

Control of oxidative stress in hepatocellular carcinoma: Helpful or harmful?

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Abstract

Oxidative stress is becoming recognized as a key factor in the progression of chronic liver disease (CLD) and hepatocarcinogenesis. The metabolically important liver is a major reservoir of mitochondria that serve as sources of reactive oxygen species, which are apparently responsible for the initiation of necroinflammation. As a result, CLD could be a major inducer of oxidative stress. Chronic hepatitis C is a powerful generator of oxidative

stress, causing a high rate of hepatocarcinogenesis among patients with cirrhosis. Non-alcoholic steatohepatitis is also associated with oxidative stress although its hepatocarcinogenic potential is lower than that of chronic hepatitis C. Analyses of serum markers and histological findings have shown that hepatocellular carcinoma correlates with oxidative stress and experimental data indicate that oxidative stress increases the likelihood of developing hepatocarcinogenesis. However, the results of antioxidant therapy have not been favorable. Physiological oxidative stress is a necessary biological response, and thus adequate control of oxidative stress and a balance between oxidative and anti-oxidative responses is important. Several agents including metformin and L-carnitine can reportedly control mechanistic oxidative stress. This study reviews the importance of oxidative stress in hepatocarcinogenesis and of control strategies for the optimal survival of patients with CLD and hepatocellular carcinoma.

Key words: Liver cancer; Liver cirrhosis; Hepatitis B; Hepatitis C; Non-alcoholic steatohepatitis; Reactive oxygen species

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Core tip: Oxidative stress is a key biological response that correlates with the progression of chronic liver disease. However, oxidative stress is an essential survival mechanism and thus to erase it is an unsuitable approach to disease control. As hepatocarcinogenesis is closely associated with increased oxidative stress *via* viral proteins or chronic inflammation and lipids, controlling oxidative stress should be effective against progressive liver disease. Agents that can control oxidative stress might represent a more effective approach than reactive oxygen species-scavenging agents.

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INTRODUCTION

The risk and pathogenesis of hepatocellular carcinoma (HCC) has been investigated in detail because about 80% of this type of cancer is due to chronic infection with hepatitis B (HBV) and C (HCV) viruses^[1]. Hepatocellular carcinoma differentiates from low- and high-grade dysplastic nodules and sequentially advances into well-, moderately- and poorly differentiated HCC. Such multistep carcinogenesis associated with chronic inflammation suggests that numerous complex pathogeneses are involved in hepatocarcinogenesis. Whole exome sequencing has revealed that HCC contains many oncogene and tumor suppressor gene mutations^[2]. The most common pathways can be p53-, Wnt- and RB1-dependent^[3]. Poor outcomes of HCC include p53 signaling-related genes such as the protein kinase TTK^[4]. Activation of the Wnt-catenin pathway is frequently anomalous in HCC and high expression levels correlate with poor outcomes^[5]. Mutations in RB1 are associated with cancer-specific and recurrence-free survival after resection^[6]. Such changes could be induced *via* HBV, HCV and lipid-induced cellular stress and chronic inflammation.

The pathogenic mechanisms of HBV- and HCV-related chronic liver disease (CLD) and hepatocarcinogenesis include viral protein-related immune function interference, tumor initiation or suppression interference and CLD-related environmental changes^[1]. Tumor-related gene methylation is induced *via* HBV and HCV infection without inflammation and it is increased stepwise under conditions of chronic hepatitis, dysplastic nodules and HCC^[7,8]. Oxidative stress is included in this process *via* the direct effects of viral proteins or secondarily to chronic inflammation^[1].

Recent advances in HBV- and HCV-targeted anti-viral therapy have enabled control of HBV and HCV^[9,10]. So far, the HBV load can only be decreased by nucleos(t)ide analogues that cannot eradicate the virus^[11]. Pegylated interferon (IFN) combined with ribavirin and HCV NS3 protease inhibitor, can eradicate HCV in nearly 80% of therapy recipients. However, patients with liver cirrhosis have difficulties enduring the many side-effects of these drugs such as anemia, leukocytopenia, and skin rash^[12]. New treatment modalities with IFN-free oral direct-acting antiviral agents combined with other types of therapy do not generate many of the side effects that are associated with IFN, but they are not indicated for decompensated liver cirrhosis because metabolism is decreased in patients with defective liver function^[13]. Therefore, the direct hepatocarcinogenic activities of HBV and type C liver cirrhosis remain a major threat in endemic areas.

