AIH) has gradually become an important public health concern. The pathogenesis of AIH is quite complicated, and involves a variety of cellular processes. *N*-acetylcysteine is currently used as the main antidote for APAP poisoning, but its therapeutic effect is limited. Therefore, there is an urgent need to comprehensively clarify the molecular mechanisms underlying AIH and to explore novel therapeutic strategies. This work summarizes the important cellular events involved in AIH and discusses potential therapeutic targets against AIH to provide new ideas for AIH intervention.

Abstract

Acetaminophen (APAP), a widely used antipyretic and analgesic drug in clinics, is relatively safe at therapeutic doses; however, APAP overdose may lead to fatal acute liver injury. Currently, *N*-acetylcysteine (NAC) is clinically used as the main antidote for APAP poisoning, but its therapeutic effect remains limited owing to rapid disease progression and the general diagnosis of advanced poisoning. As is well known, APAP-induced hepatotoxicity (AIH) is mainly caused by the toxic metabolite *N*-acetyl-*p*-benzoquinone imine (NAPQI), and the toxic mechanisms of AIH are complicated. Several cellular processes are involved in the pathogenesis of AIH, including liver metabolism, mitochondrial oxidative stress and dysfunction, sterile inflammation, endoplasmic reticulum stress, autophagy, and microcirculation dysfunction. Mitochondrial oxidative stress and dysfunction are the major cellular events associated with APAP-induced liver injury. Many biomolecules involved in these biological processes are potential therapeutic targets for AIH. Therefore, there is an urgent need to comprehensively clarify the molecular mechanisms underlying AIH and to explore novel therapeutic strategies. This review summarizes the various cellular events involved in AIH and discusses their potential therapeutic targets, with the aim of providing new ideas for the treatment of AIH.

Keywords: Acetaminophen, hepatotoxicity, molecular mechanisms, target, liver injury, oxidative stress

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Introduction

Acetaminophen (APAP) is a widely used antipyretic and analgesic drug in clinics, which is relatively safe at therapeutic doses. APAP is currently contained in many prescription and over-the-counter medicines. Due to the misunderstanding of dosing instructions, inadvertent combination of more than one APAP-containing preparation, or continuous medication, patients may overdose. However, when APAP is taken in excess, it may lead to fatal acute liver injury.[1] APAP is one of the most common drugs causing acute liver injury and has become a leading cause of acute liver failure in developed countries, such as Europe and the United States.[2] *N*-acetylcysteine (NAC), a known antioxidant, is clinically used as the main antidote for APAP poisoning, but is only effective in the early stage of APAP intoxication.[3] NAC is a precursor of glutathione (GSH), which can improve oxidative stress by promoting the synthesis of GSH, and thus alleviate acute liver injury. NAC is likely to cause a variety of adverse reactions during NAC administration, including skin rash, allergic reaction, bronchospasm, hypotension, and even death.[4] In addition, NAC must be administered at high doses due to its low bioavailability, but high-dose NAC may increase the risk of adverse reactions.[5] More importantly, if the early and/or the most treatable stage is missed, liver transplantation is the only option to improve the survival of patients with acetaminophen-induced hepatotoxicity (AIH).[6,7]

AIH has received increasing public attention, and great efforts have been made to recognize the pathogenesis of APAP-induced liver toxicity. At its therapeutic doses, only a small amount of APAP is metabolized by the hepatocyte cytochrome P450 enzyme system into *N*-acetyl-*p*-benzoquinone imine (NAPQI), a major toxic metabolite.[8] NAPQI is efficiently scavenged by hepatic GSH stores; thus, no liver damage occurs. However, during APAP overdose,

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the generation of NAPQI increases significantly, leading to rapid depletion of GSH in the liver. Excessive NAPQI binds to the cysteine residues of various essential proteins in cells, thus undermining their normal biological function.[9] Protein binding of NAPQI occurs predominantly in centrilobular hepatocytes, which eventually undergo oxidative stress, mitochondrial dysfunction, and necrotic cell death.[10] Remarkably, mitochondrial oxidative stress and dysfunction have been identified as the main cellular events in APAP-induced hepatic injury by a large number of studies.[11–13] Apart from mitochondrial oxidative stress and dysfunction, other cellular processes are also implicated in the pathogenesis of AIH. These processes include liver metabolism, sterile inflammation, endoplasmic reticulum stress (ERS), autophagy, and microcirculatory dysfunction. Some biomolecules involved in these processes have been determined to play key regulatory roles in AIH and provide promising targets for intervening APAP-induced liver injury. Given the prevalence of APAP use, as well current treatment limitations, the elucidation of the molecular mechanisms and the search for new therapeutic targets have become crucial issues in AIH research. In this review, we summarize the various cellular events involved in AIH and discuss possible therapeutic measures targeting these cellular events.

Molecular mechanisms of AIH

Liver metabolism of APAP

APAP metabolism mostly occurs in liver microsomes. Most APAP (85–90%) is catalyzed by UDP-glucuronosyltransferase (UGT) or sulfonyltransferase (SULT) enzymes to produce non-toxic glucuronosylated or sulfated metabolites, respectively.[14] Glucuronic acid conjugates are rapidly eliminated from hepatocytes and mostly (>75%) excreted into bile.[15] The remaining APAP-glucuronides (<25%) are secreted into the plasma through basolateral transporter multidrug resistance–associated protein 3 (MRP3).[16] Most APAP-sulfates are excreted into bile by multidrug resistance–associated protein 2 (MRP2), and a few are excreted into bile by human breast cancer resistance protein 1 (BCRP1).[17] Only approximately 2% of APAP is excreted into the urine.[18] The remaining APAP (5–9%) is oxidized by the cytochrome P450 enzymes, mainly cytochrome P450 2E1 (CYP2E1), into NAPQI, which leads to AIH.[18] At the therapeutic doses of APAP, NAPQI loses its toxicity by binding with GSH in the liver. In addition, NAPQI can directly modify the cysteine sulfhydryl group of the cytosolic kelch-like ECH associated protein 1 (Keap1), allowing the separation of nuclear factor erythroid 2-related factor 2 (Nrf2) from Keap1 by deubiquitination. This activates the Keap1-Nrf2 pathway and upregulates antioxidant enzymes to promote metabolic inactivation of APAP.[19] Once APAP is overdosed, a large amount of NAPQI accumulates, thereby resulting in a rapid depletion of GSH in the liver. As a result, the physiological regulation of Keap1-Nrf2 is lost, and free NAPQI reacts with protein sulfhydryl groups to form APAP adducts (Figure 1). The mitochondria are the main site of the formation of protein adducts, which may block the synthesis of mitochondrial DNA by impairing related enzymes.[20] It is worth noting that gene polymorphisms of liver metabolic enzymes, such as *UGT*, *SULT*, and *CYP2E1*, and some co-existing diseases, such as viral hepatitis, acute and chronic malnutrition, and nonalcoholic fatty liver disease, may increase the susceptibility to APAP-induced liver injury, even at a therapeutic dose.[21,22] This phenomenon probably correlates with the increase in NAPQI caused by these factors.

