Review Article

Naturally Occurring Nrf2 Activators: Potential in Treatment of Liver Injury

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Oxidative stress plays a major role in acute and chronic liver injury. In hepatocytes, oxidative stress frequently triggers antioxidant response by activating nuclear erythroid 2-related factor 2 (Nrf2), a transcription factor, which upregulates various cytoprotective genes. Thus, Nrf2 is considered a potential therapeutic target to halt liver injury. Several studies indicate that activation of Nrf2 signaling pathway ameliorates liver injury. The hepatoprotective potential of naturally occurring compounds has been investigated in various models of liver injuries. In this review, we comprehensively appraise various phytochemicals that have been assessed for their potential to halt acute and chronic liver injury by enhancing the activation of Nrf2 and have the potential for use in humans.

1. Introduction

Liver has an extraordinary capacity to detoxify compounds that have potential to induce liver injury. As a consequence, the liver is also vulnerable to injury. Although liver injury is a major cause of morbidity and mortality, medical therapies to prevent hepatocyte loss or protect hepatocytes are limited. One example is N-acetylcysteine (NAC), which is used for the treatment of acetaminophen- (APAP-) induced liver injury to promote recovery and reduce the need for liver transplantation. Hence, it is imperative to identify better medical therapies that are hepatoprotective and have minimal side effects.

Naturally occurring compounds have long been used as potential hepatoprotective agents. Drafts such as Ayurveda and traditional Japanese, Chinese, and Kampo (traditional Chinese medicine but adapted to the Japanese culture) medicine recommended the use of formulations of specific plants and fruits in the treatment of liver diseases [1–4]. In recent years, technological advances led to the isolation of active phytochemicals, which are now available as potential therapeutic agents [5, 6].

Oxidative stress is a major factor in the mechanism underlying liver diseases. It contributes to the initiation as well as progression of liver injury [7]. Factors such as alcohol, drugs, heavy metals, and high-fat diet are now identified as inducers of hepatic oxidative stress [8]. In liver injury, hepatocytes, the key parenchymal cells, suffer oxidative stress the most. In response to oxidative stress, Kupffer cells produce a variety of cytokines which contribute to hepatocyte apoptosis [9]. Oxidative stress also induces proliferation of stellate cells and collagen synthesis, thus promoting fibrosis and cirrhosis. In response to overwhelming oxidative stress, there is a significant use of antioxidant proteins, along with an increase in lipid peroxidation. However, to maintain redox homeostasis, hepatocytes have a sophisticated antioxidant system comprising antioxidant proteins, enzymes, and transcription factors to combat oxidative stress. Hence, regulation of hepatic oxidative stress can play a critical role in the treatment of various liver diseases.

Nuclear erythroid 2-related factor 2 (Nrf2), a transcription factor of the cap'n'collar basic leucine zipper family [10], is a key regulator of oxidative stress in numerous cell types including hepatocytes [11–16]. Nrf2 is primarily regulated

by Kelch-like ECH-associated protein 1 (Keap1), a substrate adaptor for a cul3-containing E3 ubiquitin ligase [17]. In the absence of oxidative stress, Nrf2 is located in the cytoplasm where it interacts with Keap1 and is rapidly degraded by the ubiquitin-proteasome pathway [18, 19]. However, under oxidative stress, phosphorylation of Nrf2 leads to its dissociation from Keap1 and subsequent translocation to the nucleus [14, 15, 19]. Herein, it binds to antioxidant response element (ARE) sequences and, in partnership with other nuclear proteins, enhances the transcription of ARE-responsive genes such as hemeoxygenase-1 (HO-1), NAD(P)H:quinone oxidoreductase 1 (NQO1), glutathione-S-transferases (GST), glutamate-cysteine ligase modifier subunit (GCLM), glutathione peroxidase (GPX), and glutamate-cysteine ligase catalytic subunit (GCLC) to mount strong antioxidant and cytoprotective responses [20, 21].

Numerous studies have shown that natural products regulate oxidative stress in the liver by modulating Nrf2-ARE pathway to render hepatoprotective effect. This review discusses the importance of Nrf2-ARE in regulating liver injury and the role of natural product based activators (phytochemicals) of Nrf2-ARE pathway in treating liver injury (Table 1).

