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Oxidative stress during acetaminophen hepatotoxicity: Sources, pathophysiological role and therapeutic potential

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ABSTRACT

Acetaminophen (APAP) hepatotoxicity is characterized by an extensive oxidative stress. However, its source, pathophysiological role and possible therapeutic potential if targeted, have been controversially described. Earlier studies argued for cytochrome P450-generated reactive oxygen species (ROS) during APAP metabolism, which resulted in massive lipid peroxidation and subsequent liver injury. However, subsequent studies convincingly challenged this assumption and the current paradigm suggests that mitochondria are the main source of ROS, which impair mitochondrial function and are responsible for cell signaling resulting in cell death. Although immune cells can be a source of ROS in other models, no reliable evidence exists to support a role for immune cell-derived ROS in APAP hepatotoxicity. Recent studies suggest that mitochondrial targeted antioxidants can be viable therapeutic agents against hepatotoxicity induced by APAP overdose, and re-purposing existing drugs to target oxidative stress and other concurrent signaling events can be a promising strategy to increase its potential application in patients with APAP overdose.

1. Introduction

Acetaminophen (APAP) hepatotoxicity is the leading cause of acute liver failure in many Western countries [1,2]. A key mechanism of the toxicity is the cytochrome P450-catalyzed metabolic activation of APAP, which generates the reactive metabolite N-acetyl-p-benzoquinone imine (NAPQI) and initiates toxicity in both rodents and humans [3]. Excessive NAPQI formation after APAP overdose depletes cellular glutathione (GSH), adducts proteins including mitochondrial proteins, and induces mitochondrial oxidant stress and dysfunction. This results in nuclear DNA fragmentation and necrotic cell death and a subsequent inflammatory response, including the release of pro-inflammatory cytokines and activation of immune cells [3]. Currently, APAP-induced liver injury has served as the most popular, mechanistically well studied and clinically relevant model for testing of phytotherapeutics and other hepato-protective interventions. In spite of significant evidence pointing towards the existence of a general oxidative stress during APAP hepatotoxicity, the cellular or intracellular sources and the nature of the ROS in this context remain debatable. This has led to controversial conclusions and ultimately jeopardized the translation of new therapeutic approaches to the human pathophysiology [4,5]. In particular, the pathophysiological role of ROS in the mechanism of toxicity has not been clearly discussed and still requires further investigation. This review will provide an updated overview of the potential sources of ROS in APAP hepatotoxicity. The corresponding pathophysiological role of each source in the toxicity will be also summarized and discussed. We propose that mitochondrial targeted antioxidants or re-purposing of existing drugs which target mitochondrial ROS may have therapeutic potential for APAP poisoning in animals and potentially in humans.

2. Sources and relevance of oxidant stress in acetaminophen hepatotoxicity

2.1. Cytochrome P450-mediated oxidant stress

In the 1980's, it was recognized that cytochrome P450-mediated drug metabolism in microsomes can generate ROS (mainly superoxide and hydrogen peroxide) [6]. Cytochrome P450 2E1-mediated ROS production has been suggested to play a role in alcohol-induced liver injury [7], although recent evidence suggests that mitochondria and immune cell-derived oxidant stress can also contribute to the progression of the injury (Fig. 1) [8–10]. A mitochondrial oxidant stress has also been implicated in the progression of I/R injury [11]. Since APAP is also metabolized by P450 enzymes in microsomes, it was assumed that P450-mediated metabolism of APAP generated ROS in APAP hepatotoxicity, leading to subsequent lipid peroxidation and liver injury [12]. This hypothesis was mainly based on the use of inducers

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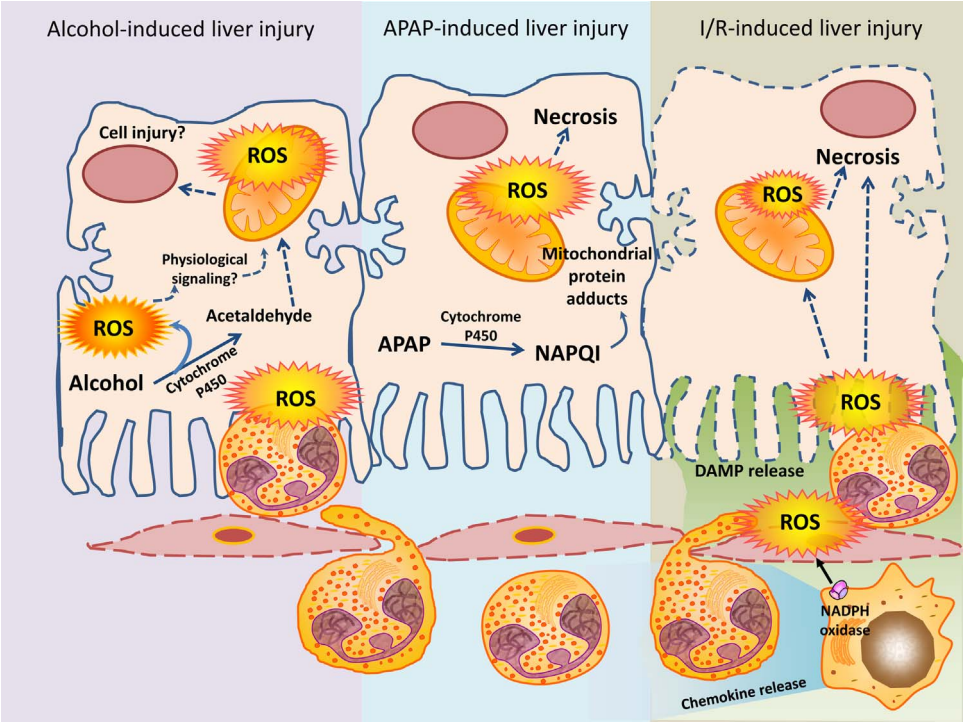

