Chapter 10
Role of Oxidative Stress in Alcoholic/Non-Alcoholic Liver Diseases

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Abstract Oxidative stress is the shift in the balance between oxidants and antioxidants in favor of oxidants. Reactive oxygen species (ROS) play a central role in inducing oxidative stress. Mitochondria are the main site of cellular ROS production, and simultaneously have a well-organized antioxidant system. Therefore, mitochondria have evolved multiple systems of quality control to ensure that the requisite number of functional mitochondria is present to meet the demands of the cell. The liver also is the major iron storage organ in the body and therefore mild to moderate degrees of hepatic iron accumulation are sometimes involved in chronic liver diseases. Iron overload, especially excess divalent iron can be highly toxic, mainly via the Fenton reaction producing hydroxyl radicals. The liver is often a target of injury by oxidative stress. Oxidative stress has been shown to be present in alcoholic liver diseases, non-alcoholic steatohepatitis, and chronic hepatitis C to a greater degree than in other inflammatory liver diseases. This chapter highlights iron overload in the liver and mitochondrial ROS production through reduced mitochondrial quality control as important causative factors for inducing oxidative stress in chronic liver diseases, especially focusing on alcoholic liver disease, non-alcoholic steatohepatitis, and chronic hepatitis C.

Keywords Reactive oxygen species · Iron · Mitochondria · Mitochondria quality control · Non-alcoholic steatohepatitis · Chronic hepatitis C

10.1 Introduction

Reactive oxygen species (ROS) are produced by living organisms as a result of normal cellular metabolism and environmental factors. ROS are highly reactive molecules and can damage cell structures such as carbohydrates, nucleic acids,

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lipids, and proteins and alter their functions. However, since the body is able to remove ROS to a certain degree, these reactive species are not necessarily a threat to the body under physiological conditions [1, 2]. ROS are required at a certain level in the body to perform its important physiological functions. The shift in the balance between oxidants and antioxidants in favor of oxidants is termed "oxidative stress." Oxidative stress contributes to many pathological conditions and diseases. The liver is often a target of injury by oxidative stress. Many risk factors, including alcohol, drugs, environmental pollutants, and irradiation, may induce oxidative stress in the liver, which in turn results in severe liver diseases. Oxidative stress has been shown to be present in alcoholic liver disease, non-alcoholic steatohepatitis (NASH), and chronic hepatitis C to a greater degree than in other inflammatory liver diseases. Interestingly, these diseases have iron overload and mitochondrial injury in the liver in common. This chapter will review and discuss the role of oxidative stress in liver diseases, especially focusing on hepatic iron overload and mitochondrial ROS production.

10.2 Oxidative Stress in the Liver

In mammals, an organized antioxidant system has developed to maintain the redox homeostasis in the liver. Both enzymatic and non-enzymatic antioxidant systems are essential for cellular responses in order to deal with oxidative stress under physiological conditions. Antioxidant enzymes such as catalase, superoxide dismutase (SOD), and glutathione peroxidase and non-enzymatic electron receptors such as glutathione (GSH) are affected and used as indexes to evaluate the level of oxidative stress [3]. Erythroid 2-related factor 2 (Nrf2) is a major regulator of cellular redox balance [3, 4]. Nrf2 physiologically binds to kelch-like ECH-associated protein-1 (Keap1) in the cytoplasm, and is inactivated and easily degraded. Under oxidative stress Nrf2 dissociates from Keap1 via Keap1 modification or Nrf2 phosphorylation and is activated. The activated Nrf2 translocates into the nucleus and interacts with the antioxidant response element, promoting the expression of cytoprotective target genes, including antioxidant enzymes and phase II detoxifying enzymes [3, 5–7].

When there are excessive ROS, the homeostasis is disturbed, resulting in oxidative stress, which plays a critical role in liver diseases and other chronic and degenerative disorders. This oxidative stress triggers hepatic damage by inducing alterations of lipids, proteins, and DNA contents and modulating pathways that control normal biological functions. Since these pathways regulate the transcription of genes, protein expression, cell apoptosis, and hepatic stellate cell activation, oxidative stress is considered to be a pathological mechanism that results in the initiation and progression of various liver diseases, such as alcoholic liver disease, NASH, and chronic viral hepatitis [3].