The incidence of non-alcoholic steatohepatitis (NASH)

has recently increased and it progresses to HCC. Non-alcoholic steatohepatitis is an advanced stage of non-alcoholic fatty liver disease (NAFLD) that is involved in metabolic syndrome, a condition that becoming increasingly prevalent^[14]. Both NASH and related HCC require urgent investigation because obesity is widespread in first-world countries. Oxidative stress is a key factor in NASH progression and NASH-related hepatocarcinogenesis^[15,16]. The standard treatment for NASH is supplementation with the representative antioxidant, vitamin E^[17].

The administration of antioxidant therapies for diseases involving oxidative stress is controversial because reactive oxygen species (ROS) are essential for maintaining defense by anti-pathogenic microorganisms, or by anti-carcinogenic mechanisms. Although many antioxidants are already on the market, their proven antioxidant activities *in vitro* have not been confirmed *in vivo*^[18]. Many studies of cerebrovascular diseases and mortality have associated vitamin E with unfavorable outcomes^[14]. Therefore, the notion of controlling oxidative stress in this manner requires re-evaluation. This article reviews current understanding of oxidative stress in viral hepatitis- and NASH-related HCC and the controversy surrounding antioxidant therapy for these diseases.

OXIDATIVE STRESS INVOLVEMENT IN HEPATOCARCINOGENESIS

The mechanisms of hepatocarcinogenesis include several common functions such as oncogene activation, oxidative stress and tumor suppressor function attenuation, but the upstream functions differ. HBV related HCC can be found in non-cirrhotic carriers, whereas HCV-related HCC is found mainly in patients with cirrhosis^[19]. The incidence of NASH related HCC is increasing and more clinical evidence is needed.

Oxidative stress could be induced *via* chronic hepatic inflammation regardless of etiology. Acute liver injury and hepatic inflammation induce ROS *via* neutrophils and Kupffer cell activation. As these cells invade liver parenchyma, hepatocytes could also be affected by the induced ROS. Although the free diffusion of superoxide is blocked by the plasma membrane, superoxide dismutase in the membrane can convert superoxide anions to H₂O₂ after internalization into hepatocytes^[20]. The ROS in mitochondria are by-products of the beta oxidation pathway for fatty acid metabolism and they are generated *via* electron leakage from mitochondrial electron transport resulting in the activation of oncogenic pathways^[21].

Mechanisms of HBV-related HCC and oxidative stress involvement

Enveloped HBV is a DNA virus containing a relaxed circular DNA genome enclosed by envelope protein^[22,23]. After envelopment and the release of mature virions, HBV converts into a covalently closed circular DNA

that persists in the nucleus of infected cells as mini-chromosomes that are difficult to eradicate^[24]. After initial infection, HBV persists in the liver for life, even if a patient achieves a clinical cure with seroclearance of HBV envelope antigen (HBsAg) and the emergence of anti-HBs antibody^[25]. Chronic inflammation and liver fibrosis caused by chronic HBV infection contributes to the development of HCC^[26]. However, in addition to these host factors, HBV itself plays a direct role in the development of HCC^[27,28]. A significant proportion of HBV-related HCC arises in otherwise normal livers^[19,29], and animal models transfected with this virus genome develop HCC, which confirms the oncogenic potential of HBV in the liver^[27,30]. Gene expression profiles of HBV-related HCC indicate that several genes related to signal transduction, transcription and metastasis play direct hepatocarcinogenic roles^[31]. Despite a considerable amount of research, the molecular basis of HBV-related hepatocarcinogenesis remains unclear^[32,33].