2. Nrf2 Signaling in Acetaminophen-Induced Hepatotoxicity

APAP is one of the most widely used over-the-counter analgesics. APAP is safe when taken at therapeutic doses but causes severe liver injury when ingested in higher-than-recommended doses. Acute liver failure due to APAP overdose is associated with high mortality [22]. In the United States, the incidence of APAP overdose is over 100,000 cases each year [23]. When ingested in therapeutic doses, APAP is mainly metabolized by sulfation and glucuronidation, leaving only a small fraction to be metabolized by cytochrome p4502E1 (CYP2E1) [24]. However, upon overdose, glucuronidation and sulfation pathways get saturated leading to APAP's metabolism by CYP2E1, resulting in generation of N-acetyl-p-benzoquinone imine (NAPQI), a toxic intermediate metabolite capable of inducing oxidative stress. NAPQI depletes hepatic glutathione (GSH) content and binds to cellular proteins, with subsequent activation of c-Jun N-terminal kinase (JNK). JNK activation results in overproduction of reactive oxygen species (ROS) and reactive nitrogen species (RNS) resulting in mitochondrial dysfunction and hepatocyte death [25–27]. Within 60 minutes of administration of APAP in mice, there is an increase in Nrf2 nuclear translocation in hepatocytes with concomitant increase in the expression of several Nrf2 target genes [28, 29]. Studies using Nrf2-knockout mice showed that even lower doses of APAP could induce mortality due to suppressed GSH synthesis pathway [30]. Contrary to this, hepatocyte-specific deletion of Keap1 activates Nrf2 and confers resistance against APAP toxicity [31]. Interestingly, in Nrf2-knockout mice, the elimination of APAP metabolites is also decreased as a result of reduced expression of multidrug resistance proteins (MRPs) [32]. And, in Keap1-knockout mice, MRP expression is increased, which enhanced efflux of APAP metabolites [29].

These observations indicate that, in APAP-induced hepatotoxicity, Nrf2 modulates injury not only by regulating antioxidant response but also by modulating APAP elimination.

Protein tyrosine phosphorylation is pivotal in cell survival. In this context, the balance between tyrosine kinases and phosphatases determines cell fate. One of the protein tyrosine phosphatases, protein tyrosine phosphatase 1B (PTP1B), is widely expressed and inactivates many tyrosine kinase family members by dephosphorylation. During APAP toxicity in mice, PTP1B expression is significantly increased. PTP1B deficient mouse hepatocytes are protected against APAP-induced GSH depletion and oxidative stress by prolonged Nrf2 nuclear accumulation [33].

Various classes of phytochemicals have been shown to activate Nrf2 pathway and reduce APAP toxicity (Table 1). Sauchinone, a polyphenol, reduces the impact of APAP overdose by enhancing Nrf2 phosphorylation via protein kinase C-δ (PKCδ) and decreasing interaction amongst Nrf2 and Keap1 [34]. Salvianolic acid B reduces APAP-induced liver injury via phosphoinositide-3-kinase- (PI3K-) and PKC-mediated Nrf2 activation, resulting in enhanced HO-1 and GCLC expressions [35]. Sulforaphane (an isothiocyanate found in cruciferous vegetables, namely, cauliflower, broccoli, kale, cole crops, cabbage, collards, brussels sprouts, and mustard) and oleanolic acid (a triterpenoid found in olives) both reduce APAP-induced oxidative stress and liver injury via Nrf2 and related antioxidant gene activation [36, 37]. A recent study from our laboratory evaluated the hepatoprotective potential of withaferin A, an active ingredient of *Withania somnifera*. We observed that withaferin A treatment 1 h *after* APAP intoxication activates Nrf2 responsive genes such as NQO1 and GCLC to reduce oxidative stress and subsequent liver injury [38]. An in vitro study, using rat hepatocytes, reported that ginsenoside Rg3 (a ginseng saponin) upregulates antioxidant genes (GCLC, GCLM) and basolateral MRPs via Nrf2 activation [39].

Currently, NAC is the only FDA-approved antidote for APAP hepatotoxicity and is most effective within 8–10 h after APAP overdose. Various studies that utilized phytochemicals to reduce APAP toxicity provide a strong proof of concept that naturally existing activators of Nrf2 can be used to reduce APAP-induced hepatotoxicity. However, these studies have to be interpreted carefully. Many studies have used a preventive experimental strategy, where the phytochemical was administered before inducing APAP overdose, or simultaneously, both unlikely clinical scenarios; patients seek care after APAP overdose. Moreover, such strategies suggest that phytochemicals may interfere with APAP metabolism by suppressing CYP2E1 activity and preventing generation of NAPQI, thus making the interpretation of these studies difficult [40, 41]. In our recent work, we employed an experimental strategy that was clinically relevant, that is, administering WA 1 h after APAP overdose, which provides sufficient time for APAP's metabolism into NAPQI and generation of protein adducts to adequately mimic clinical APAP hepatotoxicity in humans [38, 42]. Therefore, additional studies that not only employ clinically relevant strategies but also use a combination of Nrf2 activators and NAC to combat APAP toxicity are warranted.

TABLE 1: Various Nrf2 activator phytochemicals and their role in liver injury.

