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REVIEW ARTICLE

Role of free radicals in liver diseases

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Abstract Reactive oxygen and nitrogen species (ROS and RNS) are produced by metabolism of normal cells. However, in liver diseases, redox is increased thereby damaging the hepatic tissue; the capability of ethanol to increase both ROS/RNS and peroxidation of lipids, DNA, and proteins was demonstrated in a variety of systems, cells, and species, including humans. ROS/RNS can activate hepatic stellate cells, which are characterized by the enhanced production of extracellular matrix and accelerated proliferation. Cross-talk between parenchymal and nonparenchymal cells is one of the most important events in liver injury and fibrogenesis; ROS play an important role in fibrogenesis throughout increasing platelet-derived growth factor. Most hepatocellular carcinomas occur in cirrhotic livers, and the common mechanism for hepatocarcinogenesis is chronic inflammation associated with severe oxidative stress; other risk factors are dietary aflatoxin B_1 consumption, cigarette smoking, and heavy drinking. Ischemia–reperfusion injury affects directly on hepatocyte viability, particularly during transplantation and hepatic surgery; ischemia activates Kupffer cells which are the main source of ROS during the reperfusion period. The toxic action mechanism of paracetamol is focused on metabolic activation of the drug, depletion of glutathione, and covalent binding of the reactive metabolite *N*-acetyl-*p*-benzoquinone imine to cellular proteins as the main cause of hepatic cell death; intracellular steps critical for cell death include mitochondrial dysfunction and, importantly, the formation of ROS and peroxynitrite. Infection with hepatitis C is associated with increased levels of ROS/RNS and decreased antioxidant levels. As a consequence, antioxidants have been proposed as an adjunct therapy for various liver diseases.

Keywords Oxidative stress · Liver damage · Liver injury · ROS · RNS · Cancer · Fibrosis · Paracetamol · HCV

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Introduction

Oxygen toxicity

Oxygen is lethal to mammals within a few days when dioxygen is inhaled at 1 atm, whereas survival time at 5 atm is approximately 1 h. Oxygen toxicity is associated with the capacity of this molecule to oxidize organic molecules and to produce free radical species according to the general reactions:

$$RH_2 + O_2 \rightarrow RH^{\bullet -} + \text{(1-electron transfer)}$$

$$RH_2 + O_2 \rightarrow R + H_2O_2 \text{ (2-electron transfer)}$$

For these reactions to occur at significant rates, transition metal catalysts are required.

Properties of free radicals

All molecules have electrons as their outermost components. The behavior of these electrons determines the properties of the molecule. Modern quantum-mechanical theory describes electrons as having an intrinsic tendency to spin, thereby generating an electromagnetic field, the effect of which can be canceled by a similar charge spinning in the opposite direction. Thus, the most stable configuration of