Mitochondrial oxidative stress and dysfunction

During AIH, mitochondrial proteins such as housekeeping proteins, GSH peroxidase, and adenosine triphosphate (ATP) synthase are the binding targets of NAPQI. In addition, NAPQI can also interfere with complexes I and II in the mitochondrial electron transport chain (ETC), resulting in electron leakage to form superoxide radicals.[23] A variety of antioxidant enzymes – such as superoxide dismutase 2 (SOD2), catalase (CAT), and GSH peroxidase (GPX) – usually scavenge superoxides from mitochondria. A previous study showed that APAP-induced liver injury was significantly aggravated in SOD2-deficient mice.[24] Except for being decomposed into H_2O_2 and O_2, excess superoxides can also react with nitric oxides (NOs) to synthesize highly reactive peroxynitrites in the mitochondria.[25] Normally, GSH is able to effectively remove these peroxynitrites. When exposed to a large amount of NAPQI, GSH would be exhausted to trigger the tyrosine nitration of mitochondrial proteins. These nitrated mitochondrial proteins give rise to mitochondrial DNA damage and the opening of the mitochondrial permeability transition pore (MPTP), eventually inducing cell necrosis (Figure 2).[26] Mitochondrion is the most important organelle of energy metabolism in cells, which synthesize most of their ATP pool through oxidative phosphorylation. ATP is essential for maintaining normal cellular activity. Several metabolomics studies have confirmed that APAP-induced mitochondrial oxidative stress disrupted the metabolic processes of citric acid cycle and fatty acid β-oxidation, thus blocking ATP synthesis and accelerating hepatocyte death.[27–29] Currently, three major signaling proteins – including c-Jun N-terminal kinase (JNK), Nrf2, and p53 – have been found to be involved in mitochondrial oxidative stress. Notably, all of these proteins can serve as targets for alleviating APAP-induced mitochondrial oxidative stress.

JNK

An early result of APAP-induced mitochondrial oxidative stress is the activation of JNK in the cytoplasm. The massive NAPQI levels deplete GSH in hepatocytes, resulting in the release of superoxides from the mitochondria into the cytosol. These superoxides can oxidize thioredoxins and detach the apoptosis signal-regulating kinase 1 (ASK-1) from them, thus triggering the self-activation of ASK-1.[30,31] In addition, mitochondrial oxidative stress may also trigger the phosphorylation of glycogen synthase kinase-3β (GSK-3β), which activates the mixed lineage kinase 3 (MLK3) protein.[25,32] Activated ASK1 and MLK3 then stimulate mitogen-activated protein kinase kinase kinase 4/7 (MKK4/7) and activate JNK to initiate a cascade effect.[33,34] JNK translocates to mitochondria, binds to SH3 homology–associated Bruton's tyrosine kinase (BTK) binding protein (Sab) on the mitochondrial