Phytochemicals	Effective doses	Experimental procedure (injury model)	Outcomes	References
Acetaminophen toxicity				
Ginsenoside Rg3	3 μg/mL	Rat hepatocytes treated with 200 μM NAPQI	Repletion of GSH content and enhanced expression of Mrp expression	[39]
Oleanolic acid	90 mg/kg	Mice injected with 330 μmol/kg APAP into the right femoral vein	Enhanced antioxidant response to reduce hepatocyte necrosis	[37]
Salvianolic acid B	25 and 50 mg/kg	Mice treated with single dose of 300 mg/kg APAP (i.g.)	Antioxidant response and phase II enzyme induction via activation of PI3K/Akt and PKC signaling to reduce liver injury	[35]
Sauchinone	30 mg/kg	Mice treated with single dose of 500 mg/kg APAP (i.p.)	Induction of antioxidant genes to reduce hepatocyte necrosis	[34]
Oleanolic acid	5 mg/kg	Mice treated with single dose of 300 mg/kg APAP (i.p.)	Reduction of ROS generation, GSH depletion, and lipid peroxidation coupled with upregulation of antioxidant genes	[36]
Withaferin A	40 mg/kg	Mice treated with single dose of 250 mg/kg APAP (i.p.)	Reduced hepatocyte injury by reducing GSH depletion	[38]
Inflammatory injury				
Ellagic acid	5, 10, and 20 mg/kg	Mice treated with single dose of 800 mg/kg Gal + 50 μg/kg LPS (i.p.)	Reduced LPS/GalN-induced NF-κB activation and increased antioxidant genes	[43]
Linalool	10, 20, and 40 mg/kg	Mice treated with single dose of 800 mg/kg Gal + 50 μg/kg LPS (i.p.)	Reduced LPS/GalN-induced NF-κB activation and induction of cytoprotective genes	[44]
Mangiferin	5, 10, and 20 mg/kg	Mice treated with single dose of 800 mg/kg Gal + 50 μg/kg LPS (i.p.)	Reduced liver injury by activating antioxidant pathway and inhibiting NLRP3 inflammasome activation	[45]
Oroxylin A	15, 30, and 60 mg/kg	Mice treated with single dose of 800 mg/kg Gal + 50 μg/kg LPS (i.p.)	Decreased liver injury by activating antioxidant genes and inhibiting TLR4 signaling-mediated inflammation	[46]
Chemical toxicity				
Tungtungmadic acid	5 and 20 μM	Hepa1c1c7 cells treated with 250 μM t-BHP	HO-1 induction via the PI3K/Akt signaling pathway to reduce hepatocyte death	[47]
Antcin C	20 μM in cells, 100 mg/kg in mice	HepG2 cells treated with 10 mM AAPH, mice treated with single dose of 80 mg/kg AAPH (i.p.)	Induction of antioxidant response via increase of JNK1/2 and PI3K/Akt activities	[48]
Butein and phloretin	25 μM in vitro, 30 mg/kg in vivo	Mouse hepatocytes treated with 0.5 mM t-BHP, rats treated with single dose of 1 mL/kg CCl$_4$ (i.p.)	Upregulation of HO-1 and GCLC expression through ERK2 pathway	[49]
Carthamus red	10 and 20 mg/kg	Mice treated with two doses of 2 mL/kg CCl$_4$-olive oil mixture (1 : 1)	Upregulation of Nrf2, GSTα, and NQO1 expressions associated with decreased hepatocyte injury and ALT levels	[50]
Curcumin	200 mg/kg	Mice treated with single dose of 20 mg/kg DEN (i.p.)	Nrf2-mediated HO-1 induction and amelioration of hepatocyte injury	[51]
Diallyl disulfide	50 and 100 mg/kg	Rats treated with single dose of 2 mL/kg CCl$_4$ (i.g.)	Induction of antioxidant and detoxifying enzyme activities and suppressing of inflammatory cytokines production by reducing NF-κB activation	[52, 53]
Ginsenoside Rg1	20 and 40 mg/kg	Rats treated with 2 mL/kg of 50% CCl$_4$ (s.c.) twice a week for 8 weeks	Reduced liver fibrosis by augmented antioxidant systems	[54]
Glycyrrhetinic acid	25 and 50 mg/kg	Mice treated with 6.4 g/kg CCl$_4$ (s.c.) for 30 days	Enhanced antioxidant genes expression to reduce hepatocyte injury	[55]
Hesperidin	40 and 80 μM	LO-2 cells treated with 150 μM t-BHP	ERK-mediated nuclear translocation of Nrf2 to induce HO-1 gene expression and antioxidant response	[56]

TABLE 1: Continued.