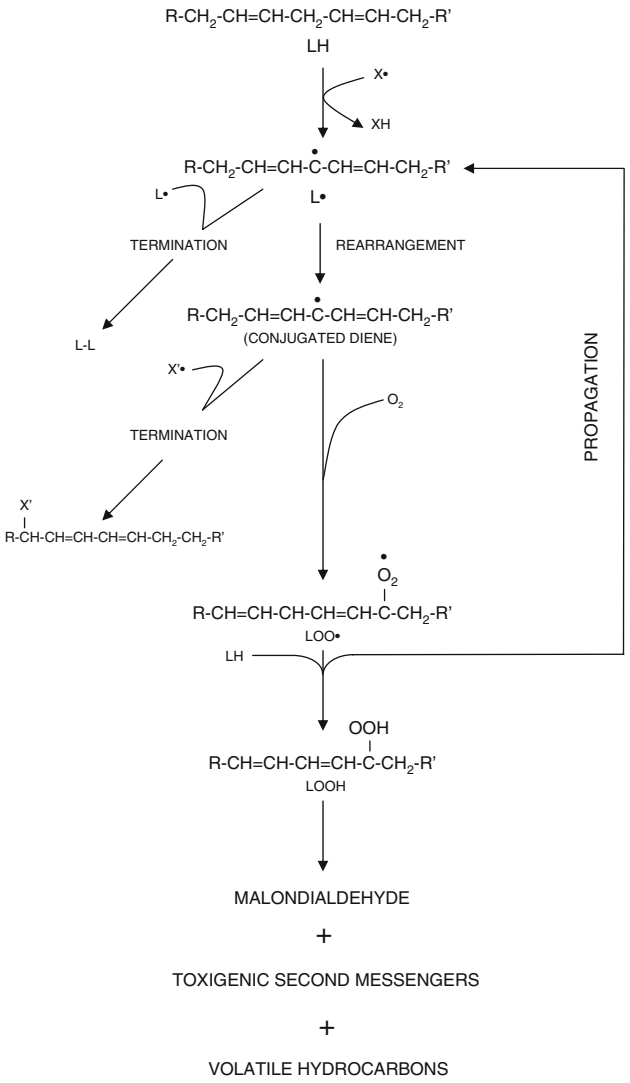

Fig. 1 Lipid peroxidation (LPO). X′ and X′• are free radicals, causing initiation and termination of the LPO sequence, respectively. L′•, lipid radical; LOO•, lipid peroxide; LOOH, lipid hydroperoxide

electrons is a paired one in which each member has opposite spins. Given this requirement for pairing, any situation in which species are generated with an unpaired electron will result in a potentially reactive entity known as a free radical. Therefore, a stable molecule contains an even number of electrons and a free radical is formed by gaining or losing one or more electrons. In order to have significant activity as a free radical, a molecule must have an unpaired electron and sufficient redox potential to be reactive. Free radicals can be generated in biological systems through a variety of processes. A major question in free radical biology is what they do after they have been formed.

Polyunsaturated lipids are essential to the entire supporting system of cells, including cell membranes, endoplasmic reticulum, and mitochondria. Disruption of their structural properties can, therefore, have dire consequences for cellular function. Peroxidation of lipids has traditionally been a major effect of free radicals. As a result of this, many of the assay methods to establish free radical-induced injury have measured by-products of the reaction of these molecules with lipids (Fig. 1).

Reactive oxygen and nitrogen species (ROS and RNS, respectively; Fig. 2) are produced by normal cellular metabolism with beneficial effects such as cytotoxicity against bacteria and other pathogens. In fact, there are enzymes whose functions are to produce ROS/RNS, such as nicotinamide adenine dinucleotide phosphate (NAD(P)H) oxidases, nitric oxide synthases (NOS), and myeloperoxidases. Since these free radicals may also damage normal tissue, the balance between antioxidants and prooxidants is critical for normal function. An imbalance favoring prooxidants is defined as oxidative stress. Oxidative stress is proposed to be critical in various diseases including liver diseases.

Alcoholic liver disease and free radicals

The World Health Organization has reported recently that alcohol-related diseases are the third cause of death and disability in most developed countries and are one of the leading causes in several of the developing countries of Central and South America, Eastern Europe, and East Asia [1]. It is interesting to note that the pharmacological treatment of alcohol liver disease is associated with free radicals.

Di Luzio [2] in 1966 was the first to observe lipid peroxidation after alcohol exposure; this was confirmed by other researchers [3]. The capacity of ethanol to increase both ROS/RNS and peroxidation of lipids, DNA, and proteins was demonstrated in a variety of systems, cells, species, including humans (Fig. 3). A lot was learned about alcohol metabolism, the various pathways and enzymes involved, and how alcohol directly via its solvent action affects cellular membranes or indirectly via its metabolism alters cell function. A major mechanism is lipid peroxidation and oxidative stress in alcohol toxicity. Several routes have been suggested to play a key role in the mechanism of alcohol-induced oxidative stress [4, 5]. It is likely that many systems contribute to the ability of alcohol to induce oxidative stress.