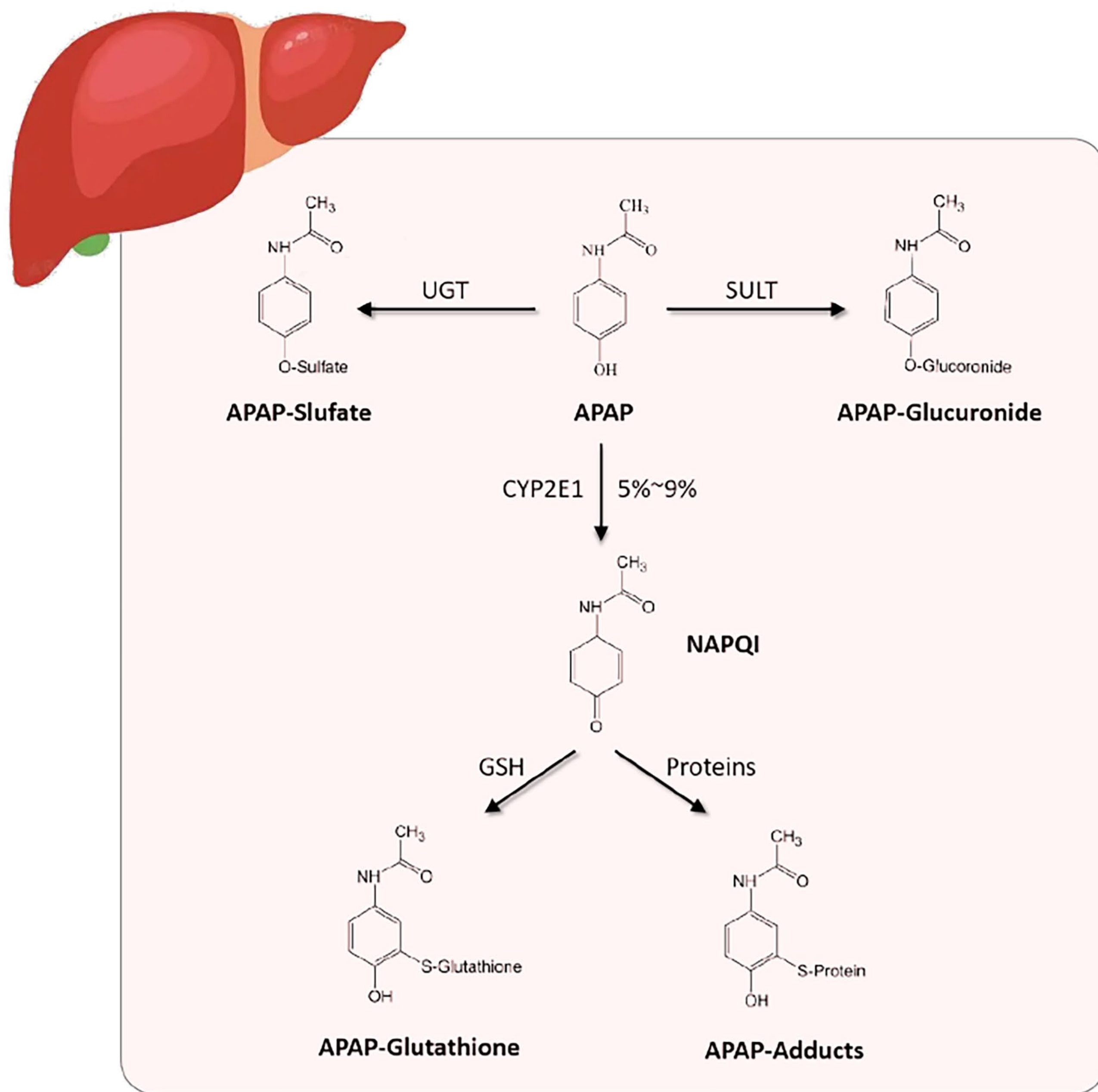

Figure 1. The metabolic ways of acetaminophen.

membrane, and subsequently inactivates a proto-oncogene tyrosine-protein kinase (Src) in mitochondria, eventually leading to the dysfunction of the ETC and the increased release of reactive oxygen species (ROS).[35] ROS activates the upstream mitogen-activated protein kinase (MAPK), which phosphorylates JNK. Sustained activation of JNK can further promote the generation of mitochondrial ROS, thus forming a self-sustaining activation loop. Furthermore, activated JNK translocates the mitochondria, and recruits cytoplasmic Bax, which triggers the opening of MPTP, thereby leading to the loss of membrane potential and the depletion of ATP.[36] The induction of MPTP will eventually cause the release of multiple important mitochondrial proteins into the cytoplasm, such as apoptosis-inducing factor (AIF), endonuclease G (Endo G), and cytochrome c (Cyt C). These proteins are transferred to the nucleus, where they induce DNA fragmentation and cell death (Figure 2).[37]

Nrf2

Nrf2 is an intracellular transcription factor that protects cells from oxidative stress by regulating the expression of protective genes. Under physiological conditions, Nrf2 binds to an inhibitor, keap1, and remains inactive in the cytoplasm. When oxidative stimulation occurs, Nrf2 releases from keap1, and activates itself. Activated Nrf2 binds to antioxidant response elements (AREs) and translates to the nucleus, ultimately initiating the expression of downstream target genes, such as heme oxygenase-1 (HO-1), NAD(P)H:quinone oxidoreductase 1 (NQO1), and glutamate–cysteine ligase catalytic

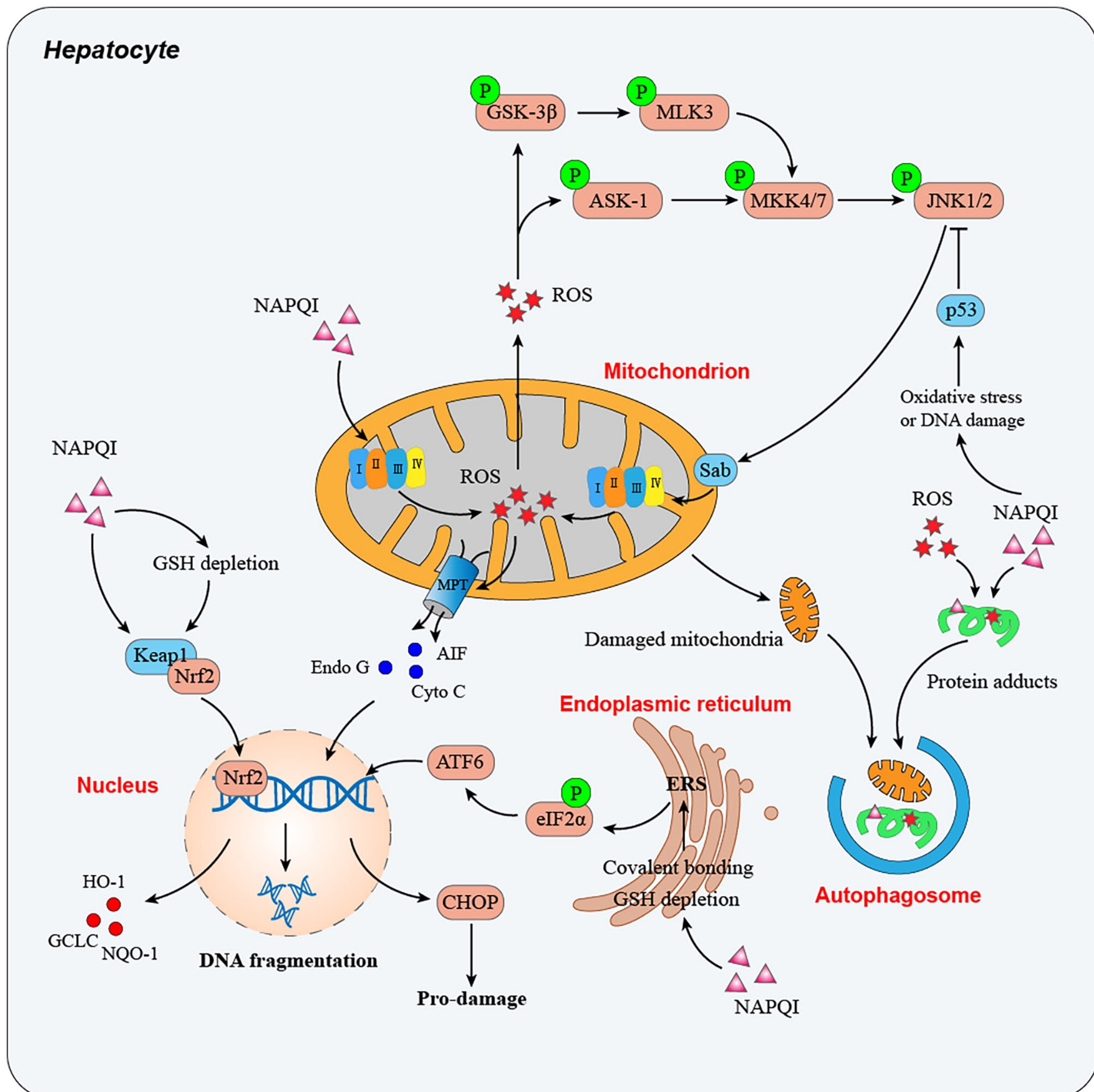

Figure 2. The major cell events of acetaminophen-induced hepatotoxicity.