Phytochemicals	Effective doses	Experimental procedure (injury model)	Outcomes	References
Isoorientin	5 μg/mL	HepG2 cells treated with 200 μM t-BHP	Upregulation of antioxidant enzyme expression through PI3K/Akt pathway	[57]
Naringenin	50 mg/kg	Rats treated with 2 mL/kg CCl_4-olive oil mixture (1:1) on days 2 and 5 (i.p.)	Increase in Nrf2 and HO-1 expression to reduce liver injury	[58]
Oxyresveratrol	10 μM for in vitro study, 10 and 30 mg/kg for in vivo study	200 μM t-BHP treatment to HepG2 cells, ice treated with single dose of 0.5 mL/kg CCl_4 (i.p.)	ERK phosphorylation-mediated induction of antioxidant pathway to protect hepatocytes against oxidative stress, mitochondrial damage, and resultant cell death	[59]
Puerarin	100 μM	500 μM t-BHP treatment to Hepa1c1c7 and HepG2 cells	Augmentation of cellular antioxidant defenses through Nrf2-dependent HO-1 induction via PI3K pathway	[60]
Resveratrol	50 and 75 μM	Primary rat hepatocytes treated with 500 μM t-BHP	Reduced hepatocyte death by improving antioxidant status	[61]
Schisandrin B	15 μM	AML12 cells treated with 20 μM menadione for 1 h	Induction of ERK/Nrf2 signaling to enhance glutathione-mediated antioxidant response to protect hepatocytes against menadione-induced apoptosis	[62]
Metal toxicity				
Curcumin	200 mg/kg	Exposure of mice to $NaAsO_2$ (100 mg/L) in drinking water	Induction of antioxidant genes and enhanced methylation and elimination of arsenic	[63]
Lutein	40 mg/kg	Mice treated with 4 mg/kg As_2O_3 (i.g.)	Reduced liver injury by induction of antioxidant response	[64]
S-Allylcysteine	100 mg/kg	Mice treated with single dose of 17 mg/kg $K_2Cr_2O_7$ (s.c.)	Induction of antioxidant response to reduce liver injury	[65]
Alcohol toxicity				
Lucidone	1, 5, and 10 μg/mL	HepG2 cells treated with 100 mM ethanol	Induction of HO-1 via Nrf2 signaling pathway to enhance antioxidant response	[66]
Quercetin	100 μM	Primary human hepatocytes treated with 100 mM ethanol	ERK- and p38-mediated Nrf2 nuclear translocation and subsequent induction of HO-1 activity	[67, 68]
Quercetin	50 μM	LO-2 cells treated with 100 mM ethanol	Preventing hepatotoxicity by inducing p62 expression and induction of antioxidant response	[69]
Sulforaphane	50 mg/kg	Mice treated with 3 g/kg ethanol (30%) for 5 days (i.g.)	Decreased hepatocyte lipid accumulation and injury without altering CYP2E1 expression	[70]
Nonalcoholic steatohepatitis				
Baicalein	10 mg/kg	Rats fed with MCD diet for 8 weeks	Reduction in inflammation and oxidative hepatocyte injury	[71]
Curcumin	50 mg/kg	Rats fed with HFD for 6 weeks	Reduced hepatocyte lipid accumulation and improved insulin resistance and anti-inflammatory and antioxidant effects	[72]
Gastrodin	10, 20, and 50 mg/kg	HL-7702 cells treated with 0.6 mM of OA for 24 h, mice fed with HFD for 10 weeks	AMPK-mediated induction of Nrf2 pathway to enhance expression of antioxidant enzymes	[73]
Lycopene	15 mg/kg	Mice fed with HFD for the next 6 weeks following a single dose of 30 mg/kg DEN injection	Reduction in hepatocyte injury by induction of antioxidant pathway along with a decrease in CY2E1 expression	[74]
Cholestatic liver injury				
Oleanolic acid	20 mg/kg	Mice treated with 125 mg/kg LCA (i.p.)	Upregulation of Mrp2, Mrp3, and Mrp4 to reduce cholestatic liver injury	[75]
Oleanolic acid	20 mg/kg	Bile duct ligation in mice	Induction of Mrps and FXR antagonism to reduce cholestatic liver injury	[76]

TABLE 1: Continued.

Phytochemicals	Effective doses	Experimental procedure (injury model)	Outcomes	References
Paeoniflorin	200 mg/kg	Rats treated with 50 mg/kg ANIT for 4 days (i.g.)	Enhanced GSH synthesis by activating Nrf2 through PI3K/Akt-dependent pathway	[8]
Sulforaphane	50 mg/kg	Mice treated with 3 g/kg ethanol (30%) for 5 days (i.g.)	Decreased hepatocyte lipid accumulation and injury without altering CYP2E1 expression	[70]
Sulforaphane	25 mg/kg	Bile duct ligation in mice	Antifibrotic response by inhibition of TGF-β/Smad signaling pathway	[77]

AAPH: 2,2′-azobis(2-amidinopropane) dihydrochloride; MCD: methionine and choline deficient; CCl$_4$: carbon tetrachloride; DEN: dimethylnitrosamine; HFD: high-fat diet; Gal: galactosamine; LPS: lipopolysaccharide; OA: oleic acid; NAPQI: N-acetylbenzoquinoneimine; i.p.: intraperitoneal; s.c.: subcutaneous; i.g.: intragastric; t-BHP: tert-butyl hydroperoxide; APAP: acetaminophen; LCA: lithocholic acid; ANIT: alpha-naphthylisothiocyanate; Nrf2: nuclear factor (erythroid-derived 2)-like 2; JNK1/2: c-Jun N-terminal kinases 1/2; PI3K/AKT: phosphoinositide 3-kinase/protein kinase B; HO-1: heme oxygenase-1; GCLC: glutamate-cysteine ligase catalytic subunit; ALT: alanine transaminase; GST: glutathione S-transferase; NQO1: NAD(P)H quinone dehydrogenase 1; AMPK: 5′ AMP-activated protein kinase; GSH: glutathione; CYP2E1: cytochrome P450 2E1; NLRP3: NLR family pyrin domain containing 3; Mrp: multidrug resistance-associated protein; TLR4: Toll-like receptor 4; Keap1: Kelch-like ECH-associated protein 1.