Fig. 1. Sources of ROS in APAP hepatotoxicity in comparison to other forms of liver injury. The cytochrome P450 enzyme system can generate free radicals during metabolism of various compounds. Cyp2E1-mediated ROS formation during metabolism of alcohol has been show to play an important role in alcohol-induced liver injury, while the involvement of mitochondria- and immune cell-derived ROS has also been implicated. With regards to acetaminophen (APAP)-induced liver injury, the reactive intermediate NAPQI generated during APAP metabolism forms mitochondrial protein adducts, which cause oxidative stress within the organelle and subsequently initiate signaling cascades resulting in programmed necrosis. In contrast to APAP injury, immune cells mediated oxidative stress is critical in liver injury-induced by conditions such as ischemia-reperfusion, where damage associated molecular patterns (DAMPS) released from hepatocytes instigate free radical and chemokine production from Kupffer cells in the sinusoids, which then results in infiltration of neutrophils into the space of Disse and further oxidant stress close to the hepatocyte, which ultimately produces mitochondrial oxidant stress and results in cell necrosis.

and inhibitors of cytochrome P450, which enhanced and attenuated APAP-induced lipid peroxidation, respectively [12]. However, this conclusion was soon challenged by the fact that glutathione disulfide (GSSG) formation, a sensitive indicator of hepatic oxidant stress, was not increased during APAP metabolism in a rat model [13], and this was subsequently confirmed in a mouse study [14]. The original observations in the rodent models were later supported *in vitro*. In primary mouse hepatocytes, 2′,7′-dichlorofluorescein fluorescence, a marker of intracellular oxidant stress, did not change during drug metabolism but rapidly increased after APAP was metabolized and GSH was depleted [15]. Furthermore, in metabolically competent human HepaRG cells, ROS and peroxynitrite were only detectable after 6 h post-APAP, which is after APAP metabolism [16]. Another interesting finding is that although in rats P450-mediated metabolism of APAP also causes GSH depletion and even significant protein adducts formation, no increased oxidant stress or liver injury was detected [17]. Taken together, these studies did not find direct evidence of APAP-induced oxidant stress during the metabolism phase. However, recent fluorescence measurements using highly sensitive semiconducting polymer–based nanosensors provided evidence for H_2O_2 formation during the metabolism phase of APAP *in vivo* [18]. The fact that this signal could be attenuated by P450 inhibitors suggests that the oxidant stress was dependent on the drug metabolism [18]. However, given the extensive evidence that many interventions are highly effective when given after the metabolism phase indicate that these nanosensors are detecting a sub-toxic oxidant stress that may have limited relevance for cell death.

2.2. Lipid peroxidation as mechanism of cell death

Lipid peroxidation (LPO) has been a frequently invoked mechanism in ROS-induced cell death and liver injury [19–21]. Common initiators of the peroxidation process are HO·and HOO·, which can be generated through the Fenton Reaction. In addition, LPO can be caused by peroxynitrite and heme dependent lipid peroxide decomposition [22]. The abstraction of H· from lipid molecules (mostly from polyunsaturated fatty acids) produces fatty acid radicals. This initiates a free-radical chain reaction that triggers the peroxidation of a large number of target molecules, which would ultimately severely destroy the integrity of cell membranes, damage the function of membrane bound enzymes and even impair nuclear DNA. The involvement of LPO in the pathophysiology of cell injury is typically assumed when parameters of LPO (*e.g.*, malondialdehyde, ethane and pentane exhalation, formation of hydroxyfatty acids, hydroxynonenal, *etc.*) are increased in the injured tissue, but decreased after an intervention that is thought to act as an antioxidant. This is normally accompanied by improved liver injury, leading to the conclusion that LPO is responsible for the cell death [23–30]. Experimental evidence for the hypothesis that APAP hepatotoxicity is caused by LPO was first suggested by Wendel and coworkers [31]. The recognition that the P450-mediated metabolism of xenobiotics can release ROS in microsomes [6,32], and the fact that inducers and inhibitors of P450 enzymes modulate APAP-induced LPO and liver injury in mice *in vivo* supported the idea that P450 enzymes were responsible for the oxidant stress [12]. Although there was no doubt that there was severe LPO in the livers of these animals as indicated by extensive ethane and pentane exhalation and high levels of thiobarbituric acid-reactive substances (TBARs) [12,31,33]), the widely overlooked fact was that the animals were fed a vitamin E-deficient diet high in polyunsaturated fatty acids, which made them exquisitely sensitive to LPO induced by APAP or allyl alcohol [12,31,33,34]. Importantly, the massive LPO that occurred under these conditions (> 50-fold increase of ethane exhalation and TBARS levels) caused massive liver injury and acute liver failure within 4 h after APAP overdose [12,33], and pretreatment with vitamin E or iron chelators