(GCLC) subunit.[38] These detoxification-related proteins promote metabolic inactivation of APAP, thus protecting the liver from damage induced by NAPQI (Figure 2).[39] Current studies have confirmed that Nrf2-deficient mice have a high sensitivity to AIH, while Nrf2-activated mice have a high resistance to AIH.[40,41] The Nrf2-keap1 pathway may be regulated through a variety of mechanisms. In addition to being directly activated by NAPQI, protein tyrosine phosphatase 1 B (PTP1B),[42] fibroblast growth factor 21 (FGF21),[43] and the M1 muscarinic receptor (M1R)[44] have also been found to modulate the Nrf2 signaling pathway.

P53

p53 participates in the regulation of many important biological processes, such as the cell cycle, cell apoptosis, and DNA damage repair. p53 is activated by a variety of stimuli including oxidative stress and DNA damage. Notably, p53 plays a dual role in oxidative stress: in mild and moderate oxidative stress, it promotes the repair of genetic damage and cell survival by mediating cell cycle arrest and DNA repair; in severe oxidative stress, it triggers cell aging and apoptosis.[25] Huo *et al.*[45] observed that p53 was activated in AIH mice. Furthermore, when p53 was inhibited, JNK phosphorylation was enhanced to induce more severe liver injury in the APAP model. In addition, p53-deficient mice were found to be more vulnerable to APAP-induced liver toxicity, indicating that p53 was a protective factor against AIH.[46] However, p53 signaling pathway was confirmed to play different roles in the development of APAP-induced liver injury and subsequent compensated liver regeneration.[47]

Sterile inflammation

APAP-induced liver toxicity may trigger extensive sterile inflammation in the liver tissue.[48] A large area of cell necrosis appears following APAP overdose. Damaged hepatocytes may release massive amounts of cell contents, such as nuclear DNA fragments, mitochondrial DNA, high mobility group box-1 (HMGB1), and ATP.[49] These cell contents, also known as damage-associated molecular patterns (DAMPs), may bind to and activate Toll-like receptors in macrophages, promote the formation of inflammasome complexes, and induce the release of pro-inflammatory cytokines. These pro-inflammatory mediators subsequently recruit neutrophils and monocytes to the damaged area of the liver to trigger sterile inflammation. After APAP overdose, cytokines, such as tumor necrosis factor-alpha (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6), and chemokines, such as monocyte chemoattractant protein-1 (MCP-1) and macrophage inflammatory protein 2 (MIP-2), were detected in the plasma of animals and patients.[50,51] The Toll-like receptor family is commonly expressed in liver macrophages and other immune cells. Toll-like receptor 4 (TLR4) and Toll-like receptor 9 (TLR9) have been confirmed to closely correlate with the development of AIH.[52,53] Furthermore, the activation of inflammasomes mainly manifests as an elevation of serum IL-1β and interleukin-18 (IL-18) and the recruitment of inflammatory cells.[54] A prior study reported that the deletion of TLR9 or Nalp3 markedly weakens APAP-induced hepatic injury, thereby increasing the survival rate of mice. The underlying mechanisms could involve the reduction of serum IL-1β and neutrophils by activating the Nalp3 inflammasome.[55] A sustained and amplified inflammatory response accelerates the release of abundant pro-inflammatory cytokines. These cytokines promote the expression of inducible nitric oxide synthase (iNOS) to increase the formation of peroxynitrites, greatly aggravating liver injury. Conversely, inflammation can erase necrotic cell debris and promote liver repair and regeneration. For example, IL-6 from the inflammatory reaction promotes the regeneration of damaged liver tissue, and this effect is achieved by increasing the expression of protective heat shock proteins with strong hepatoprotective activity.[56] It was confirmed that the aggregation of neutrophils and macrophages in the injured area was necessary to promote liver regeneration.[57] Inflammation may play an opposing role at different stages of the pathological process of AIH: it functions as a promoter in the injury stage, whereas it is a helper in the regeneration stage.[25] Thus, blocking inflammation may have a protective effect at the beginning, but is actually harmful to the final outcome. Overall, there is controversy regarding sterile inflammation as a potential therapeutic target for AIH. However, inducing or inhibiting inflammation after clarifying its role at different stages will remain a favorable treatment strategy.

ERS

The liver is the major organ involved in drug metabolism, and there are abundant endoplasmic reticula in liver cells. The endoplasmic reticulum is the main site for the synthesis, processing, and transport of various proteins in cells. An increase in misfolded or unfolded proteins in the endoplasmic reticulum can induce ERS. When ERS occurs, the cells are able to maintain intracellular homeostasis through the unfolded protein response.[58] Long-term or extreme ERS also leads to endoplasmic reticulum dysfunction and further induces cell apoptosis.[59] C/EBP homologous protein (CHOP) plays an important role in ERS-induced apoptosis, which is mainly regulated by three sensors in the inner membrane of the endoplasmic reticulum, namely, protein kinase RNA-like endoplasmic reticulum kinase (PERK), activating transcription factor 6 (ATF6), and inositol needs protein 1 (IRE1).[60] A previous study found that hepatic endoplasmic reticulum underwent severe oxidative stress in early APAP-induced liver damage.[61] Similarly, Uzi *et al.*[62] reported that ERS was observed in mice 12 h after APAP treatment, and APAP evidently induced liver injury by activating CHOP. In the development of AIH, ERS was triggered by the covalent binding of NAPQI to endoplasmic reticulum proteins.[63] The accumulated NAPQI depletes GSH in the endoplasmic reticulum to induce an oxidation–reduction imbalance, which initiates the phosphorylation of eukaryotic initiation factor 2α (eIF2α) to activate ATF6 and CHOP, eventually resulting in hepatocyte apoptosis (Figure 2).[64] The above evidence clearly shows that ERS is tightly associated with the development of AIH.