There are several studies that show that antioxidants administration or chelators of iron or reduced glutathione (GSH)-replenishing agents can ameliorate or prevent the toxic effects of ethanol. In the intragastric infusion of ethanol, liver damage was associated with enhanced lipid peroxidation, formation of 1-hydroxyethyl radical, decreased formation of protein carbonyl in GSH, and formation of lipid radicals [6–10]. Replacement of polyunsaturated lipids

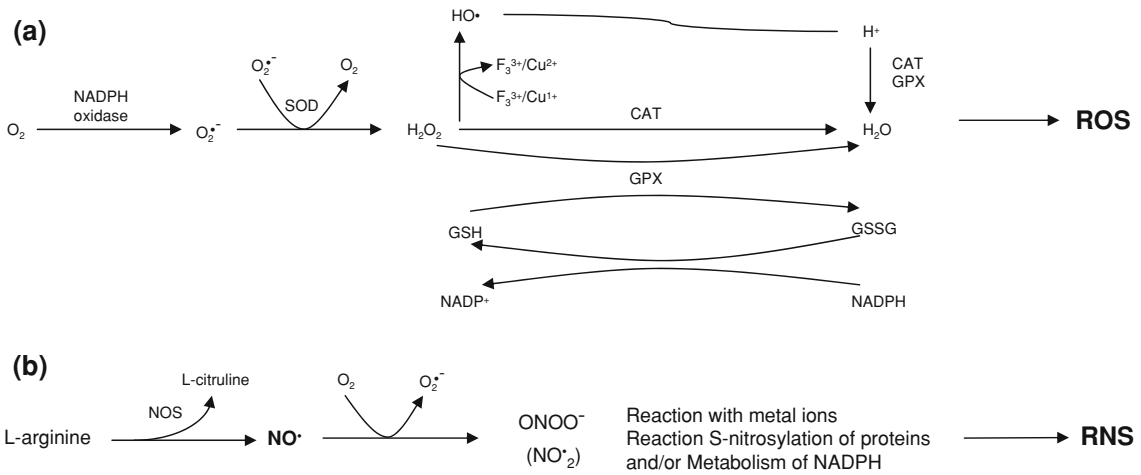

Fig. 2 **a** Main pathways for the formation of reactive oxygen species (ROS). **b** The three major mechanisms of reactive nitrogen species (RNS). CAT, catalase; GPX, glutathione peroxidase; NOS, nitric oxide synthase; $NO^{\bullet}$, nitric oxide; $ONOO^{-}$, peroxinitrite anion; $O_2^{\bullet -}$, superoxide anion

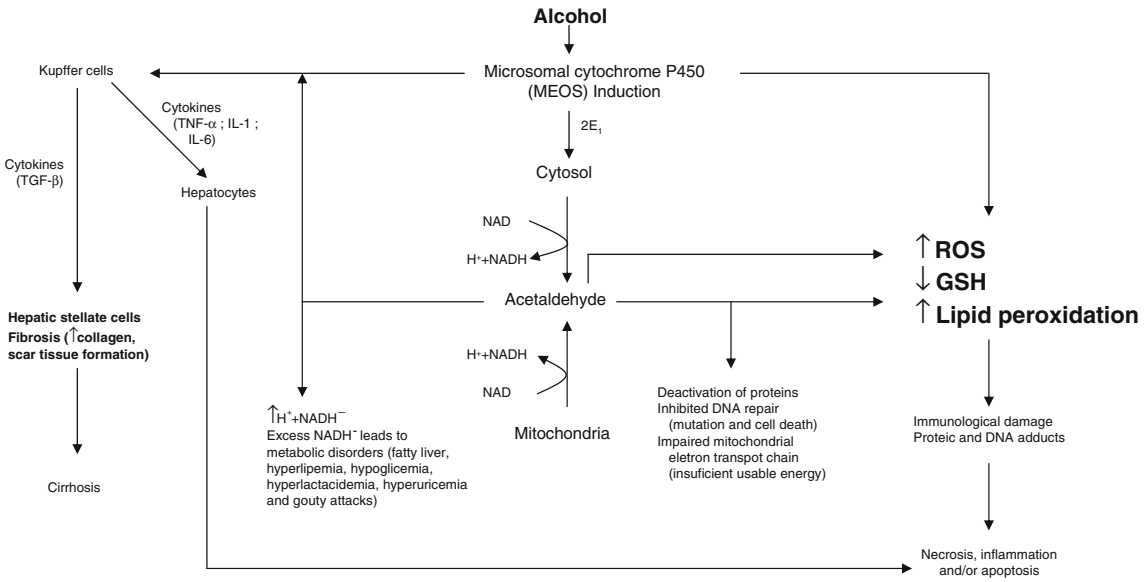

Fig. 3 Effect of alcohol that exacerbates some of the toxic effects of acetaldehyde and generates a harmful condition called oxidative stress in the cells, characterized by excess levels of reactive oxygen species (ROS)