3. Acute Inflammatory Liver Injury and Nrf2

Inflammation-mediated repeated liver injury may progress to chronic fibrosis and cirrhosis. Studies in various models of liver injury have shown that Nrf2 plays a role in inflammation-induced liver injury. Lack of an active Nrf2 signaling pathway results in severe inflammation-induced oxidative stress. Concanavalin A (ConA) treatment to mice activates and recruits T-lymphocytes and causes severe inflammation and hepatocyte apoptosis [78, 79]. Following intravenous injection of ConA, Nrf2-KO mice develop increased liver injury compared to WT mice. Hepatocyte-specific conditional Keap1-null mice and WT mice pretreated with three daily doses of CDDO-Im, a Nrf2 activator, had significantly reduced ConA-induced injury compared to Nrf2-KO mice. Similarly, in response to LPS/galactosamine treatment, Nrf2-null mice had increased liver injury compared to WT mice, and, predictably, hepatocyte-specific Keap1-KO mice were protected [80]. These results highlight the importance of Nrf2-mediated regulation of cytokine-dependent hepatocyte apoptosis [81]. In another study, investigators used a short interfering RNA (siRNA) against Keap1 to assess the role of Nrf2 in liver injury; mice were injected with Keap1- or luciferase (control)-siRNA-containing liposomes via the tail vein and, after 48 hours, with ConA. Silencing of hepatic Keap1 attenuated ConA-induced inflammation-associated liver damage [82].

Mangiferin and oroxylin A, the naturally occurring flavonoids, reduce LPS/galactosamine-induced liver injury in mice by reducing oxidative stress and inflammation. Mangiferin enhanced Nrf2/HO-1 signaling and inhibited NLR family, pyrin domain containing 3 (NLRP3) inflammasome [45]. Oroxylin A activated Nrf2 signaling and inhibited Toll-like receptor 4 (TLR4) signaling [46]. Linalool, a major volatile component of essential oils in several aromatic plant species, and ellagic acid (EA), a naturally occurring plant phenol found in certain fruits, nuts, and vegetables, have been shown to possess anti-inflammatory and antioxidant properties. Both of these phytochemicals reduced LPS/galactosamine-induced hepatocyte apoptosis by activating hepatocyte

Nrf2-mediated antioxidant response and inflammation by suppressing NF-kB pathway in the liver [43, 44]. These phytochemicals, which reduce both oxidative stress and inflammation, are potential agents for the treatment of fulminant hepatitis in humans.

4. Role of Nrf2 in Chemical-Induced Liver Injury

Carbon tetrachloride (CCl$_4$) is a potent hepatotoxin known to cause centrilobular hepatic necrosis in rodent models of liver fibrosis. Acute administration of CCl$_4$ at higher doses causes severe hepatocyte necrosis, while chronic administration at lower doses is used to induce hepatic fibrosis [40]. However, this model is often criticized because it affects the central zone of the hepatic acinus and some nonmetabolized intermediates of CCl$_4$ can induce lung and kidney injury [83]. In hepatocytes, cytochrome P450 metabolizes CCl$_4$ to trichloromethyl free radicals (CCl$_3$$^\bullet$ and/or CCl$_3$OO$^\bullet$), which subsequently leads to generation of ROS ($^\bullet$O$_2$$^-$, H$_2O_2$, and $^\bullet$OH) [84]. Chemical hepatotoxins such as bromobenzene, CCl$_4$, and furosemide trigger hepatic Nrf2 nuclear translocation and Nrf2-regulated gene expression [85]. In Nrf2-deficient mice, repair of liver injury after a single treatment with CCl$_4$ was severely delayed [86]. Similarly, after 1-bromopropane exposure, compared to WT mice, Nrf2-knockout mice had increased liver injury with reduced antioxidant response [87].

Lee and colleagues studied the efficacy of diallyl disulfide (DADS), a secondary component derived from garlic, in amelioration of CCl$_4$-induced acute liver injury. Pretreatment with DADS (50 and 100 mg/kg/day) significantly activated Nrf2 expression and antioxidant and phase II enzymes to reduce liver injury [52, 53]. Oxyresveratrol (OXY), an antioxidant present in mulberry fruits and twigs, ameliorated tert-butyl hydroperoxide- (t-BHP-) and CCl$_4$-induced hepatotoxicity by reducing oxidative stress possibly via extracellular signal-regulated kinase- (ERK-) mediated Nrf2 activation in hepatocytes [59]. Other flavonoids such as carthamus red and naringenin also activate Nrf2 signaling to reduce CCl$_4$

injury in rats [50, 58]. Glycyrrhetinic acid, a triterpenoid, and ginsenoside Rg1, a phytoestrogen, ameliorated CCl_4-induced liver fibrosis via nuclear Nrf2 translocation and upregulation of antioxidant enzymes to reduce oxidative stress [54, 55].