Autophagy

Autophagy is strictly regulated by the cell itself, which renews the cell by removing unnecessary cytoplasmic contents, including macromolecules, misfolded proteins, and damaged organelles. Multiple intracellular stimuli, such as ROS and ERS, can induce autophagy.[65] As a basic mechanism for maintaining cell homeostasis, autophagy is able to eliminate APAP adducts and damaged mitochondria, thus preventing APAP-induced necrosis.[66,67] PTEN-induced kinase 1 (PINK1) can act as a molecular sensor to detect the functional state of mitochondria. When mitochondria are damaged, PINK1 first accumulates on the mitochondrial outer membrane and then recruits Parkin, which is considered to be required for initiating mitochondrial autophagy *in vitro* models, to initiate the mitochondrial autophagy. The double deletion of PINK1 and Parkin severely impairs mitochondrial autophagy and aggravates the liver damage caused by APAP overdose in mice.[68] Igusa *et al.*[69] further found that mouse liver cells with liver autophagy deficiency induced by the selective knockout of the *ATG7* gene were more vulnerable to AIH. In their research, APAP-induced ROS production, mitochondrial membrane depolarization, and JNK activation in hepatocytes were significantly accelerated by autophagy defect. In addition, a recent study also revealed that the liver-specific Ulk1 and Ulk2 double knockout mice, where Ulk1 and Ulk2 were key components of the unc-51-like autophagy activating kinase 1 (ULK1) complex that functions upstream of the autophagy pathway, were more resistant to APAP-induced liver injury.[70] Notably, Ulk1/2 knockdown did not affect the autophagy activity of hepatocytes but rather inhibited JNK activation by blocking the phosphorylation of MKK4/7.[70] Collectively, autophagy activation is likely to play a protective role in AIH.

Microcirculation dysfunction

Studies have shown that NAPQI-induced microvascular congestion in the centrilobular region precedes direct hepatocyte injury.[71,72] This microcirculatory damage results from the collapse of sinus wall and the flow of blood components into the space of Disse.[73,74] Liver sinusoidal endothelial cells (LSECs) are the early targets of APAP-induced hepatic injury.[72] LSEC swelling was the earliest morphological alteration that occurred 30 min after the APAP attack. This change leaded to the collapse of the sinus wall, which caused red blood cells to infiltrate the Disse space. This reduced sinusoidal perfusion, thus exacerbating the development of APAP-induced liver injury.[73] It has also been reported that the increased matrix metalloproteinase (MMP) levels that occurred during APAP intoxication was closely associated with hepatic microcirculatory dysfunction, including impaired sinusoidal perfusion, and infiltration of erythrocytes in the Disse space.[75] Notably, a previous study found that damaged LSECs could result in liver congestion, and APAP might be directly toxic to the LSECs isolated from mice through the depletion of GSH.[76] In particular, a clinical study also identified an abnormal function of LSECs in patients with AIH using serum hyaluronic acid, which strongly supported the viewpoint that APAP-induced hepatic microcirculation disorder was involved in the formation of AIH.[77] Furthermore, Ganey *et al.*[78] found that LSECs were, indeed, damaged earlier than hepatocytes after APAP overdose. Interestingly, these impaired LSECs activated coagulation cascades to reduce the number of platelets. This subsequently disturbed the coagulation system by activating the protease-activated receptor 1 (PAR-1) signaling pathway, which ultimately promoted the development of AIH.

Therapeutic targets and strategies for AIH

Targeting APAP liver metabolism

Metabolic studies on APAP found that NAPQI was reduced by increasing the activity of phase II metabolic enzymes, which was beneficial for alleviating APAP-induced liver toxicity. The activation of liver X receptors (LXRs) also greatly improved AIH mainly by enhancing the catalytic capacity of phase II metabolic enzymes.[79] In addition to enhancing these enzymes, AIH can also be markedly mitigated by regulating the expression or activity of cytochrome P450 enzymes. Notably, *Phyllanthus urinaria* extract can treat AIH by inhibiting the expression of CYP2E1 in mice.[80] Tea polyphenols have also been shown to protect mice from AIH by inhibiting the activities of cytochrome P450 1A2 (CYP1A2) and CYP2E1.[81] In addition, the pregnane X receptor (PXR), an important nuclear receptor in the metabolism field, can modulate the expression of cytochrome P450 enzymes.[82] PXR knockout mice were found to be less sensitive to AIH, which implied that PXR mainly played an active role in regulating the cytochrome P450 enzyme system.[83] Interestingly, AIH was also obviously reduced in mice with 5-lipoxygenase (5-LO) deletion, which was related to the inhibition of cytochrome P450 3A11 (CYP3A11).[84] On the whole, these aforementioned treatments do have certain anti-AIH effectiveness. Patients with AIH often have liver injury and are beyond the time frame for early medical intervention. Therefore, compared to preventive interventions in the metabolic stage, targeted therapy is more clinically significant.

Targeting mitochondrial oxidative stress and dysfunction

Currently, clinical treatment of AIH mainly focuses on mitochondrial oxidative stress. NAC is the major clinical treatment for AIH, which may reduce oxidative stress by supplementing GSH to alleviate acute liver injury. However, owing to the narrow therapeutic window, NAC needs to be used within 8 h after APAP poisoning to achieve good therapeutic efficacy. It is worth noting that some adverse reactions, such as nausea, vomiting, and allergy, may occur during NAC treatment.[85] It was reported that the antioxidant Mito-Tempo targeting mitochondrial could effectively prevent AIH after 3 h of APAP treatment. This suggests that Mito-Tempo is a potential treatment option for patients with advanced APAP poisoning.[86] Methylene blue is another existing drug that has been proven to contain hepatoprotective effects in an AIH model. Methylene blue may serve as an electron carrier to effectively restore ETC function and maintain mitochondrial bioenergetic homeostasis, thus protecting mice from AIH.[87] Moreover, clofibrate and docosahexaenoic acid showed remarkable efficacy in protecting against AIH, which was closely correlated with the upregulated expression of peroxisome proliferate–activated receptor α (PPARα).[88,89] Patterson *et al.*[88] found that the activated PPARα could induce the expression of its target gene uncoupling protein 2 (UCP2) and increase the activity of fatty acid β-oxidation-related enzymes and peroxidase in mitochondria. This altogether improved fatty acid catabolism and antioxidant processes to resist APAP-induced liver injury.