10.3 Iron and Oxidative Stress in Liver Diseases

The liver is the major iron storage organ in the body and therefore mild to moderate degrees of hepatic iron accumulation are sometimes involved in chronic liver diseases [8–12]. Iron overload, especially excess divalent iron can be highly toxic, mainly via the Fenton reaction producing hydroxyl radicals [13]. This is particularly relevant for liver diseases with mild to moderate iron overloaded such as alcoholic liver disease, NASH, and chronic hepatitis C, in which oxidative stress has been proposed to be a major mechanism of liver injury. Oxidative stress and increased iron levels strongly favor DNA damage, genetic instability, and tumorigenesis. Indeed, a significant correlation between 8-hydroxy-2′-deoxyguanosine (8-OHdG), a marker of oxidatively generated DNA damage [14] and hepatic iron excess has been shown in iron overloaded liver diseases.

10.3.1 Iron Overload in Alcohol Liver Disease

Patients with alcoholic liver disease frequently exhibit iron overload in association with increased hepatic fibrosis. Even moderate alcohol consumption elevates body iron stores. Hepcidin, a 25 amino-acid peptide synthesized in the liver, is a key mediator of iron metabolism, and acts to attenuate both intestinal iron absorption and iron release from reticuloendothelial macrophages [15, 16]. As one of the mechanisms underlying alcohol-induced iron overload, alcohol metabolism-mediated oxidative stress has been shown to regulate hepcidin transcription via a transcription factor, CCAAT/enhancer-binding protein α (C/EBPα), which in turn leads to increased duodenal iron transport [17].

When hepatocytes accumulates excess iron in clinical alcohol abuse or in an experimental model of combined iron and alcohol hepatotoxity, there is evidence for synergy among the putative pathways of oxidative stress. How excess hepatocytic iron accumulates in alcoholic excess is unknown, but when the usual safe harbor for intracellular iron, namely the endosomal-lysosomal compartment, is compromised, it becomes a potent source of free, chelatable pro-oxidant iron. In this regard excess iron in alcohol-induced liver damage and alcohol excess in iron overload disease are powerful cocktails promoting subcellular organelle damage leading to cell death and fibrogenesis [18].

10.3.2 Iron Overload in NASH

Non-alcoholic fatty liver disease (NAFLD) is present in 10–30% of the world's population. A recent large cohort study showed that 35% of subjects enrolled in the NASH Clinical Research Network had stainable hepatic iron [19]. In animal models

of fatty liver, iron loading is associated with the development of hepatic inflammation and fibrosis [20]. Early reports on the association of hepatic iron with NAFLD and NASH were controversial. However, more recent studies have strongly suggested a causative role for iron in the development of NASH, demonstrating that increased hepatic iron contributes to progression of NAFLD [9, 21]. In addition to the induction of oxidative stress, iron has been implicated as a cofactor in the pathogenesis of insulin resistance, which is universal among individuals with NAFLD and is implicated in the progression of liver injury [22]. Iron also appears to be a risk factor for the development of HCC in patients with NASH. Iron overload in patients with NASH-related cirrhosis is reported to be potentially associated with HCC development [23].

The precise mechanisms by which some patients with NASH are prone to hepatic iron accumulation remain elusive. However, several mechanisms have been proposed (Table 10.1). Aigner et al. suggested an impaired release of iron from liver cells as an underlying mechanism for iron accumulation in NAFLD [32]. They found down-regulation of the *ferroportin-1* and *hemojuvelin* (*Hjv*) genes, probably due to an increase in tumor necrosis-alpha (TFN-α). This also explains the characteristic pattern of iron deposition in NAFLD, which is different from the pattern seen in hereditary hemochromatosis; i.e., hepatic and sinusoidal deposition without a zonal gradient. Transgenic mice expressing the retinoic acid receptor alpha-dominant

Table 10.1 Proposed mechanisms by which hepatic iron accumulates in NAFLD/NASH

Factor(s) related to iron metabolic disorders	Intermediary molecules	Molecules responsible for iron accumulation	Affected pathway in iron transport	References
Increased TNF-α[a] production	Hemojuvelin	Ferroportin-1	Iron release from hepatocytes and Kupffer cells	[24]
Impaired retinoic acid signaling	Hemojuvelin, TfR2[b]	Ferroportin-1	Iron release from hepatocytes and Kupffer cells	[25, 26]
Erythrocytes		Phosphatidylserine	Erythrocyte phagocytosis by Kupffer cells	[27]
Undetermined humoral factor(s)	IRP1	Dmt1[c]	Duodenal iron absorption	[28]
Copper deficiency	Ceruloplasmin	Ferroportin-1	Iron release from hepatocytes and Kupffer cells	[29]
ROS[d] production	Ceruloplasmin	Ferroportin-1	Iron release from hepatocytes and Kupffer cells	[30]