dramatically reduced those LPO parameters and protected against the injury [34–36]. Based on this evidence, it was concluded that APAP triggers massive LPO, which is responsible for liver injury. However, this only applies to animals with vitamin E-deficiency and cell membranes high in polyunsaturated fatty acids. In contrast, when animals were fed regular rodent chow, APAP caused severe liver injury within 6–24 h, which was accompanied by no or very limited LPO [36]. Importantly, vitamin E treatment did not protect, suggesting that LPO is not a relevant mechanism of cell death in APAP toxicity under normal conditions [36].

Nevertheless, many recent studies on protection of natural products with antioxidant effects, still conclude that the LPO detected is responsible for APAP-induced liver injury [23–30,37–39]. However, since LPO is generally very limited in these studies, alternative explanations for the protection can be off-target effects of the natural product, *e.g.* inhibition of drug metabolism [40]. Thus, under relevant *in vivo* conditions, endogenous antioxidant defense systems including vitamin E, glutathione peroxidase (GPx), thioredoxins and iron chelation are generally sufficient to limit LPO after APAP overdose.

2.3. Mitochondrial oxidant stress

In recent years, mitochondria have been gradually recognized as the main source of oxidant stress after an APAP overdose (Fig. 1) [3]. It is well established that excessive NAPQI formation after APAP overdose depletes GSH and binds to sulfhydryl groups of cellular proteins [41,42]. Comparison of the subcellular protein adduct formation between APAP versus its non-toxic regioisomer, 3′-hydroxyacetanilide (AMAP) identified mitochondrial proteins as critical targets of NAPQI in the initiation of toxicity, as indicated by the presence of mitochondrial adducts after APAP treatment but not after non-toxic AMAP exposure in mice [43–45]. This concept is further supported by the observation that AMAP toxicity in human hepatocytes is correlated with mitochondrial adducts [46]. A number of mitochondrial proteins, including GPx and ATP synthase α-subunit, have been shown to be adducted by NAPQI (Fig. 2) [47]. Modification of GPx reduced the enzyme activity by 60% [48] while adduction of the ATP synthase α-subunit might impair the ATP synthase function and cause the cessation in ATP synthesis [48–50]. Though insufficient to cause direct cell death, cellular protein binding, especially mitochondrial protein adduction, impairs mitochondrial respiration and causes oxidant stress (Fig. 2) [49,51,52]. Although the direct molecular events that trigger mitochondrial oxidant stress remain to be identified in APAP hepatotoxicity, it is known that they interfere with the mitochondrial electron transport chain (ETC), which causes leakage of electrons from the chain and thus results in ROS formation (Fig. 2) [53]. Mitochondrial complex I is a crucial site of ROS formation in mitochondria [54–56]. Interestingly, it was found that an overdose of APAP significantly increased complex I activity in mitochondria in APAP-treated mice, and there was a strong positive correlation between complex I activity and the severity of liver injury [57]. Metformin significantly inhibited complex I activity and resulted in a reduced oxidative stress and injury [57]. In HepaRG cells, it was also observed that APAP induced proton leakage from the ETC and impaired coupled respiration, and both were inhibited by metformin treatment [57]. These observations support the hypothesis that complex I is a critical source of ROS leakage in APAP hepatotoxicity.

Electrons released from the ETC react with oxygen to form superoxide, which could either be dismutated to hydrogen peroxide and molecular oxygen by manganese superoxide dismutase (MnSOD, SOD2) in mitochondria or react with nitric oxide (NO) to form peroxynitrite. The hydrogen peroxide produced by MnSOD can directly react with GSH [58,59] or more likely be enzymatically detoxified by a number of antioxidant enzymes in hepatocytes, such as catalase, GPx or peroxiredoxins [60]. In contrast, peroxynitrite serves as a much more potent oxidant and nitrating species in APAP hepatotoxicity [58,61,62]. Consistent with this, a selective APAP-induced mitochondrial oxidant stress has been supported by multiple lines of evidence including a specific increase of mitochondrial GSSG levels [49,50,62] and the selective formation of nitrotyrosine protein adducts in mito-