Several other compounds such as 2,2′-azobis(2-amidino-propane) dihydrochloride (AAPH), menadione (MEN), and carboxymethyllysine (CML) induce free radical-induced damage, and each of them is a potent inducer of hepatotoxicity. Phytocompounds such as antcin C [48], dimerumic acid [88], and schisandrin B [62] have been shown to ameliorate AAPH-, CML-, and MEN-induced liver injury, respectively. Dimethylnitrosamine (DMN) is a semivolatile organic chemical produced as a byproduct of industrial processes and is also known to cause oxidative liver injury. In DMN-administered rats, oral administration of curcumin enhanced nuclear translocation and ARE binding of Nrf2, producing robust antioxidant response thereby reducing hepatotoxicity [51].

In laboratories, t-BHP is a commonly used inducer of oxidative stress in vitro [53, 89, 90]. In hepatocytes, t-BHP is metabolized by CYP450 into free radical intermediates such as t-butoxyl and t-methyl radicals [91], which induce lipid peroxidation and glutathione (GSH) depletion resulting in organelle damage and cell death [92]. Hence, t-BHP-induced hepatotoxicity model is widely used for evaluating merits of Nrf2-ARE signaling in hepatocytes. Various plant flavonoids possess excellent antioxidant potential and confer hepatoprotection. Flavonoids such as hesperidin, butein, and phloretin reduce t-BHP toxicity in hepatocytes via ERK phosphorylation [49, 56], whereas another member of the same class, isoorientin, induces Nrf2 activation via PI3K/Akt pathway [57]. Additionally, phenolics such as 3-caffeoyl, 4-dihydrocaffeoyl quinic acid, and resveratrol ameliorate t-BHP-induced hepatocyte oxidative stress via Nrf2 induction [47, 61]. Another phytoestrogen, puerarin, reduces t-BHP-induced oxidative stress in Hepa1c1c7 cells via PI3K/Akt pathway [60]. These findings underscore the importance of phytochemicals-mediated regulation of Nrf2 activity in diverse models of hepatocyte toxicity, but many of these results need to be validated in animal models of liver injury.

5. Metal Toxicity and Nrf2

Heavy metals, such as cadmium (Cd), chromium (Cr), copper (Cu), lead (Pb), and mercury (Hg), pose health concerns via exposure through water, food, or environmental waste [93]. In experimental models, these heavy metals cause severe oxidative stress and hepatotoxicity. Since these metals induce ROS, it is conceivable that they might induce Nrf2 activation in hepatocytes. A study using Nrf2-KO, Keap1-KO, and hepatocyte-specific Keap1-knockout mice showed that Cd caused extensive liver damage in Nrf2-null mice, whereas Keap1-KO and Keap1-HKO mice were resistant to injury [94].

Arsenic (a metalloid) is a known pollutant for drinking water and has provoked public health concern worldwide. Arsenic modulates Nrf2 signaling in vivo and in vitro [46, 95, 96]. Lutein (a carotenoid) and curcumin (a diarylheptanoid) have been shown to reduce arsenic toxicity via Nrf2 activation and subsequent antioxidant gene expression [63, 64].

Additionally, in mice, curcumin promoted methylation of arsenic and accelerated its excretion [63].

Several studies demonstrated that Cr(VI) induces hepatotoxicity by increasing ROS generation [97, 98]. In Hepa1c1c7 cells, Cr(VI) induces ROS production and in turn triggers activation of Nrf2 pathway [99]. Garlic and its derivative S-allylcysteine (SAC) reduced Cr(VI)-induced hepatotoxicity [65].

In this industrialization era, metal toxicity is a growing concern, and Nrf2 presents a potential target that can be explored to discover novel hepatoprotective agents. These studies suggest that the use of phytochemicals may be helpful in negating the hepatotoxic effects of heavy metals.

6. Nrf2 and Alcoholic Liver Injury

Chronic alcohol consumption is known to result in liver injury-associated deaths [100]. Alcohol abuse leads to increased production of ROS, depletion of hepatocytes antioxidant levels, and enhanced oxidative stress [101, 102]. However, it also leads to an increase in Nrf2 mRNA and protein in the livers [103]. Gong and Cederbaum speculated that the increase in Nrf2 expression was dependent on ethanol-mediated induction of CYP2E1. Compared to WT mice, ethanol-administered Nrf2-KO mice had depletion of total and mitochondrial GSH in their livers resulting in increased liver failure and mortality, while ethanol-fed Keap1-HKO mice were protected [104]. Keap1 knockdown in mice, which induces Nrf2 activation, also blunted ethanol-mediated increase in serum triglycerides and hepatic free fatty acids [105].