Chronic inflammation is a common feature of chronic hepatitis B and C that results in induction of oxidative stress. The HBV evades immune surveillance resulting in altered viral-specific and non-specific immune responses^[34]. Kupffer cells or macrophages exert both immunostimulatory and immunoregulatory activities upon exposure to HBV. The addition of HBV particles and HBsAg induces production of the proinflammatory cytokines interleukin (IL)-1 β , IL-6, CXCL-8 and tumor necrosis factor (TNF)- α by human CD68⁺ macrophage-enriched cells *via* nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) activation^[34]. However, another study did not find cytokine production with immunoregulatory cytokine transforming growth factor- β production^[35]. The immune system activates Kupffer cells to eradicate HBV, while HBV evades the Kupffer cell-related pathway to reduce the inflammatory pathway and render the environment favorable for survival. Such persistent atypical cytokine production and the resulting ROS affect hepatocarcinogenesis.

The HBV genome encodes several gene products such as DNA polymerase (Pol), capsid protein (core), envelope proteins L, M and S, and the multifunctional protein HBx. Among these products, the oncogenic potential of HBx protein has been analyzed in detail. Transactivating HBx protein stimulates viral gene expression and replication, and also protects virally-infected cells against immune-mediated destruction^[36]. High and low levels of HBx protein are expressed in the cytoplasm and nucleus, respectively, of hepatocytes infected with HBV^[37]. HBx protein regulates some oncogenes and affects several apoptosis-related signaling pathways^[36]. Through binding to transcription factors such as CREB, RPB5, TFIIB, XAP-1, C/EBP α and XAP3, HBx can upregulate oncogenes such as Rab18 or Yes-associated protein^[38,39]. HBx induces apoptosis by upregulating FasL protein through activating MLK3/MKK7/JNKs signaling and interacting with Bcl-2/CED-9 signaling^[40,41].

Concentrated cytoplasmic HBx co-localizes with mitochondria that are sources of ROS^[42]. The C-terminal

region of HBx produced by HBx truncation is required for ROS production^[37] and it is found in 46% of HCC, but not in non-tumor tissues^[43]. The C-terminal truncated HBx found in HCC suggests that ROS are involved and that it is significantly associated with increased venous invasion and metastasis. The stable expression of C-terminal truncated HBx *in vitro* results in increased C-Jun kinase transcriptional activity and the enhanced invasiveness of hepatoma cell lines.

Integration of the HBV gene into the house genome is an important mechanism that is responsible for HCC development. The frequency of integration is reportedly higher in tumors than in adjacent liver tissues (86.4% vs 30.7%). Several cancer-related genes such as *TERT*, *MLL4*, and *CCNE1* are integrated by HBV, especially in tumors^[44] and among these, HBx is most frequently integrated into the human genome.

Several studies have analyzed mutations within the HBV genome that might be associated with HCC. Genotypic diversity is related to differences in clinical and virological characteristics. For example, patients infected with genotype C have more severe chronic liver disease, including cirrhosis and HCC^[29,45]. Deletions and point mutations, which are more subtle genetic variations than genotypes, have been identified, such as codon 38 in the X gene, the core promoter region, G1613A and C1653T, basal core promoter region A1762T/G1764A mutations and deletions of pre-S and X protein^[46-49]. The analytical findings of HCC-related HBV mutations emphasized the importance of HBx and suggested the involvement of oxidative stress. The envelope protein pre-S is also involved in HCC development. The pre-S region might affect the pathway of hepatocarcinogenesis *via* oxidative stress.

The HCC-associated HBV variant pre-S has been identified in the pre-S2 start codon and/or in deletions of the 5'-terminal of the pre-S2 region and pre-S1 mutation with deletions of the 3'-terminal of pre-S1^[50,51]. These pre-S region mutant products induce the accumulation of mutated L protein in the endoplasmic reticulum (ER) of hepatocytes. The ER plays a major role in the synthesis, folding and trafficking of secretory and membrane proteins that correlate with the cellular response known as ER stress. A considerable amount of experimental data have confirmed the potential pro-oncogenic role of pre-S mutated gene products *via* accumulation in the ER with enhanced ER stress and ROS^[51]. The pre-S mutated proteins accumulating in the ER can trigger c-Raf-1/Erk2 signaling, which results in AP-1 and NF- κ B activation, enhanced proliferative activity of hepatocytes and an increased incidence of liver tumors in transgenics^[52]. Pre-S2 mutated proteins also have non-ER related functions such as interacting with Jun activation domain-binding protein 1, which results in cyclin-dependent kinase inhibitor p27 degradation and cell cycle progression^[53].