Adopted from Table 7.1 in "The Liver in Systemic Diseases" edited by Ohira H [31]
[a]Tumor necrosis factor alpha
[b]Transferrin receptor 2
[c]Divalent metal transporter
[d]Reactive oxygen species

negative form in hepatocytes develop steatohepatitis and liver tumors [24]. Hepatic iron accumulates in these mice, and retinoid treatment decreases hepatic iron content through suppression of Hjv expression [25]. These results suggest that impaired retinoic acid function is responsible for hepatic iron accumulation in NASH. Otogawa et al. indicated that the engulfment of phosphatidylserine-externalized, apoptotic signal-positive erythrocytes by hepatic macrophages might lead to the accumulation of iron derived from hemoglobin in the liver of NASH [26]. Interestingly, a recent study has demonstrated that duodenal iron absorption increases through upregulation of Dmt1, regardless of elevation of the serum Hepcidin level in patients with NASH [27]. Undetermined humoral factor(s) contained in sera of NASH patients activated IRP1, which subsequently up-regulates Dmt1 expression through the IRP/IRE system [27].

Iron accumulation may be linked to copper homeostasis. One study reported that copper status was linked to iron homeostasis in NAFLD, suggesting that low copper bioavailability causes increased hepatic iron stores via decreased ferroportin-1 expression and ceruloplasmin ferroxidase activity, thus blocking liver iron export in copper-deficient subjects [28]. In addition, oxidative stress/ROS in hepatic cells has been demonstrated to down-regulate ceruloplasmin via a novel mRNA decay mechanism that may contribute to hepatic iron accumulation by decreasing hepatic iron release [29].

10.3.3 Iron Overload in Chronic Hepatitis C

Based on the assumption that one-third of iron stores are normally in the liver, this would translate to a normal median hepatic iron content of 0.27 g for men and 0.13 g for women [30]. Extensive studies reported median hepatic iron concentrations of 396 [range: 0–2105] and 458 [range: 114–2190] μg/g dry weight liver tissue in patients with chronic hepatitis C [33, 34]. These results suggest that the hepatic iron content in patients with chronic hepatitis C is approximately 0.50–0.69 g, equivalent to 2–5 times the normal hepatic iron content if the liver weight is estimated to be 1500 g.

The role of *HFE* mutations in chronic hepatitis C has been well reviewed [35]. In general, patients with chronic hepatitis C seem to have no difference in the prevalence of heterozygosity for *HFE* mutations as compared with a control population. The levels of hepatic hepcidin mRNA and serum Hepcidin, that is, the 25 amino acid bioactive hepcidin, are reported to be lower in in patients with chronic hepatitis C than in those with chronic hepatitis B or controls despite a significant correlation between hepcidin and serum ferritin or the histological iron score in both groups [36, 37]. Thus, the relatively decreased synthesis of hepcidin in chronic hepatitis C contrasts with the absolute deficit or lack of hepcidin synthesis observed in hereditary hemochromatosis and may account for the mild to moderate hepatic iron overload observed in some patients with chronic hepatitis C. The mechanisms underlying hepatitis C virus (HCV)-related hepatic iron overload appear to have some similarities

with alcohol-induced iron overload in terms of disrupted hepcidin transcription through suppressed activity of C/EBPα due to ROS [17, 38].

Hepcidin is potentially regulated through the bone morphogenic protein (BMP)/sons of mothers against decapentaplegic (SMAD) cascade by both circulating transferrin-bound iron and intracellular iron stores in chronic hepatitis C. Taking into account the significant correlation between hepcidin expression and serum ferritin or the histological iron score [36, 37], hepcidin transcription seems to be properly regulated in response to the iron concentration in chronic hepatitis C. Thus, the opposing effects of HCV-induced hepcidin-suppressive factors and iron-load-induced hepcidin-stimulation factors potentially regulate hepcidin transcription in chronic hepatitis C. Inflammation also regulates hepcidin transcription. Proinflammatory cytokines such as IL-6 mediate this response by inducing transcription of hepcidin mRNA via signal transducer and activator of transcription (STAT)3, which binds to a STAT-responsive element within the hepcidin promoter [39]. Serum levels of IL-6 have been shown to be elevated in patients with HCV-related chronic liver disease [40], which raises the possibility that IL-6 acts to stimulate hepcidin expression through the STAT3 pathway. This would be expected to counteract the decrease in hepcidin transcription caused by HCV-induced ROS. On the other hand, chronic inflammation with production of proinflammatory cytokines has the potential to deliver an additional burden of ROS, which would be expected to reinforce the decrease in hepcidin transcription. Most likely, during chronic inflammation states in vivo like chronic hepatitis C, the regulation of hepcidin is more complex and may depend on many variables, including the particular stage of systemic and/or hepatic inflammatory disease. This might explain the variations in hepatic iron concentrations reported among patients with HCV-related chronic liver disease. The schematic outline in Fig. 10.1 depicts the assumed mechanisms underlying the hepatic iron accumulation in chronic hepatitis C.