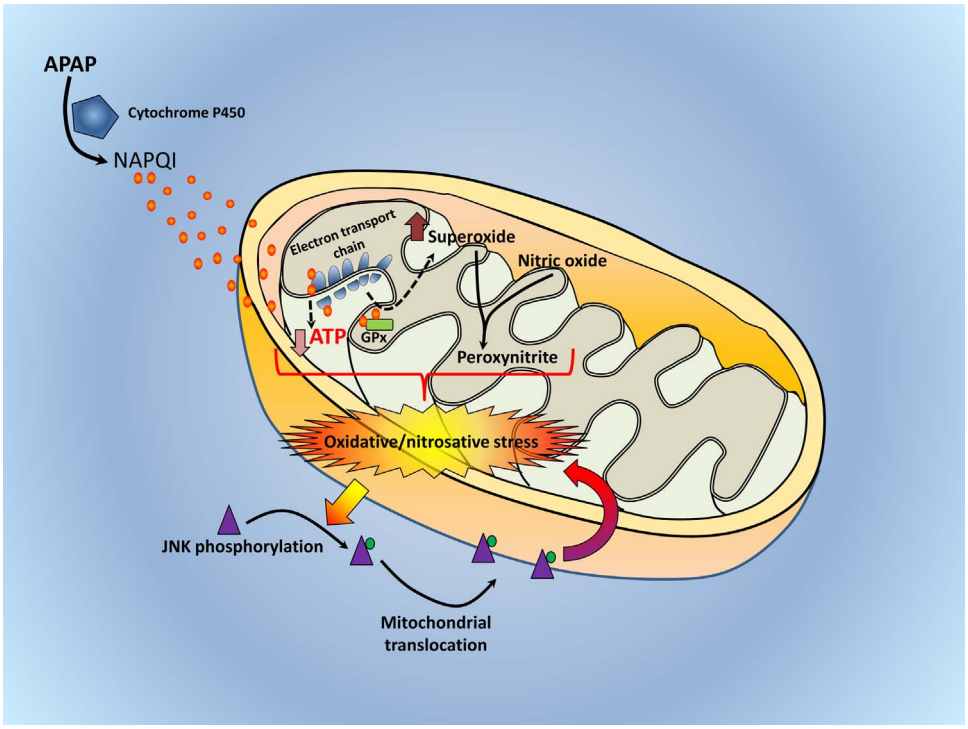

Fig. 2. Mitochondrial oxidative stress and signaling in APAP hepatotoxicity. Metabolism of APAP forms the reactive metabolite NAPQI, which targets proteins, especially mitochondrial proteins. Adduction of ATP synthase and glutathione peroxidase compromises generation of ATP through the electron transport chain and interferes with mitochondrial anti-oxidant capacity. The enhanced generation of superoxide results in its reaction with nitric oxide to produce peroxynitrite, which ultimately produces oxidative/nitrosative stress. This then activates the MAP kinase c-jun-N-terminal kinase (JNK), resulting in its phosphorylation and translocation to the mitochondria, which amplifies the initial oxidative stress.

chondria and the oxidative loss of mitochondrial DNA in APAP-treated mice [63]. In addition, MitoSox Red staining, which specifically detects mitochondrial superoxide, was observed in primary mouse hepatocytes and in metabolically competent human HepaRG cells treated with APAP [16,64]. Furthermore, isolated mitochondria from APAP-treated mice were also shown to have elevated superoxide formation [57]. These observations together strongly support the existence of a selective mitochondrial oxidant stress after APAP exposure.

The pathophysiological relevance of this mitochondrial oxidant stress has also been extensively documented. It was shown that female mice were less susceptible to APAP hepatotoxicity compared to males due to the faster recovery of GSH in mitochondria, which detoxifies ROS and peroxynitrite resulting in less mitochondrial dysfunction [65]. Promoting the replenishment of hepatocellular GSH levels including the mitochondrial content with exogenous GSH or its precursors was shown to enhance the detoxification of peroxynitrite and hydrogen peroxide and effectively protect against hepatotoxicity after APAP overdose [50,58,66,67]. In contrast, mice with partial MnSOD-deficiency ($MnSOD^{+/-}$) showed a much higher susceptibility to APAP toxicity with increased peroxynitrite and protein carbonyl formation [68,69]. Interestingly, MnSOD was shown to be nitrated and partially inactivated by peroxynitrite after APAP [70], which might concurrently increase the susceptibility of hepatocytes to ROS damage, especially considering that the antioxidant GSH is severely depleted shortly after APAP overdose [42]. Furthermore, the mitochondrial targeted antioxidant Mito-Tempo, which serves as a MnSOD mimetic [71], was highly effective in preventing APAP-induced oxidant stress and liver injury [72]. Scavenging peroxynitrite with the natural product resveratrol also effectively protected against APAP hepatotoxicity [73]. Furthermore, iron translocation from disrupted lysosomes into mitochondria promotes the oxidant stress-induced mitochondrial permeability transition pore opening and necrotic cell death [74,75]. Taken together, this suggests that targeting mitochondrial oxidant stress can be a promising therapeutic target for treatment of APAP overdose in the clinic. Indeed, one of the protective mechanisms of NAC, which is the only currently available antidote for APAP overdose patients, is thought to be by detoxifying ROS and reducing the oxidant stress in the liver [50]. Other effective antioxidants, especially mitochondrial targeted antioxidants such as Mito-Tempo, would certainly be worth further investigation in the clinical setting if its safety in humans can be verified.