Quercetin (a plant flavonoid), a known antioxidant and free radical scavenger, reduces ethanol toxicity in hepatocytes which is Nrf2-mediated. Quercetin upregulates HO-1 via the MAPK/Nrf2 pathway to confer hepatoprotection [67, 68]. A recent study showed that quercetin interacts with Keap1 and blocks its binding to Nrf2 [69]. Sulforaphane (an isothiocyanate) is a known activator of Nrf2 pathway. Zhou et al. showed that sulforaphane induces Nrf2 activation to reduce lipid accumulation and oxidative stress in hepatocytes, an effect independent of CYP2E1 [70]. Lucidone (a naturally occurring cyclopentenedione in *Lindera* sp.) reduces ethanol-induced oxidative stress in HepG2 cells [66] by inducing Nrf2-mediated antioxidant response via profound upregulation of HO-1.

Despite its known detrimental effects, alcohol remains a major cause of liver injury. These observations from preclinical studies indicate that targeting Nrf2 has merits in treating alcoholic liver injury. These natural Nrf2 activators need to be studied in clinical settings to determine the impact on alcohol abuse-mediated liver injury burden.

7. Nrf2 and Nonalcoholic Steatohepatitis

Nonalcoholic fatty liver disease (NAFLD) is becoming the most common cause of chronic liver disease [106, 107]. In NAFLD, multiple mechanisms operate simultaneously which results in hepatocyte apoptosis, inflammation, and fibrosis,

that is, nonalcoholic steatohepatitis (NASH) [107, 108]. In NASH, excessive lipid accumulation and subsequent generation of ROS from impaired mitochondrial respiratory chain result in GSH depletion [109, 110]. It is interesting to note that two research groups showed in mice that high-fat diet (HFD) can increase or decrease hepatic Nrf2 [111]. However, upon HFD feeding, Nrf2-null mice suffered from severe liver injury compared to wild-type mice [112]. Livers of methionine-choline deficient diet- (MCD-) fed Nrf2-knockout mice had higher levels of oxidative stress, iron accumulation, fibrosis, and inflammation than wild-type mice [113–115]. It is suggested that Nrf2 activation protects against NAFLD and NASH also via controlling inflammation [115]. The overall merits of Nrf2-mediated prevention of oxidative damage and progression of NAFLD imply its potential use in patients suffering from NASH.

Studies have shown the beneficial role of phytochemicals in treating NASH in preclinical and clinical settings [116–118]. Lycopene (a carotenoid derived from tomato) is shown to reduce HFD-induced steatohepatitis in rats [119]. Wang et al. showed that lycopene-induced inhibition of NASH and hepatocarcinogenesis in rats is partially due to induction of Nrf2 and HO-1 genes [74]. Gastrodin (GSTD, a natural compound isolated from *Gastrodia elata* BI, a traditional Chinese herbal medicine) ameliorates oxidative stress and proinflammatory response in cellular and animal models of NAFLD. GSTD-induced AMPK activation phosphorylates Nrf2, which in turn enhances its nuclear translocation and expression of antioxidant genes (HO-1 and SOD1) [73]. In rats, baicalein (a flavonoid) has been shown to ameliorate MCD-diet-induced NASH by activating multiple pathways including Nrf2-ARE pathway [71]. Similar results have been reported in studies with curcumin where Nrf2 activation ameliorated hepatotoxicity and reduced NASH [72]. Scientific data available till date indicate that the role of natural compounds in Nrf2 activation and alleviation of NASH in experimental models warrants additional human studies. Owing to the multifactorial nature of NASH, a combination of Nrf2 activator and lipid-lowering drugs may translate into an effective therapeutic strategy.

8. Nrf2 and Cholestatic Liver Injury

Cholestasis, a reduction in bile flow, results in a dramatic increase in both liver and serum bile acid concentrations leading to acute liver toxicity, proliferation of bile ducts, and eventually cirrhosis [120]. Bile duct ligated- (BDL-) Nrf2-deficient mice showed reduced elimination of bile acids and higher intrahepatic accumulation of toxic bile acids leading to GSH depletion and injury [121, 122]. BDL-Keap1-KO mice had sustained activation of Nrf2 and increased expression of MRP efflux transporters, detoxifying enzymes, and antioxidant genes [121, 123]. Interestingly, following BDL, Nrf2-KO mice developed into a cholestatic phenotype but liver injury was not different from WT mice undergoing BDL, suggesting that Nrf2 plays a role in the regulation of bile acid homeostasis in the liver [121]. Nrf2-KO mice treated with lithocholic acid (LCA, a toxic bile acid) compared to wild-type mice had severe multifocal liver necrosis and increased inflammation and serum ALT levels [124].