(necessary for peroxidation of lipids to occur) with medium-chain triglycerides or saturated lipids in the diets fed to rats intragastrically prevented or lowered the peroxidation of lipids and the alcohol-induced hepatic damage [9, 11]. Therefore, polyunsaturated lipids plus alcohol are needed for the production of liver damage. Iron is known both to produce $^{\bullet}OH$ and to induce liver damage; importantly, addition of iron to these diets exacerbated hepatic damage [12]. Interestingly, administration of antioxidants, such as ebselen, vitamin E, superoxide dismutase (SOD), and precursors of GSH prevented alcohol-induced hepatic damage in rats [8]. Since ethanol-induced liver injury was linked to oxidative stress, Cederbaum and co-workers [13, 14] investigated the effect of a compromised antioxidant defense system, copper–zinc SOD1 knockout mice, in an alcohol-induced hepatic damage model. A moderate ethanol consumption induced oxidative stress and liver damage in these mice, indicating that compromised antioxidant defense exacerbates alcohol liver damage.

The previous in vivo studies were confirmed by in vitro studies with hepatocytes. Studies with isolated hepatocytes from long-term ethanol-fed rats and corresponding controls showed that ethanol metabolism via alcohol dehydrogenase leads to increased ROS production, hepatocyte damage, and apoptosis; these reactions were prevented by antioxidants [15, 16]. Studies with HepG2 cell lines expressing CYP2E1 indicated that addition of iron, polyunsaturated fatty acids, or ethanol or depletion of GSH resulted in

hepatocytes toxicity, increased oxidative stress, and mitochondrial injury, events blocked by antioxidants [17]. CYP2E1 plays an important role in ethanol-induced oxidant stress—topic reviewed in depth recently by Lu and Cederbaum [10].

The role of free radicals in alcohol-induced hepatic injury and the capacity of ethanol to promote oxidative stress are important areas of research, in particular, because such information may possess very important therapeutic significance to prevent the hepatotoxic effects of ethanol by antioxidants, inhibitors of CYP2E1, iron chelators, or GSH replenishment among others.

Fibrosis/cirrhosis and free radicals

Liver fibrosis is the result of an exacerbated wound-healing process after chronic hepatic damage and is characterized by the activation of hepatic stellate cells (HSC) and excess production of extracellular matrix (ECM) components by these cells. The activation of HSC involves the transdifferentiation from a quiescent state into myofibroblast-like cells. The activated HSC are characterized by the enhanced production of ECM and accelerated proliferation.

Hepatic stellate cells

The embryologic origin of stellate cells has been elusive. Currently, the bulk of evidence supports their origin from either the endoderm or the septum transversum, as it forms from cardiac mesenchyme during invagination of the hepatic bud [18]. A separate issue pertains to whether stellate cells and sinusoidal endothelial cells derive from a common precursor cell, a likely possibility given their shared mesenchymal phenotype, close proximity in situ, and joint expression of some angiogenic factors, for example, vascular endothelial cell growth factor [19].

The source of activated stellate cells and myofibroblast in liver injury has provoked extensive study and debate [20], specially the notion that bone marrow contributes a substantial fraction of these cells.

ROS generated within cells or, more generally, in a tissue environment may easily turn into a source of cell and tissue injury. ROS and other oxidative stress-related intermediates contribute to death, the perpetuation of chronic inflammatory responses, fibrogenesis, with a major focus on hepatic chronic wound healing and liver fibrogenesis [21, 22].