10.4 Mitochondria-Derived Oxidative Stress

The mitochondrial electron transport system consists of several multipolypeptide protein complexes (I-V) embedded in the inner mitochondrial membrane that receive electrons from reducing equivalents (i.e., nicotinamide adenine dinucleotide [NADH] and flavin adenine dinucleotide [$FADH_2$]) generated by dehydrogenases (e.g., pyruvate dehydrogenase, α-ketoglutarate dehydrogenase, acyl-CoA dehydrogenase, etc.). These electrons flow through the complex I, the ubiquinone cycle (Q/QH_2), complex III, cytochrome c, complex IV, and to the final acceptor O_2 to form H_2O. Electron flow through complexes I, III, and IV results in the pumping of protons to the outer surface of the inner membrane, establishing a membrane potential that is used by adenosine triphosphate (ATP) synthetase to drive the rephosphorylation of ADP. Several of the redox couples within the electron transport chain transfer single rather than two electrons and are therefore susceptible to leaking electrons directly to surrounding O_2 to form the free-radical superoxide ($O_2^{\bullet -}$).

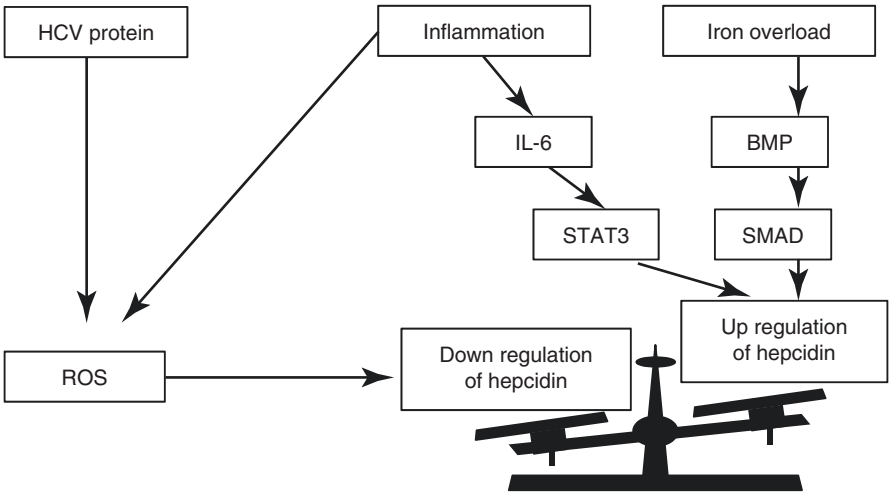

Fig. 10.1 Schematic diagram depicting the assumed mechanisms underlying the hepatic iron accumulation in patients with chronic hepatitis C. Hepcidin transcription in chronic hepatitis C may be potentially regulated by the opposing effects of HCV-related ROS-induced hepcidin suppression and iron load-induced hepcidin stimulation. Inflammation may also have the opposing effects of stimulation and suppression of hepcidin transcription through the IL-6/STAT pathway and ROS pathway, respectively. Consequent relative suppression of hepcidin expression is potentially one of the mechanisms underlying the hepatic iron accumulation in patients with chronic hepatitis C. *HCV* hepatitis C virus, *ROS* reactive oxygen species, *IL-6* interleukin 6, *STAT* signal transducer and activator of transcription, *BMP* bone morphogenic protein, *SMAD* sons of mothers against decapentaplegic. Adopted from Fig. 7.1 in "The Liver in Systemic Diseases" edited by Ohira H [31]

The detoxification of ROS is an important function of the cellular redox homeostasis system. Cells rapidly convert $O_2^{\bullet-}$ into the two-electron nonradical hydrogen peroxide (H_2O_2) via manganese SOD (MnSOD). H_2O_2 in turn can be further reduced to H_2O in the mitochondrial matrix by GSH or the thioredoxin/peroxiredoxin systems, or can freely diffuse out of the mitochondria where it again is buffered by GSH [41].