SP600125, a classical ATP-competitive inhibitor of JNK, has been reported to have protective effects against AIH *in vivo* and *in vitro*. In particular, the delayed administration of this inhibitor for 5 h was more effective than NAC in AIH patients.[90] Interestingly, the antirheumatic drug leflunomide also clearly protected mice from AIH by suppressing the JNK-mediated activation of mitochondrial permeabilization.[91] Considering the possible protective effects of JNK on liver regeneration, the strategy of directly or indirectly inhibiting JNK may also reduce the potential benefits of JNK and restrict the therapeutic application of JNK inhibitors.[92] Recently, antcin H isolated from *Antrodia camphorata* has also been shown to prevent AIH by suppressing the interaction between phosphorylated JNK and Sab.[93] Since antcin H interferes with the self-maintaining activation loop of JNK rather than directly inhibiting JNK, the molecule could allow a greater beneficial effect.[94] Therefore, antcin H may have promising clinical applications in the future. More importantly, metformin, a commonly used antidiabetic drug, has also been shown to treat AIH by upregulating growth arrest and DNA-damage inducible 45 (Gadd45) expression and inhibiting JNK phosphorylation.[95] Due of its multiple pharmacological effects, metformin is likely to be one of the most promising agents for treating AIH.

Considering the important regulatory role of Nrf2 signaling in liver oxidative stress, a large number of bioactive components have been found to prevent AIH by modulating Nrf2 (Table 1). For example, schisandrol B isolated from *Schisandra sphenanthera* exhibited a remarkable protective effect against AIH, partly through the activation of the Nrf2/ARE pathway.[96] In addition, tanshinone IIA, the main active component of *Salvia miltiorrhiza*, could also protect the liver from APAP-induced hepatic injury by activating the Nrf2 pathway.[97] Moreover, it was found that caffeic acid could effectively prevent APAP-induced liver toxicity by activating the Keap1-Nrf2 antioxidative defense system. More specifically, caffeic acid decreased Keap1 expression, activated Nrf2 by inhibiting the binding of Keap1 to Nrf2, and thus upregulated the expression of HO-1 and NQO1.[98] Furthermore, esculentoside A, with satisfactory antioxidant activities, could enhance the Nrf2-mediated survival mechanisms through the AMP-activated protein kinase (AMPK)/Akt/glycogen synthase kinase-3 beta (GSK3β) pathway, thus effectively alleviating APAP-induced liver damage.[99] Importantly, these natural compounds possess a remarkable antioxidant activity, and this effect is realized by activating the Keap1-Nrf2 pathway. Oxidative stress is the major pathogenic factor of AIH, and the Keap1-Nrf2 pathway is the main defense mechanism against oxidative stress. Therefore, activating the Keap1-Nrf2 pathway may be an effective therapeutic strategy for APAP-induced liver injury. Although these natural compounds can improve AIH, these findings are all based on animal models. Therefore, further studies are required to determine their clinical efficacy. In addition, because any influence on the accumulation of NAPQI may interfere with the subsequent progression of liver injury, it is necessary to elucidate the effects of these natural products on the metabolic activation of APAP.

One study has reported that resveratrol significantly reduced APAP-induced JNK activation and mitochondrial oxidative damage. Besides that, resveratrol treatment also induced SIRT1 and negatively regulated p53 signaling to increase cell proliferation–related proteins, which promoted hepatocyte proliferation.[100] In a mouse model of AIH, Huo *et al.*[45] explored the relationship between p53 and JNK, a key mediator of APAP-induced mitochondrial oxidative stress. They found that activated p53 suppressed JNK activation (Figure 2), thereby protecting the liver. It was also reported that the doxorubicin-mediated activation of p53 attenuated AIH by enhancing APAP transport and metabolism and inhibiting oxidative damage.[46] These findings suggest that activated p53 is helpful in preventing APAP-induced liver injury. However, in the process of liver repair in the late stage of liver injury, the activation of p53 delays liver regeneration.[47] Since p53 is stage-specific in the pathogenesis of liver injury, p53 may not be an ideal target for treating AIH.

Targeting sterile inflammation

Whether sterile inflammation promotes the progression of liver injury or serves as a means of cellular defense against toxicity remains controversial.[118] Despite this, aseptic inflammation may still be used as an intervention target to protect against APAP-induced liver injury. Benzyl alcohol has been proven to reduce the release of IL-1 and IL-18 in a TLR4-dependent manner, thus preventing AIH.[101] However, its clinical application is limited by mitochondrial toxicity.[119] Atractylenolide I also displayed an obvious protective effect against AIH. This active compound is likely to inhibit the activation of nuclear factor-κB (NF-κB) through the TLR4/MAPKs/NF-κB pathways, thus downregulating the expression of pro-inflammatory cytokines, including IL-1β, IL-6, and TNF-α.[103] Furthermore, blocking neutrophil recruitment is also a feasible treatment approach. Resolvin was found to attenuate AIH by restraining the entry of neutrophils into liver tissues.[102] In contrast, inflammation also helps erase dead cells and stimulate later liver repair and regeneration. Therefore, research on the role of inflammatory response in the liver later regeneration is also essential. IL-6 has been shown to promote liver regeneration by activating signal transducer and activator of transcription-3 (STAT3).[120] In addition, Masubuchi *et al.*[56] reported that IL-6-deficient mice were more susceptible to APAP-induced liver injury, which was related to the insufficient expression of hepatic heat shock proteins 25, 32, 40, and 70 after APAP treatment. These findings suggest that IL-6 may protect against APAP-induced liver injury through multiple biological mechanisms. Lactoferrin is a multifunctional protein that modulates the function of immune cells and exerts hepatoprotective effects by regulating inflammatory responses.[104] Overall, as the exact role of inflammation in AIH remains unclear, its use as a therapeutic target is controversial.