Ursodeoxycholic acid (UDCA), a secondary bile acid, is reported to be hepatoprotective [125, 126]. UDCA treatment of WT mice significantly increased nuclear Nrf2 expression and that of hepatic Mrp2, Mrp3, and Mrp4 [127]. In patients with biliary cirrhosis, UDCA treatment enhanced hepatic Nrf2 expression with an upregulated hepatic thioredoxin and thioredoxin reductase 1 expression [128]. These studies indicate a role of Nrf2 and its activation as a viable target in treating cholestatic liver injury.

Oleanolic acid (OA), a natural triterpenoid, has been shown to reduce LCA- and BDL-induced cholestatic injury in mice [75, 76]. Protection of both of these models is thought to be due to Nrf2-mediated upregulation of Mrps. However, high doses of OA induce cholestatic liver injury in mice by downregulation of hepatic transporters and disruption of bile acid uptake and metabolism [129]. Sulforaphane also reduces cholestasis injury in mice by hepatocyte Nrf2 activation [77]. Alpha-naphthylisothiocyanate (ANIT) is used to induce hepatotoxicity in animal models, which mimics drug-induced cholestatic hepatic injury in humans [130]. Chen et al. demonstrated that paeoniflorin (a monoterpene glycoside) ameliorates ANIT-induced cholestasis in rats by activating Nrf2 via PI3K/Akt pathway [8]. Since Nrf2 regulates both antioxidant genes and multidrug resistance-associated proteins, it has great implication in treating cholestasis.

9. In Vitro Nrf2-ARE Pathway Activation by Phytochemicals

Various hepatocyte cell lines and primary hepatocytes have been used to demonstrate mechanisms of phytochemicals-induced Nrf2 activation. A handful of phytochemicals have been reported to activate Nrf2 signaling in hepatocytes and related cell lines even in the absence of exogenous oxidative stress stimuli. For example, compared to vehicle, phytochemicals such as isothiocyanate (6-methylthiohexyl isothiocyanate), capsaicinoids (capsaicin), terpenoids (carnosol and maslinic acid), polyphenols (eckol, quercetin, and phloretamide), chalconoids (xanthohumol), and flavonoids (epicatechin) induce Nrf2 activation in hepatocytes [131–139]. Although these studies comprehensively demonstrate the ability of phytochemicals to induce Nrf2 activation in hepatocytes, one of the merits of these compounds under conditions of oxidative stress, both in vitro and in vivo, is the prevailing lacunae, which leaves many questions unanswered.

10. Cytotoxicity of Phytochemicals

Consumption of herbal mixtures or bioactive molecules can lead to adverse events as well as hepatotoxicity [140]. Phytochemicals found naturally in many plants, fruits, and vegetables are generally believed to be devoid of major side effects [140]. However, safety studies are lacking and many phytochemicals that are marketed as food supplements have already been shown to have adverse effects. Polyphenols and flavonoids, which are popular for their beneficial

effects, have been reported to induce adverse events. For example, consumption of polyphenols inhibits nonheme iron absorption and may lead to iron depletion [141]. At higher doses, flavonoids have been reported to act as mutagens, prooxidants, and inhibitors of key enzymes involved in hormone metabolism [142]. Oleanolic acid, which is a known inducer of Nrf2 pathway, has been shown to cause cholestasis at higher doses [129, 143].

Medical literature is littered with various reports of herb-induced hepatotoxicity [144, 145]. For example, black cohosh used for menopausal symptoms can lead to liver failure. We refer the reader to recent articles [146–149]. Safety studies for most phytochemicals discussed here are lacking. The FDA is not authorized to review dietary supplement products for safety and effectiveness before they are marketed (http://www.fda.gov). Hence, there is a big gap in the knowledge regarding the safety profile of phytochemicals and toxicity studies will be needed before they are considered safe for human usage.

11. Concluding Remarks

Almost all modes of liver injury are associated with increased oxidative stress and an overwhelmed antioxidant defense system. Since Nrf2 activation is associated with the enhancement of endogenous antioxidant system, it can be an ideal therapeutic target for reducing oxidative stress. Nrf2-ARE pathway boosts hepatocyte antioxidant defense system but may not be able to reduce pathogenesis of liver injury. Hence, a synchronal use of natural Nrf2 activators in combination with other pharmacological agents can be a potential multipronged approach to reduce liver injury. The search for treatments for liver injury is ongoing and potential candidates include whole herbal extracts as well as purified phytochemicals. The literature on preclinical use of phytochemicals to treat various modes of liver injury is ever-growing. However, very few phytochemicals have been tested in clinical settings. Some of the key limitations in this regard are (1) poor choice of experimental models and their clinical relevance, (2) lack of a specific identified mechanism of action, and (3) lack of comprehensive time point data for better interpretation of preclinical findings. These limitations have resulted in weakened significance of these compounds as hepatoprotective agents. Therefore, further studies are needed to explore the full potential of these compounds.

Competing Interests

The authors declare that there are no competing interests regarding the publication of this paper.

Acknowledgments

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