Cross-talks between parenchymal and nonparenchymal cells are the most important event in liver injury and fibrogenesis. Soluble factors such as cytokines [23] and ROS are the most important factors in these cross-talks and are possible targets for therapeutic consideration.

ROS are involved in necrosis and apoptosis of hepatocytes and HSC activation [24, 25]. Several major classes of free radical scavengers, such as catalase, superoxide SOD, and glutathione peroxidase (GSH-P), were investigated in various types of liver damage, and they afforded effective protection against the oxidative insults to hepatic parenchyma [26].

High levels of ROS, from phagocytic cells, such as Kupffer cells (KC), protect the organism from external pathogens; however, lower amounts of ROS mainly from HSC actively participate in the regulation of intracellular signaling [25, 27]. Platelet-derived growth factor (PDGF) is the most potent mitogen of HSC and is, therefore, likely to be an important mediator during liver fibrogenesis [28]. Interestingly, NAD(P)H is expressed in HSC and produce ROS, which, in turn, induces the production of PDGF; again, this molecule increases mitosis of HSC [27]. These results strongly suggest that ROS play an important role in fibrogenesis increasing PDGF throughout.

Hepatocellular carcinoma and free radicals

Hepatocellular carcinoma (HCC) is one of the most malignant and frequent worldwide spreading diseases; it is the third most common cause of cancer deaths [29, 30]. This disease occurrence is increasing in developed Western countries such as the United States, with an incidence ratio of 2.8 and 6.2 (whiteand African American, respectively) per 100,000 habitants [31]; it is endemic in Korea, Taiwan, China, and sub-Saharan Africa [32]. The major risk factors for HCC are chronic hepatitis B and C viruses (HBV and HCV), accounting for 80% of HCC cases; other risk factors are dietary aflatoxin B_1 consumption, cigarette smoking, and heavy drinking [33].

Most HCC occur in cirrhotic livers, and the common mechanism for hepatocarcinogenesis is chronic inflammation associated with severe oxidative stress [34]. There is a large body of evidence indicating that ROS play a pathogenetic role in carcinogenesis [35]. During the initiation phase of this process, ROS may interact directly with DNA, damaging specific genes that control cell growth and differentiation, among others [36]. They can also increase the activity of carcinogenic xenobiotics by facilitating their activation to reactive compounds [37]. During the progression phase of carcinogenesis, ROS can directly stimulate the growth of cancer cells [38]. The hydroxyl radical is, among all the ROS produced during the inflammation phase, the most damaging; it has been proved that it is responsible for a number of base modifications, including the formation of thymine and thymidine glycol, 8-hydroxydeoxyguanosine, and 5-hydroxylmethyluracil [35]. 8-Hydroxydeoxyguanosine is a guanine modification that

induces a point mutation in the daughter DNA strand and is commonly used as an indicator of DNA damage [39].

Chronic alcohol exposure elicits hepatocyte hyperegeneration due to the activation of survival factors and interference with retinoid metabolism. Direct DNA damage results from acetaldehyde, which can bind to DNA, inhibit DNA repair systems, and lead to the formation of carcinogenic exocyclic DNA ethenoadducts. Long-term alcohol abuse also interferes with the methyl group transfer and may alter gene expression [40].

The network linking HCV infection, inflammation, free radical production, and carcinogenesis applies very well to HCV-mediated chronic liver damage, just as it applies to any chronic inflammatory condition [41]. Research into the role of structural and nonstructural proteins of HCV and the changes induced in cytokine expression oncogenes, antioncogenes, and intracellular kinases shows that HCV is by itself and not only through inflammation able to induce ROS, an effect specific to this virus [42]. This free radical production, accompanied by oxidative genomic injury, constitutes the first step of a cascade of genomic and postgenomic events that play an important role in HCC [43]. More information is necessary from recently introduced technologies for proteomics that will hopefully close the gap between hypothesis and understanding.