Targeting ERS

CHOP was confirmed to be a critical regulator of APAP-induced ERS.[62] It was reported that simvastatin could protect mice from AIH by inhibiting the expression of CHOP.[105] Ozagrel hydrochloride also significantly reduced hepatocyte death by downregulating CHOP expression.[106] In addition, 4-phenyl-butyric acid, a chemical chaperone that aids in the correct folding of proteins, could prevent APAP-induced liver injury in mice. The underlying mechanisms most likely involve the reduction of ERS-induced apoptosis by suppressing CHOP expression.[107] Therefore, suppressing CHOP may be a potential treatment strategy for patients with AIH. Kahweol, derived from coffee, alleviated APAP-induced hepatocyte death, and its cell protection was related to the inhibition of ERS.[108] Moreover, the SPHK1 inhibitor PF543 also effectively relieved ERS by reducing the phosphorylation of eIF2α and activating ATF6, thus remarkably improving AIH.[109] Interestingly, guanabenz, a well-known antihypertensive drug, also showed liver protection in AIH mice, which resulted from the reduction of ERS by inhibiting the proteolysis of ATF6.[110]

Targeting autophagy

Adiponectin has been proven to prevent AIH by activating AMPK- and ULK1-mediated autophagy.[111] Rapamycin also effectively weakened the AIH by inducing autophagy.[67] In addition, cardamonin-induced autophagy by activating the NFE2L2 signaling pathway, thus resulting in the protection against AIH.[112] Interestingly, a recent study also found that fisetin extracted from fruits or vegetables promoted

Table 1. The major bioactive substances targeting different cell events against APAP-induced hepatotoxicity.

Cell event	Bioactive substance	Animal model/APAP dose	Major therapeutic mechanisms	Reference
APAP liver metabolism	TO1317	Mice, 200 mg/kg, p.o.	Inducing phase II conjugating enzymes and suppressing phase I P450 enzymes by activating LXR.	79
	Phyllanthus urinaria extract	C57Bl6 mice, 550 mg/kg, i.p.	Inhibiting CYP2E1 expression.	80
	Tea polyphenols	Kunming mice, 500 mg/kg, p.o.	Reducing CYP2E1 and CYP1A2 expression.	81
	Pregnenolone 16α-carbonitrile	Mice, 350 mg/kg, i.p.	Inducing CYP3A11 expression by activating PXR.	82
	Zileuton	C57BL/6J mice, 200 mg/kg, p.o.	Suppressing CYP3A11 by inhibiting 5-LO.	84
Mitochondrial oxidative stress and dysfunction	Mito-Tempo	C57BL/6J mice, 300 mg/kg, i.p.	Specifically attenuating mitochondrial oxidant stress and preventing mitochondrial dysfunction.	86
	Methylene blue	C57BL/6J mice, 450 mg/kg, i.p.	Restoring ETC function and maintaining mitochondrial bioenergetic homeostasis by serving as an electron carrier of damaged complex II.	87
	Fenofibrate	C57BL/6J mice, 400 mg/kg, i.p.	Activating PPARα.	88
	Docosahexaenoic acid	CD-1 mice, 800 mg/kg, p.o.	Activating PPARα.	89
	SP600125	C57BL/6 mice, 350 mg/kg, i.p.	Directly inhibiting JNK.	90
	Leflunomide	C57BL/6 mice, 750 mg/kg, i.p.	Suppressing the JNK-mediated activation of mitochondrial permeabilization.	91
	Antcin H	C57BL/6NHsd mice, 300 mg/kg, i.p.	Suppressing the interaction between phosphorylated JNK and Sab.	93
	Metformin	C57BL/6J mice, 400 mg/kg, i.p.	Inhibiting JNK phosphorylation by upregulating Gadd45 expression.	95
	Schisandrol B	C57BL/6 mice, 400 mg/kg, i.p.	Activating Nrf2/ARE pathway.	96
	Tanshinone IIA	C57BL/6J mice, 300 mg/kg, i.p.	Upregulating the levels of GCLC, HO-1and NQO1 by activating Nrf2 pathway.	97
	Caffeic acid	ICR mice, 400 mg/kg, p.o.	Upregulating the expression of HO-1 and NQO1 by activating Keap1-Nrf2 pathway.	98
	Esculentoside A	BALB/c mice, 400 mg/kg, i.p.	Activating Nrf2 through AMPK/Akt/GSK3β pathway.	99
	Doxorubicin	C57BL/6 mice, 400 mg/kg, i.p.	Activating p53 to enhance APAP transport and metabolism and to inhibit oxidative damage.	
	Nutlin 3a	C57BL/6N mice, 300 mg/kg, i.p.	Suppressing JNK activation.	45
	Resveratrol	C57BL/6 mice, 400 mg/kg, i.p.	Promoting hepatocyte proliferation by regulating SIRT1-p53 signaling pathways.	100
Sterile inflammation	Benzyl alcohol	C57BL/6 mice, 400 mg/kg, i.p.	Reducing the release of IL-1 and IL-18 in TLR4-dependent way.	101
	Resolvin	C57BL/6 mice, 400 mg/kg, i.p.	Inhibiting the adhesion of neutrophils to endothelial cells.	102
	Atractylenolide I	C57BL/6 mice, 500 mg/kg, i.p.	Downregulating the expression of pro-inflammatory cytokines, including IL-1β, IL-6, and TNF-α, by inhibiting NF-κB through TLR4/MAPKs/NF-κB pathway.	103
	Lactoferrin	C57BL/6J mice, 300 mg/kg, i.p.	Modulating inflammatory responses.	104
Endoplasmic reticulum stress	Simvastatin	C57BL/6 mice, 400 mg/kg, i.p.	Inhibiting CHOP expression.	105
	Ozagrel hydrochloride	ICR mice, 330 mg/kg, i.p.	Downregulating CHOP expression.	106
	4-Phenylbutyric acid	C57BL/6 mice, 450 mg/kg, i.p.	Suppressing CHOP expression.	107
	Kahweol	C57BL/6N mice, 400 mg/kg, i.p.	Inhibiting ERS.	108
	PF543	C57BL/6J mice, 200 mg/kg, i.p.	Relieving the ERS by reducing SPHK1-mediated eIF2α phosphorylation and ATF4 level.	109
	Guanabenz	Swiss Webster mice, 370 mg/kg, i.p.	Reducing ERS by inhibiting the proteolysis of ATF6.	110
Autophagy	Adiponectin	C57BL/6J mice, 500 mg/kg, i.p.	Activating AMPK- and ULK1-mediated autophagy.	111
	Rapamycin	C57BL/6 mice, 500 mg/kg, i.p.	Inducing autophagy.	66
	Cardamonin	C57BL/6 mice, 400 mg/kg, i.p.	Enhancing autophagy by activating NFE2L2 signaling pathway.	112
	Fisetin	C57BL/6 mice, 400 mg/kg, p.o.	Promoting autophagy by increasing ATG5 expression.	113
	Glycycoumarin	C57BL/6 mice, 300 mg/kg, i.p.	Decreasing JNK phosphorylation and mitochondrial oxidative stress by activating sustained autophagy.	114
Microcirculation dysfunction	V-PYRRO/NO	CD-1 mice, 600 mg/kg, i.p.	Maintaining hepatic vasculature by releasing NO.	115
	2-((4-Biphenylsulfonyl) amino)-3-phenyl-propionic acid	C57BL/6 mice, 600 mg/kg, p.o.	Attenuated microvascular injury by inhibiting MMP.	75
	Prazosin	CD-1 mice, 3.5 mmol/kg, i.p.	Reducing hepatic erythrocyte accumulation.	116
	Heparin	C57BL/6J mice, 400 mg/kg, i.p.	Inhibiting coagulation system.	78
	Dabigatran	C57BL/6 mice, 300 mg/kg, i.p.	Inhibiting coagulation system.	117