2.4. Immune cell-derived oxidant stress

Another potential source of oxidant stress could be resident macrophages (Kupffer cells) and inflammatory cells recruited into the liver (monocytes/macrophages, neutrophils) (Fig. 1). Macrophages and neutrophils mainly kill targets by reactive oxygen using NADPH oxidase (NOX2) to generate superoxide and hydrogen peroxide, and, in the case of neutrophils, use of myeloperoxidase to form the very potent oxidant hypochlorous acid [76,77]. Direct cytotoxicity of immune cell-derived ROS including hypochlorite has been implicated in many liver pathologies, *e.g.*, hepatic ischemia-reperfusion injury [78,79], endotoxemia [80], and obstructive cholestasis [81]. Many of these pathologies involve a sterile inflammatory component [82]. Similarly, the extensive necrotic cell death after APAP overdose also triggers the release of damage-associated molecular patterns (DAMPs), which includes high-mobility group box 1 protein (HMGB1) [83,84], mitochondrial DNA and nuclear DNA fragments [85], heat shock proteins [84], uric acid [86] and others. DAMPs mediate the activation of resident macrophages (*e.g.*, Kupffer cells) *via* toll-like receptors, which lead to increased cytokine and chemokine formation and immune cell accumulation in the liver (*e.g.*, neutrophils and monocyte-derived macrophages) [77,87]. Kupffer cells reside in sinusoids and the generated ROS (*e.g.*, NOX2-derived superoxide and hydrogen peroxide) can cause cell injury in stressed hepatocytes [78]. However, an involvement of Kupffer cells in the pathophysiology of APAP hepatotoxicity has been highly controversial. Although it was originally reported that mice pretreated with gadolinium chloride ($GdCl_3$), an intervention that inactivates Kupffer cells, had reduced peroxynitrite formation and were protected against APAP-induced liver injury [88], neither of these results could be reproduced by subsequent studies [89–91]. In support of these later reports, mice functionally deficient in NADPH oxidase activity ($gp91phox^{-/-}$ mice) did not show a decreased oxidative stress or reduced liver injury [92,93]. In addition, even complete elimination of Kupffer cells by clodronate liposomes could not protect against the injury [89]. Further evidence against the importance of a Kupffer-derived oxidant stress during APAP toxicity would be the fact that the most active Kupffer cells reside around the periportal area [94], which cannot be responsible for cell death after APAP where the necrotic cells are exclusively located in the centrilobular areas of the liver. Taken together, these very diverse approaches consistently argue against an involvement of a Kupffer cell-induced oxidant stress in the pathophysiology.

Another potential source of oxidant stress after APAP overdose is the infiltrating neutrophil. These phagocytes are the first immune cells to respond to the extensive APAP-induced necrosis [95]. However, neutrophils do not only produce superoxide and hydrogen peroxide by NADPH oxidase, but due to the presence of myeloperoxidase, these cells can generate hypochlorite. Furthermore, in contrast to Kupffer cells, neutrophils are mobile and can extravasate from sinusoids and adhere to the targeted hepatocytes to be fully activated [96]. The adherence to hepatocytes triggers a long-lasting oxidant stress in close proximity to the target, which allows oxidants such as hydrogen peroxide [97] and hypochlorous acid [79–81] to diffuse into hepatocytes and induce cell death (Fig. 1). The assumption for the involvement of neutrophils in APAP hepatotoxicity came from studies showing that the neutropenia-inducing antibody Gr-1 protected against APAP hepatotoxicity [98,99]. However, it was recognized that the protection was due to additional effects of the antibody when the animal was pretreated for 24 h [100]. Importantly, there was no direct evidence for a neutrophil-derived oxidant stress in APAP hepatotoxicity [101]. Hypochlorite-modified proteins, which are markers of neutrophil-derived oxidant stress, were not detectable after APAP in the liver [101]. The hepatic or peripheral neutrophils were not activated or primed for ROS formation at 6 h after APAP in mice [102] or 24–48 h in humans [93], at which time liver injury is in full progress or has even peaked. In addition, neither NADPH oxidase inhibitors nor deletion of gp91phox, a subunit of NADPH oxidase, protected against APAP hepatotoxicity [92,93,101]. Furthermore, neither increasing neutrophil infiltration by administration of endotoxin or IL-1β nor preventing its infiltration with antibodies against β_2 integrins (CD18), or use of mice deficient in adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1) or CD18, affected APAP hepatotoxicity [92,95,101–103]. Together, these data suggest that neutrophils, despite their early infiltration, are unlikely a relevant source of the oxidant stress after APAP overdose.