10.4.1 ROS Production in Alcoholic Liver Disease

Alcohol metabolism occurs mainly in the liver, and alcohol is metabolized via both oxidative and non-oxidative pathways. Oxidative pathways are the predominant mechanism for alcohol metabolism. The most common pathway for oxidative metabolism in the liver is characterized by alcohol dehydrogenase (ADH), which metabolizes alcohol into acetaldehyde. Alcohol can also be oxidized into acetaldehyde by cytochrome P450 2E1 (CYP2E1) and catalase. Acetaldehyde is further metabolized into acetate and acetyl-CoA for use in metabolic pathways by aldehyde

dehydrogenase (ALDH), which has two isoforms: cytosolic ALDH1 and mitochondrial ALDH2 [42]. The deleterious ethanol-mediated effects have been largely attributed to ethanol-induced oxidative stress and the subsequent damaging effects on mitochondria and other cellular compartments. ROS-producing proteins causing ethanol-mediated tissue injury include CYP2E1, inducible nitric oxide synthase (iNOS), nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, xanthine oxidase, and mitochondrial complexes [43]. CYP2E1 is indeed suggested to induce its damaging effects in the liver following ethanol exposure due to its ability to produce oxidative radicals such as hydrogen peroxide and superoxide anions. There are several lines of evidence to support the location of CYP2E1 not only in endoplasmic reticulum but also in the mitochondria in vivo and in vitro. Direct damage of mitochondrial DNA by oxidative radicals and/or post-translational protein modifications of many mitochondrial proteins largely contributes to the oxidative stress-mediated hepatic injury. Thus, CYP2E1 plays direct and/or permissive roles in promoting mitochondrial dysfunction and hepatotoxity.

10.4.2 ROS Production in NAFLD

In the setting of obesity and hepatic insulin resistance, the existing nutrient and hormonal milieu is altered, favoring increased hepatic triglyceride accumulation [44, 45]. In this environment, ectopic fat accumulation in the liver seems secondary to chronic free fatty acid overload from insulin-resistant, dysfunctional adipose tissue, together with higher rates of hepatic de novo lipogenesis [45], and is often associated with hepatic insulin resistance and hepatocyte death. Recent evidence suggests that continuous adaptation or remodeling of mitochondrial energetics, gene expression, morphology, and content play a key role in the pathogenesis of simple steatosis/NASH [46, 47]. Mitochondrial oxidative energetics encompasses multiple pathways that include β-oxidation, hepatic tricarboxylic acid (TCA) cycle, ketogenesis, respiratory chain activity, and ATP synthesis, all of which work in concert to maintain cellular homeostasis. These multiple pathways have been reported to be induced in several mouse models of nutritional overload, as well as in human subjects, when obesity and simple steatosis are present [48]. As generation of acetyl-CoA through β-oxidation and its terminal oxidation through the hepatic TCA cycle are major sources of energy generation, induction of TCA cycle flux could be obligatory for high energy-demanding processes (e.g., gluconeogenesis and lipogenesis) during states of substrate overload, including simple steatosis or NASH in obesity or type 2 diabetes mellitus. However, sustained induction of TCA cycle flux, and its uncoupling from mitochondrial respiration and ATP synthesis, may bring about the unwanted effect of fueling ROS generation and the development of tissue inflammation [48]. Indeed, defects in mitochondrial morphology, the electron transport chain, and ATP production have been documented in NAFLD along with high levels of ROS and other mediators of inflammation. Satapati et al. illustrated how a modest elevation

of free fatty acid delivery into normal liver was enough to upregulate the mitochondrial oxidative machinery [49]. During obesity and hepatic insulin resistance, chronic free fatty acid overload and the sustained induction of mitochondrial TCA cycle flux can act as a metabolic mechanism that hastens oxidative stress, inflammation, and progression to NASH.

10.4.3 ROS Production in Chronic Hepatitis C

Schwer et al. have demonstrated that HCV core protein associates with the mitochondria-associated membrane (MAM) fraction, a point of close contact between the endoplasmic reticulum and mitochondrion [50]. Direct interaction of HCV core protein with mitochondria potentially modifies mitochondrial ROS production and scavenging, subsequently inducing oxidative stress. When mitochondrial electron transport activity is inhibited by HCV core protein [51, 52], electrons are likely to leak from the electron transport chain transfer, accelerating mitochondrial $O_2^{\bullet -}$ production and/or H_2O_2 emission.