APAP: acetaminophen; LXR: liver X receptor; ETC: electron transport chain; JNK: Jun N-terminal kinase; GCLC: glutamate cysteine ligase catalytic; NQO1: NAD(P)H:quinone oxidoreductase 1; CHOP: C/EBP homologous protein; ERS: endoplasmic reticulum stress; MMP: matrix metalloproteinase; p.o.: per os; i.p.: intraperitoneal injection; CYP2E1: cytochrome P450 2E1; CYP1A2: cytochrome P450 1A2; PXR: pregnane X receptor; 5-LO: 5-lipoxygenase; PPARα: peroxisome proliferate–activated receptor α; Gadd45: growth arrest and DNA-damage inducible 45; ARE: antioxidant response element; HO-1: heme oxygenase-1; Nrf2: nuclear factor erythroid 2-related factor 2; AMPK: AMP-activated protein kinase; GSK3β: glycogen synthase kinase-3 beta; NFE2L2: nuclear factor erythroid 2-related factor 2; eIF2α: eukaryotic initiation factor 2α; ATF4: activating transcription factor 4; NF-κB: nuclear factor-κB; interleukin: interleukin; TNF-α: tumor necrosis factor-alpha; MAPK: mitogen-activated protein kinase.

autophagy by increasing the expression of ATG5, thereby inhibiting the development of AIH.[113] Moreover, globular adiponectin was also found to prevent APAP-induced hepatocyte death, which was achieved partly through the inhibition of ERS and inflammasome activation by inducing autophagy.[121] Mo *et al.*[122] discovered that AMPK-dependent autophagy is involved in the liver protection of IL-22 against APAP-induced liver injury. Furthermore, glycycoumarin remarkably decreased APAP-induced JNK phosphorylation and mitochondrial oxidative stress through the sustained activation of autophagy.[114] It is noteworthy that excess autophagy is detrimental to organisms, as it can initiate programmed cell death that is similar to apoptosis or necrosis.[123,124] Therefore, inducing moderate autophagy can be considered as a potential therapeutic strategy against AIH.

Targeting microcirculation dysfunction

NO plays an important role in maintaining an adequate blood supply for hepatic microvessels by affecting the expression of leukocytes, platelets, and endothelial cell adhesion molecules.[125] L-NMMA, an NOS inhibitor, indirectly suppressed NO synthesis and aggravated liver microcirculation disturbances, suggesting that NO could stabilize hepatic microcirculation.[126] A prior study reported that NO donor maintained the normal operation of the hepatic vascular system to prevent congestion, thus effectively blocking AIH in mice.[115] In addition, MMP inhibitor was confirmed to significantly attenuate APAP-induced parenchymal and microvascular injury, which implied MMP as a promising intervention target.[75] Elevated catecholamine levels contribute to AIH pathophysiology by impairing hepatic perfusion. Alpha(1)-adrenoceptor antagonists have also been shown to improve hepatic microvascular dysfunction by significantly reducing hepatic erythrocyte accumulation, thereby effectively alleviating APAP-induced liver injury.[116] These evidences strongly indicate that microcirculatory disturbance plays a key role in AIH pathogenesis, and its prevention may be a critical therapeutic approach. In particular, it has been reported that the anticoagulant effect of heparin can alleviate AIH in the early stage, that is, 6 h after APAP treatment.[78] Another anticoagulant, dabigatran, also showed the same curative effects on AIH. Despite the benefits of anticoagulant treatment with dabigatran, liver injury was significantly aggravated 24 h after APAP administration due to a decrease in hepatocyte proliferation.[117] Therefore, the benefits of anticoagulant therapy remains uncertain, which need to be confirmed through in-depth studies in the future.

Summary and outlook

With the widespread use of APAP, AIH has gradually become a significant public health problem. NAC is used clinically as the primary antidote to relieve APAP-induced oxidative stress. The molecular mechanisms of AIH are rather complex and involve a series of cellular events, including liver metabolism, mitochondrial oxidative stress and dysfunction, sterile inflammation, ERS, autophagy, and microcirculation dysfunction. To date, many bioactive substances have been confirmed to be effective in preventing AIH by targeting the key nodes in these biological processes (Table 1). These bioactive substances can be exploited as potential drugs to develop the most suitable therapeutics for AIH. It should be emphasized that mitochondrial oxidative stress and dysfunction are the major cellular events associated with AIH. Therefore, targeted intervention against mitochondrial oxidative stress and dysfunction may be a promising therapeutic strategy. In addition to mitochondrial oxidative stress and dysfunction, other cellular events may also be potential therapeutic targets for AIH. This study shows that treatments targeting hepatic metabolism can provide early interventions for AIH. However, follow-up clinical studies are needed to further explore the clinical therapeutic effects and potential adverse reactions of these drugs. Treatments targeting ERS or microcirculation disorders can also prevent AIH to some extent, but the protective mechanisms need to be further clarified in future clinical studies. Notably, sterile inflammation and autophagy play distinct roles in different stages of AIH, making them contradictory regulators of AIH. Therapeutic strategies targeting these two cellular events may ultimately be ineffective. In conclusion, in the future, we need to conduct more research to further clarify the exact role of these cell events in AIH to make later-stage treatments possible in clinics.

AUTHORS' CONTRIBUTIONS

GL, LH, and ZZ wrote the manuscript. GL also designed and supervised the writing of manuscript.

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