Ischemia/reperfusion liver injury and free radicals

Interruption of blood flow of an organ with its subsequent lack of nutrient and oxygen supply is an inherent effect during various surgical processes. In hepatic surgery, there are situations in which the ischemic periods can be very long; this is the case during the resection of large hepatic tumors, vascular reconstructions, management of hepatic trauma from various origins, and hepatic procedure for transplantation [44, 45]. When the flow of oxygen and blood is re-established, reperfusion increases the damage induced during the ischemic period, worsening the injury produced at the cellular level [44, 46]. This process called ischemia–reperfusion (IR) injury affects directly on hepatocyte viability, particularly during transplantation and hepatic surgery [45, 47]. In the ischemic period, various modifications occur at the cellular level, which promote cell injury. A decline in oxidative phosphorylation leads to ATP depletion and loss of calcium homeostasis [48].

The detrimental effects of ATP catabolism are reinforced by the production of various compounds, including cytokines vasoactive agents, and specially ROS. These effects are associated by a decline in cytoprotective compounds such as prostacycline, nitric oxide, and others [49]. Liver cell death occurs due to both apoptosis and necrosis [50].

Aerobic metabolism produces ROS that are normally inactivated through diverse antioxidant mechanisms. However, during oxidative stress conditions, the balance between ROS and antioxidants shifts toward the former, resulting in liver damage [51].

Some of the process that participate both directly and indirectly in IR injury by ROS include the formation of xantine oxidase from xantine dehydrogenase (an oxygen-dependent process that releases ROS, hydrogen peroxide, and superoxide and produces uric acid) [52], induction of NADPH oxidase by activated KC and neutrophils (ROS production is blocked when NADPH oxidase is inhibited), and NO formation and its conversion to peroxynitrite (both are RNS) [53]. The cytotoxic effects of ROS and RNS in the liver translate into tyrosine residues, lipid peroxidation, inactivation of the heme group, and nitrosylation of iron–sulfur group [44, 53].

Strong evidence indicates that KC (the resident macrophages of the liver) may cause hepatic injury in various disease processes, including cold [54] and warm [55] ischemic injury. Ischemia activates KC, which are the main source of ROS during the reperfusion period [53]. Various studies show that newly recruited nonocytes and leukocytes are partially responsible for the ischemic damage. They play an important role in the synthesis of ROS such as superoxide and hydrogen species [55]. In hepatocytes, proinflammatory cytokines, such as TNF-α, IL-1, or interferon-γ, can induce the production of ROS [56]. In addition, ischemic cell damage leads to intracellular oxidant stress during reoxygenation [57]. Mitochondria are recognized as the major intracellular source of ROS that are produced by cellular respiration [57].

Since antioxidants can inhibit ROS, various studies have aimed at modulating the severity of IR damage utilizing different mechanisms, including pharmacological allopurinol [58, 59], α-tocopherol [60], *N*-acethylcysteine [61], and enzymatic catalase [62, 63] and SOD [57]. Endogenous antioxidant concentrations decrease significantly during reperfusion [60, 64]; thus, administration of antioxidants, especially in the early stages of reperfusion, may significantly diminish IR injury in transplanted livers.

Genetic, pharmacological, and surgical approaches to decrease liver IR damage have been applied and are increasingly being used. Therapeutic approaches include ischemic preconditioning and the pharmacological treatment with *N*-acetylcysteine, prostaglandins, and prostacycline [44].

Paracetamol-induced liver damage and free radicals

Paracetamol (acetaminophen; *N*-acetyl-*p*-aminophenol [APAP]) is a safe and effective analgesic and antipyretic

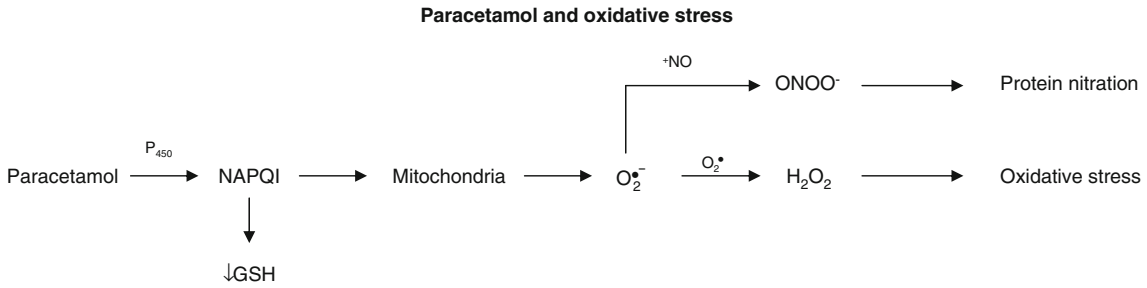

Fig. 4 Schematic representation depicting the role of $O_2^{\bullet}$ and $NO^{\bullet}$ in paracetamol toxicity