Oxidative stress must be involved to some degree in HBV-related hepatocarcinogenesis, possibly *via* HBx and pre-S-related functions. Relatively weak oxidative stress has been defined in HBV-related hepatocar-

cinogenesis *in vitro*. HBV with HBx protein expressed in mitochondria, binds to voltage-dependent anion channels 3 and alters the mitochondrial transmembrane potential resulting in ROS generation and the activation of several transcription factors^[54]. Analyses of serum from patients have shown that oxidative stress-related markers are significantly increased in HCV-, but not in HBV-related HCC^[55]. One reason for this might be that HBV has other prevailing features such as the induction of mutations in oncogenic and tumor suppressor genes by viral proteins. Anti-oxidant therapy is seemingly logical for HBV-related HCC as HBx and pre-S proteins have promising effects on oxidative stress. However the inferior outcomes of oxidative stress in human serum and *in vitro* indicate that expectations should not be too high.

Mechanisms of HCV-related HCC and involvement of oxidative stress

Since HCV, like HBV, is not a cytopathic virus, immune reactions play a central role in the development of chronic hepatitis^[56,57]. However, the clinical course and direct viral and hepatitis-related effects on hepatocarcinogenesis differ. The expression of several genes associated with detoxification and the immune response suggest that indirect and immune or detoxifying response-related hepatocarcinogenic roles are involved in HCV-related HCC^[31].

Immune systems are disrupted by HCV proteins. Antigen-presenting cells such as Kupffer cells, macrophages, or dendritic cells exhibit both immunostimulatory and immunoregulatory activities upon exposure to HCV^[58]. The HCV core and Kupffer cells affected by NS3 secrete pro-inflammatory cytokines such as IL-1 β , IL-6, TNF- α and the immunosuppressive cytokine, IL-10, *in vitro*^[59]. The release of pro-inflammatory cytokines might explain the induction and persistent inflammation of patients with chronic hepatitis C, while immunosuppressive cytokine release explains the difficulty of eradicating HCV-infected hepatocytes. The direct effects of HCV on the inflammatory signal in Kupffer cells upregulates the immunoregulatory molecule PD-L1^[60]. In addition to interference with Kupffer cell-related anti-viral activities, HCV induces a sufficient amount of inflammatory cytokines to result in chronic inflammation. Kupffer cells accumulate around inflammatory foci and express cytotoxic molecules such as granzyme B, perforin and ROS that induce inflammation and fibrosis^[61]. Sustained inflammation results in hepatocyte apoptosis and repeated regeneration cycles followed by spontaneous DNA mutation and damage resulting in HCC^[1].

HCV antigens, especially core protein, play major roles in chronic hepatitis C pathogenesis and hepatocarcinogenesis^[62]. Because microarray analysis has not suggested a direct hepatocarcinogenic pathway in HCV-related HCC, the direct effects of viral proteins are seemingly less powerful than HBV. However, HCV core protein seems to play direct roles in TNFR, PKR and

Stat3 pathways that are associated with cell proliferation, apoptosis, transformation and immortalization^[63,64]. Transgenic mice expressing HCV core protein exhibit hepatocarcinogenesis *via* fatty metamorphosis and increased oxidative stress^[65,66]. The glutathione pool is oxidized and NADPH content is decreased *via* the direct interaction of HCV core and mitochondria from the livers of transgenic mice expressing HCV proteins^[67]. Patients with HCV-related HCC have more oxidative stress in the liver and higher levels of serum oxidative stress markers such as 8-hydroxy-2'-deoxyguanosine or reactive oxygen metabolites than those with HBV-related HCC^[55,68,69]. A weak, direct carcinogenic effect of HCV core protein might coordinate with oxidative stress, chronic inflammation and damage to apoptosis regeneration cycle DNA to result in hepatocarcinogenesis at a more advanced age than patients with HBV-related infection^[70]. Oxidative stress is associated with aging that also drives hepatocarcinogenesis in patients with HCV-related HCC^[71].