Similar to neutrophils, monocytes are also recruited into the liver after APAP hepatotoxicity [104]. Monocytes are recruited to the necrotic area through an interaction of C-C chemokine receptor 2 (CCR2) on its membrane with monocyte chemoattractant protein-1 (MCP-1) generated by injured hepatocytes [105,106]. In contrast to the relatively early recruitment of neutrophils, monocytes are typically recruited into the liver within 12–24 h after an APAP overdose in mice, which is past the peak of injury [106], suggesting that the infiltrating monocytes (M2 macrophages) are not responsible for the injury but might be important for the injury resolution. In support of this, M2 macrophages were shown to have a high capacity for phagocytosis and can generate cytokines such as IL-10, which can stimulate tissue repair [76,105,107]. In addition, mice deficient in CCR2 or MCP-1 had unaffected liver injury compared to wild type animals, but experienced a reduced hepatic M2 macrophage recruitment and a substantial delay in tissue repair [105–109]. These data suggest that M2 macrophages

are involved in cell debris removal in the injury resolution phase after APAP hepatotoxicity, which is a prerequisite for liver regeneration [105,106,110]. Interestingly, this repair was not affected in gp91phox-deficient mice, suggesting that ROS generated by macrophages or neutrophils was not required for the cell debris removal process [93]. Of note, the findings in mice are consistent with clinical studies suggesting a predominant repair and regenerative role for monocyte-derived macrophages in APAP overdose patients [111]. Since it is well known that there is a close link between oxidative stress and cell proliferation [112–115], it will be particularly interesting to elucidate the pathophysiological role of oxidative stress in the injury resolution process after APAP hepatotoxicity in future investigations.

2.5. Xanthine oxidase and NAD(P)H quinone dehydrogenase 2 as sources of ROS formation

Another potential source of ROS in the liver after APAP overdose could be the enzyme xanthine oxidase. Indeed, it was observed that xanthine dehydrogenase was converted to the oxidase in the liver after APAP overdose, and mice pretreated with the xanthine oxidase inhibitor allopurinol attenuated the oxidative stress and liver injury [48,49], arguing for a potential involvement of xanthine oxidase in the pathophysiology of APAP hepatotoxicity. However, careful analysis of the data revealed that the dose of allopurinol required to protect against APAP is 5–10-fold higher than the dose needed to completely inhibit the enzyme, and a lower dose that completely inactivates the enzyme could not reduce the oxidant stress or protect against the toxicity [49]. Our follow-up studies demonstrated that the protection by the high doses was actually caused by a preconditioning effect during aldehyde oxidase-mediated metabolism of allopurinol, which induced metallothionein expression that can react with NAPQI and ROS [116,117]. This is supported by evidence that neither 1 h allopurinol pretreatment nor 18 h or 1 h oxypurinol (primary metabolite of allopurinol) pretreatment protects against APAP-induced injury [116]. These data argue against xanthine oxidase as a relevant source of ROS in APAP hepatotoxicity.

Another source of superoxide during APAP toxicity was recently proposed. The cytosolic protein NAD(P)H quinone dehydrogenase 2 (NQO2) can bind and metabolize APAP and generate superoxide thereby causing a cellular oxidant stress [118]. Since NQO2 is highly expressed in liver and kidney, it was hypothesized that this enzyme might be a relevant source of the intracellular oxidant stress induced by APAP [118]. Although these studies were performed in HeLa cells and not in hepatocytes, there are additional issues to consider. The fact that there is no biliary GSSG export during APAP toxicity argues against a relevant oxidant stress in the cytosol [14,49]. In addition, as discussed previously, the most relevant oxidant stress occurs after the metabolism phase when mitochondrial protein adducts are formed [15,49] and interventions that selectively affect mitochondrial oxidant stress are highly protective [68,69,72]. Thus, even if it can be verified that NQO2 can generate superoxide in hepatocytes, it is unlikely that NQO2 will be a pathophysiologically relevant source of oxidant stress during APAP hepatotoxicity.

3. The therapeutic potential of targeting mitochondrial oxidant stress

N-acetylcysteine (NAC) was introduced as a clinical antidote against APAP poisoning in 1970's [119]. Even today, it is still the only pharmacological treatment option for APAP overdose patients. The extensive mechanistic insights from the preclinical models have proposed multiple protective mechanisms for NAC treatment (Fig. 3). If it is given shortly after APAP overdose, i.e. within the metabolism phase of APAP, NAC supplements the GSH pool as it is a synthetic precursor and thus protects by detoxifying the reactive metabolite NAPQI [120,121]. During the injury progression phase, the newly synthesized GSH can also be transported into mitochondria, where it can detoxify ROS and peroxynitrite [58,63]. Interestingly, it was also reported that the surplus NAC in the circulation can be converted to Krebs cycle intermediates, which support mitochondrial energy metabolism and recovery of mitochondrial function [50]. These protective mechanisms are supported by the varied efficacy of NAC in overdose patients. It is very obvious that the patients who benefit most are those receiving NAC within 8 h after APAP overdose, in which the replenished GSH can detoxify NAPQI in the metabolism phase and prevent the initiation of the injury [122,123]. Although the effectiveness of NAC declines at later times, the delayed administration is still beneficial in the clinic [122,123], probably by detoxifying ROS and supporting mitochondrial energy metabolism. Unfortunately, in the clinic most