Although sufficient intraorganelle Ca^{2+} concentrations are required to stimulate metabolism by activating enzymes critical for maintenance of the TCA cycle, prolonged increases of Ca^{2+} can, in turn, interfere with the activities of these enzymes. The TCA cycle activity affects the electron transport chain activity, which in turn affects the mitochondrial membrane potential. Thus, increased Ca^{2+} influx into mitochondria induces a substrate imbalance of the TCA cycle that leads to the generation of mitochondrial ROS, probably through the inhibition of electron transport chain activity. There are several lines of evidence indicating that HCV increases mitochondrial ROS production by modulating calcium signaling. HCV core protein enhances mitochondrial Ca^{2+} uptake in response to ER Ca^{2+} release through activation of the mitochondrial Ca^{2+} uniporter, which leads to increased mitochondrial ROS production [53, 54].

10.5 Mitochondrial Quality Control as a Therapeutic Option

The role of mitochondria in energy production sensitizes them to damage owing to exposure to high levels of ROS, a by-product of energy generation that can disturb protein folding and structures and cause mitochondrial DNA mutations. Thus, the mitochondria are targets for ROS and ROS generators. Therefore, mitochondria have evolved multiple systems of quality control to ensure that the requisite number of functional mitochondria is present to meet the demands of the cell. These pathways work to eliminate damaged mitochondrial proteins or parts of the mitochondrial network via mitochondria-specific autophagy (mitophagy) and renew components by adding proteins and lipids through biogenesis, collectively resulting in mitochondrial turnover [55].

Removing damaged mitochondria by mitophagy is a protective mechanism against alcohol-induced liver injury and steatosis because it serves to maintain a healthy population of mitochondria, which prevents cell death by reducing oxidative stress and preserving respiratory chain function and mitochondrial bioenergetics for efficient energy production. Alcohol metabolism produces ROS in the liver, and mitochondria damaged by ROS release pro-apoptotic proteins. Therefore, removal of these damaged mitochondria is necessary to reduce hepatocellular death and liver injury caused by heavy alcohol consumption [42, 56].

Chronic persistence of hepatic lipid overload leads to liver injury with inflammation, cell death, and fibrosis characteristic of NASH. Some alterations in lipid metabolism are at the level of lipid mobilization, because hepatic-autophagy related protein (ATG)7 deletion decreases triglyceride break down, resulting in lipid droplet accumulation. Failure of mitochondrial quality control because of their reduced turnover through mitophagy can promote oxidative stress through ROS production and activation of downstream inflammatory pathways. The combination of lipotoxicity, oxidative stress, and chronic activation of the inflammatory response upon autophagy failure often leads to hepatocyte cell death, thus recapitulating the hallmarks of NASH (inflammation, oxidative stress, cell death, and fibrosis) [57].

As mentioned above, HCV increases mitochondrial ROS production via direct interaction of HCV proteins with mitochondria and/or modulation of mitochondrial calcium signaling. The detoxification of ROS is an important function of the cellular redox homeostasis system. Under resting cellular conditions, the intracellular redox environment is in a relatively reduced state [58]. The question is how HCV-induced mitochondrial ROS production and the subsequent oxidative stress persist in spite of ROS-detoxifying agents such as MnSOD and/or GSH or the thioredoxin/peroxiredoxin systems. Reduction of mitophagy/autophagy at least partially accounts for persistent ROS production in HCV infection because there are several lines of evidence that HCV infection suppresses autophagy flux at the step of fusion with lysosomes [59] or mitophagy by interacting with Parkin [60]. Thus, mitochondria quality control can be one of therapeutic strategies for alcoholic liver disease, NAFLD/NASH, and chronic hepatitis C.

10.6 Conclusion

Emerging evidence clearly illustrates the critical role of oxidative stress in the pathogenesis of chronic liver diseases such as alcoholic liver disease, NAFLD/NASH, and chronic hepatitis C. In this chapter, iron overload in the liver and mitochondrial ROS production through reduced mitochondrial quality control were highlighted as important causative factors for inducing oxidative stress in chronic liver diseases. Considering the robust relationship between iron overload, increased ROS production and oxidative stress in liver diseases, future studies should focus on the potential of promising therapeutic agents to attenuate iron overload and mitochondrial oxidative dysfunction.

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