drug when used at therapeutic doses. However, an overdose can induce severe liver injury both in experimental animals and in humans [65]. In the past, researchers studying the toxic action mechanism of APAP focused on metabolic activation of the drug, depletion of glutathione, and covalent binding of the reactive metabolite *N*-acetyl-*p*-benzoquinone imine (NAPQI) to cellular proteins as the main cause of hepatic cell death [66]. More recently, it was discovered that covalent binding is not sufficient to kill liver cells but is a signal for the toxicity that requires amplification in the cell [67]. Intracellular steps critical for cell death include mitochondrial dysfunction and, importantly, the formation of ROS and peroxynitrite (Fig. 4). Oxidant stress of mitochondria triggers the mitochondrial membrane permeability transition pore, loss of the membrane potential of the mitochondria, depletion of ATP, and release of intermembrane proteins that are responsible for the typical nuclear DNA fragmentation of APAP-induced cell death [67]. We have found that antioxidants, such as silymarin [68], protect the liver of rats intoxicated with APAP [69]. Reduced glutathione can effectively protect the liver both by scavenging NAPQI and by detoxifying ROS and RNS, such as peroxynitrite. This mechanism is the basis for the rational clinical use of *N*-acetylcysteine, a GSH precursor, as antidote against APAP toxicity [70].

Viral hepatitis and free radicals

Infection with HCV is associated with increased levels of ROS/RNS and decreased antioxidant levels in patients [71–74]. Patients infected with HCV show increases in lipid peroxidation levels in liver samples, serum, and peripheral blood mononuclear cells [72, 75–80]. In addition, other indicators of oxidative stress such as 4-hydroxynonenal and 8-hydroxydeoxyguanosine were found to be increased in HCV [72, 74, 80–83]. The content of GSH decreased in the blood, liver, and lymphatic system, whereas that of GSSG increased, indicating a high glutathione turnover [83].

The presence of ROS and RNS is, interestingly, more pronounced with HCV than with HBV [75]. The mechanisms for more severe increases of oxidative and nitrosative stresses during HCV disease may include chronic inflammation (i.e., phagocytic NAD(P)H oxidase activation) and overload of iron, which is more specific to HCV [72, 75, 84]. Furthermore, the production of ROS in the hepatocytes may lead to the activation of KC [85]. These cells, when activated, produce and secrete cytokines; cytokines may be proinflammatory, such as TNF-α and IL-1, or profibrotic, such as TGF-β. These proteins can further increase ROS and play important roles in the mediation of hepatic injury [23, 85–87], such as fatty liver, by inhibiting lipase of lipoprotein and adiponectin and fibrosis as a result of HSC activation [88–90].

In addition, proteins of HCV can increase ROS and RNS in the infected cells; proteins of the HCV core have been shown to augment the oxidative and nitrosative stress, lipid peroxidation, oxidized thioredoxin, and antioxidant gene expression such as that of metallothionein family proteins and manganese superoxide dismutase (MnSOD) as well as to enhance sensitivity to toxins such as ethanol and CCl_4 [81, 91–95]. HCV core gene expression diminishes the intracellular GSH levels and the mitochondrial NADPH content that are associated with increased uptake of calcium and oxidative stress generation at complex I in mitochondria, providing an action mechanism for HCV-induced ROS production [42, 91, 92, 96]. On the contrary, core protein modulates the production of cytokines and host enzymes, such as cyclooxygenase-2 and inducible nitric oxide synthase (iNOS), which can increase ROS and RNS [97–103].