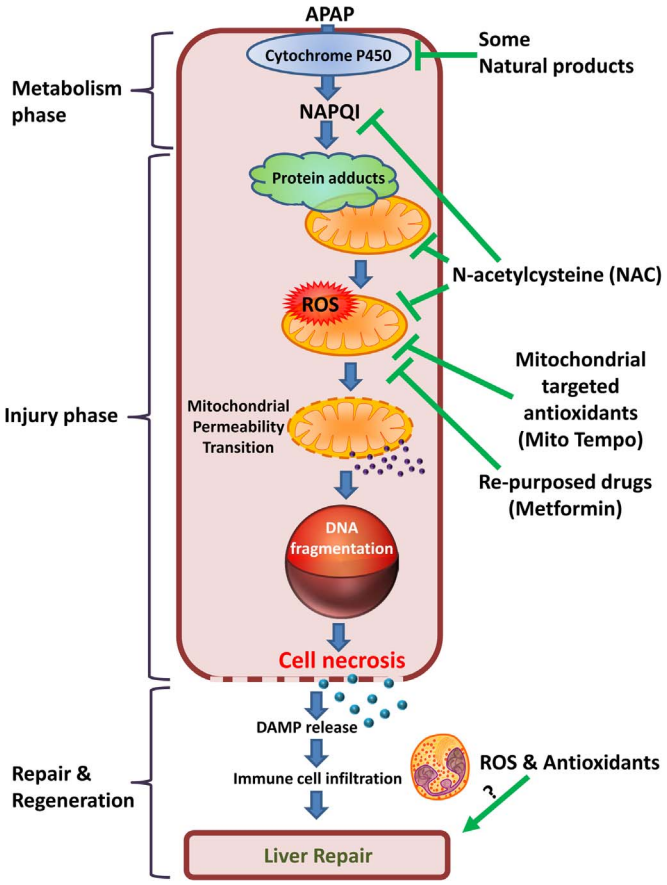

Fig. 3. Phases of hepatotoxicity after APAP overdose: Liver toxicity of APAP is initiated after a metabolism phase, where the drug is metabolized by cytochrome P450 to generate the reactive metabolite NAPQI. Excessive formation of NAPQI results in formation of protein adducts, especially on mitochondrial proteins, which initiates the injury phase. Mitochondrial protein adduct formation results in generation of reactive oxygen species (ROS) within the organelle, which ultimately leads to activation of the mitochondrial permeability transition and release of mitochondrial proteins such as apoptosis inducing factor and endonuclease G and translocation to the nucleus. This then causes nuclear DNA fragmentation and subsequently cell necrosis. Necrotic cells release damage associated molecular patterns (DAMPs), which initiate the regeneration phase, with infiltration of immune cells, and ultimately liver repair. Interventions which inhibit APAP metabolism, as seen with a number of natural products, would consequently prevent NAPQI generation and thus prevent hepatotoxicity. However, these types of therapeutics would not be clinically beneficial, since most patients present much later, i.e., at which time the injury phase has already been initiated. N-acetylcysteine (NAC), which is the current standard of care, protects by replenishing glutathione stores and scavenging NAPQI, as well as supporting mitochondrial recovery. However, since it is effective only early during the injury phase, it may not be as beneficial for patients who present late after APAP consumption. Mitochondrial targeted antioxidants such as Mito Tempo, or repurposed drugs such as Metformin, robustly prevent mitochondrial oxidative stress, which attenuates downstream signaling and cell necrosis, and could be a future therapeutic option. The effect of ROS and antioxidants on the repair phase remains unclear and deserves further investigation.

APAP overdose patients only seek medical attention during or after the peak of injury [124,125], at which time the effectiveness of NAC has already significantly decreased [122,123]. Therefore, a pharmaceutical intervention that still works during this late period would surely have therapeutic potential and greatly help those late-presenting patients..