Nonstructural proteins may also modulate the host redox status by HCV. Host antioxidant defenses, such as GSH, catalase, MnSOD, and heme oxygenase-1, are augmented, suggesting adaptation to ROS/RNS stress [92, 104, 105].

Stress produced by ROS/RNS has been implicated in HCV-induced hepatic cancer. HCV core-induced iNOS generates RNS, which may cause damage to the DNA, and augments mutations within the immunoglobulin and tumor

suppressor genes [103, 106]. The genotoxic effects of ROS/RNS may contribute to the development of B-cell lymphoma or HCC during HCV infection. In fact, this association was documented in vivo in HCV core-transgenic mice [42, 107]. Other mechanisms by which core protein increases HCC include the modulation of tumor suppressor genes and proto-oncogenes as well as the inhibition of apoptosis [83]. In this regard, it should be noted that oxidative and nitrosative stress may possess diverse effects on cell growth and apoptosis [108]. As a consequence, antioxidants have been proposed as an adjunct therapy for chronic hepatitis C [109].

Nonalcoholic fatty liver and free radicals

Oxidative stress in nonalcoholic steatohepatitis (NASH) may be associated with potential etiologic mechanism. Three factors have been proposed: lipid peroxidation, hepatic iron, and hyperinsulinemia.

Lipid peroxidation

Increased lipid peroxidation was demonstrated in both animal models of fatty liver [110–112] and patients with nonalcoholic fatty liver diseases (NAFLD) [113–117]. NASH patients have increased levels of oxidative stress as compared with patients with steatosis alone [113, 115].

In these patients, free fatty acids (FFA) are the likely source of oxidative stress. Patients with NAFLD show increased lipolysis and augmented delivery of FFA to the liver [113, 118], the concentration of which are associated with more severe liver disease [119]. Elevated FFA in the liver [120] act as ligands for the transcription factor PPAR-α, which upregulates the oxidation of FFA inside the mitochondria, microsomes, and peroxisomes [121]. The FFA oxidation products (lipid peroxides and superoxide and hydrogen peroxide radicals) can generate oxidative stress and subsequent lipid peroxidation.

Insulin

Increased insulin is a frequently occurring finding in NAFLD; however, it is frequently overlooked in its pathophysiology. Insulin can damage the liver directly and indirectly [122, 123]. Patients with long-term ambulatory dialysis develop fatty liver, but only when insulin is added to the peritoneal fluid dialysate [124–126]. The steatosis is seen only at the surface of the liver and sometimes has the histological appearance of NASH [124]. This direct effect may be due to the ability of insulin to produce ROS [123]. In addition, insulin seems to posses direct profibrogenic effects by stimulating connective tissue growth factor, especially in the presence of hyperglycemia [124]. This may explain the observation that patients with NAFLD and type 2 diabetes mellitus have a very poor prognosis [127–129]. Evidence [130] indicates that insulin may be directly involved in causing endoplasmic reticulum stress along with the unfolding protein response and apoptosis. This may exacerbate insulin resistance [131].

Iron

Some evidence [132, 133] suggests that iron is important in inducing ROS and lipid peroxidation. However, most studies do not [134–138]. On the contrary, 30% of the patients with NAFLD have elevated ferritin levels [139–141], and there is an association between insulin resistance and liver iron [142, 143]. Therefore, it sounds rationale that iron causes oxidative stress because it is a well-known prooxidant and possesses negative effects upon the mitochondria [144, 145]. However, ongoing additional studies at present indicate that iron is likely to be important in only a minority of patients with NAFLD.

Conclusions

Reactive oxygen and nitrogen species are involved in liver damage induced by several conditions such as alcohol abuse, fibrosis/cirrhosis of various etiologies, HCC, IR liver injury, paracetamol overdose, and viral hepatitis. Oxygen and NO radicals may affect the energetic, respiratory, and regenerative pathways in hepatocytes. The imbalance of proinflammatory/anti-inflammatory cytokine in immune and inflammatory cells, the expression of collagen genes, and angiogenesis in endothelial and stellate cells aggravates the disease. On the basis of these facts, antioxidant therapy alone or in combination with other pharmacological strategies appears as the most reasonable treatment of a variety of liver diseases.

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