The protective effect of NAC by detoxifying ROS encouraged the use of antioxidants for late treatment of APAP poisoning. Indeed, numerous interventions that can act as antioxidants have been shown to be protective in preclinical models of APAP toxicity. For example, delayed treatment with exogenous GSH or natural product resveratrol after APAP metabolism significantly decreased oxidative stress and protected against the injury [50,58,73]. In addition, pharmacological inhibition of the JNK signaling pathway, which amplifies the mitochondrial oxidant stress in APAP hepatotoxicity, reduced oxidant stress and the subsequent injury [126,127]. However, despite massive evidence in preclinical studies supporting a beneficial potential of antioxidants, no drugs that specifically target ROS are available clinically for APAP overdose patients. There are several reasons for this problem. First, due to the high efficacy of the current standard-of-care antidote NAC, any promising antioxidant has to be compared with NAC, and only those that can provide additive protection to NAC possess therapeutic potential. Interestingly in our recent studies, the mitochondrial targeted antioxidant Mito-Tempo, either as a late treatment alone or together with NAC offered better protection than NAC alone, which supports it as a therapeutic option for late treatment of APAP overdose in patients (Fig. 3) [72]. Comparison of its efficacy to its analog Tempo highlights not only the importance of the mitochondrial oxidant stress in the development of APAP toxicity but also the therapeutic potential of other mitochondrial targeted antioxidants [72]. It would be interesting to test the efficacy of other antioxidants, *e.g.* the mitochondria-targeting peptide Elamipretide [128], which is currently in multiple clinical trials with FDA approval pending for primary mitochondrial myopathy. Second, due to the high costs of *de novo* drug development and the limited number of patients, it is unlikely that a pharmaceutical company develops a drug specifically for APAP poisoning. Therefore, a better strategy for getting a new antidote against APAP overdose would be by re-purposing an existing drug. For example, an earlier study identified benzyl alcohol, a marketed drug for treatment of head lice, as a promising intervention in APAP hepatotoxicity [129], though a follow-up study revealed that the protection of benzyl alcohol is mainly caused by inhibition of APAP metabolism and thus is unlikely a realistic therapeutic option for treatment of APAP overdose in patients [130]. Methylene blue, a clinical used drug for diseases including methemoglobinemia and psychiatric disorders, was shown to target to mitochondria and reduce mitochondrial oxidant stress [131]. It also restored the compromised ETC function and thus protected against APAP hepatotoxicity [131]. Recently, it was reported that metformin, a first line drug for type 2 diabetes, attenuated the mitochondrial oxidant stress and protected against APAP hepatotoxicity even as a late treatment (Fig. 3) [57]. In addition, its effectiveness can be reproduced in human HepaRG cells, a clinically relevant model for APAP overdose [16], further supporting metformin as a therapeutic option for patients [57]. Nevertheless, more studies are still needed to establish its effective therapeutic dose in humans and assess potential side effects of high doses of metformin before its application in overdose patients.

4. Caveats of testing antioxidants in preclinical models

Research into natural products isolated from plants has revealed a large number of potential drug candidates that may act as antioxidants. The therapeutic potential of such interventions can only be assessed in clinically relevant experimental models such as APAP hepatotoxicity [4,132]. However, the frequent use of suboptimal preclinical models in testing the efficacy of antioxidants or other interventions has been a significant issue in recent years as experimental data from such studies can lead to controversial mechanistic conclusions and thus hampering the progress of translational research in testing antioxidants. For example, the rat is widely regarded as a poor model since even high overdoses of APAP do not cause relevant oxidant stress and liver injury in these animals [17]. However, large numbers of studies testing phytotherapeutics or other antioxidant interventions are still being conducted in this model [4,40]. Furthermore, several human hepatoma cell lines (*e.g.* HepG2, Hep3B, Huh7) are frequently used as *in vitro* models in APAP studies. However, their human pathophysiological relevance is questionable because of the lack of P450 enzymes for the metabolic activation of APAP in these cells [133], which initiates the toxicity in mice and humans. Even if an intervention, including an antioxidant, shows protection in these cell lines, it may not be therapeutically relevant for APAP overdose patients. In contrast, the mouse model closely resembles the human pathophysiology and so far has served as one of the most physiologically relevant models. The most successfully applied *in vitro* model is primary mouse hepatocytes. Although it has some limitations, such as the lack of non-parenchymal cells and loss of P450 enzyme activity over time in culture, for an acute model such as APAP-induced cell death, it generally reproduces most aspects of the *in vivo* pathophysiology [132,134]. The HepaRG cell model has been shown to closely resemble the human pathophysiology for APAP overdose (except the requirement for JNK) [16,125]. Freshly isolated primary human hepatocytes (PHH) are considered as gold standard for drug toxicity studies and a recent investigation has documented their relevance for the human pathophysiology in APAP hepatotoxicity [135]. However, due to their limited availability and high cost if commercially acquired, the use of PHH is still very limited in APAP toxicity studies. Cryopreserved PHH have been introduced to overcome these disadvantages, and currently more advanced PHH culture systems like 3D culture or sandwich culture systems are being developed to more closely represent the normal liver tissue architecture [134]. Based on these caveats, any testing of new therapeutic interventions should be performed in human pathophysiologically relevant models including primary mouse or human hepatocytes, HepaRG cells, or the *in vivo* mouse model.

5. Summary and conclusions

Current experimental evidence demonstrates that neither drug metabolism-induced oxidative stress nor extracellular ROS from inflammatory cells are relevant sources of the oxidant stress in APAP hepatotoxicity. In contrast, P450-mediated metabolism of APAP generates excessive amounts of the reactive metabolite NAPQI, which targets mitochondrial proteins for adduct formation. This impairs mitochondrial respiration and results in ROS formation, leading to an overwhelming mitochondrial oxidant stress and mitochondrial dysfunction. Despite the identification of numerous potential therapeutic targets in mechanistic studies during the last decades, only very few are likely to have clinical relevance. One of the therapeutic targets that consistently comes up in many different preclinical models including human hepatocytes is the mitochondrial oxidant stress. Thus, interventions that prevent or scavenge mitochondrial ROS and peroxynitrite are currently the most promising therapeutic targets against APAP hepatotoxicity in patients.

Conflict of interest disclosure

The authors have no conflict to disclose.

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