Serum markers and hepatocyte deposition associated with iron especially in lysosomes are frequently elevated in patients with chronic hepatitis C infection^[72]. Reticuloendothelial systems including Kupffer cells are also targets of iron deposition that might affect chronic inflammation^[73]. Excess divalent iron atoms are highly toxic, as they induce the Fenton reaction and produce highly toxic ROS, hydroxyl radicals. Because some investigators have reported that phlebotomy and a low-iron diet lowers the risk of HCC developing in patients with chronic hepatitis C infection, iron toxicity is thought to be involved in hepatocarcinogenesis^[74,75]. Oxidative stress induced by HCV reduces hepcidin transcription followed by ferroportin expression in enterocytes and increases duodenal iron uptake^[72,76]. An excess of dietary iron fed to HCV transgenic mice induces excess hepatocarcinogenesis^[77]. Iron and resultant oxidative stress closely correlate with the progress of chronic hepatitis C and related HCC development that should be incorporated as treatment options. As described above, the lipid-related, direct pro-oxidant functions of HCV proteins, especially the core and iron accumulating functions, indicate the importance of the relationship between oxidative stress and hepatocarcinogenesis in patient with chronic hepatitis C.

Mechanisms of NASH-related HCC

The pathophysiology of NASH has been considered to comprise a "two hit" theory^[78]. The first hit is hepatocyte steatosis, which is characterized by the accumulation of triglyceride in hepatocytes. The second hit consists of various types of cellular stresses, such as apoptosis, oxidative stress, ER stress, and intestinal circumstances. The recent genome-wide association study discovered that the patatin-like phospholipase 3 (*PNPLA3*) gene correlates with NASH progression^[79]. This genetic mechanism might be involved in the first hit and fatty deposition could be the second hit; nonetheless, this two-hit theory is too simple to explain all aspects of

NASH^[15,80]. Other studies have found that inflammation induces fatty deposition in hepatocytes. Such findings have recently led to a “multiple hit” theory to explain the fact that inflammation promotes steatosis or that genetic factors such as PNPLA3 correlate with disease progression^[80]. Lipid droplets were originally thought to function simply as cellular energy-storage structures. However, they are now considered to be complicated organelles that are involved in many processes such as metabolic, inflammatory and immunological responses. Lipid toxicity induces multiple hits such as oxidative stress, ER stress, and immune reactions^[14]. These cellular stresses are also involved in carcinogenesis.

Obesity-related symptoms such as hypertriglyceridemia and hypertension are established risk factors for NAFLD^[81]. Central obesity presenting as visceral fat accumulation is associated with various pathologies such as cerebrovascular diseases, type 2 diabetes, NASH and gastrointestinal cancers. Visceral obesity induces several cytokines including the inflammatory cytokine IL-17^[82], which induces neutrophil chemokine expression *via* IL-17 receptor A which is extensively expressed in the liver. Controlling the IL-17-related pathway effectively prevents NASH progression in mouse models^[83]. Elevated pre-therapy serum IL-17 levels in patients with HCC correlate with risk of early recurrence after curative hepatectomy^[84]. Co-cultured HCC cell lines and T cells producing IL-17 *in vitro* augment the proliferation of HCC cells, suggesting the importance of IL-17 for HCC pathogenesis. Neutrophil infiltration might be involved in NAFLD progression in human NASH. Therefore, visceral adipocytes that accumulate in patients with central obesity are related to such cytokines and should be involved in the pathogenesis of NAFLD.

Visceral fat accumulation correlates with increased adipokine levels, significant risks for HCC and recurrence after curative treatment^[85,86]. Adiponectin is a “good” adipokine that modulates many metabolic processes including glucose regulation and fatty acid oxidation. Adiponectin is the most abundant adipokine mainly secreted from mature, white adipose tissue, and levels of expression and secretion increase during adipocyte differentiation. Adiponectin levels inversely correlate with BMI, visceral obesity contents and insulin resistance. Serum adiponectin is significantly higher in females than in males, in whom serum androgens become more evident during puberty^[87]. Adiponectin has anti-inflammatory, anti-diabetic and anti-lipid storage effects. Furthermore, weight-loss induces adiponectin synthesis, whereas proinflammatory adipokines such as TNF- α and IL-6 suppress adiponectin^[88]. Adiponectin increases the expression of CXCL-8 in primary hepatocytes that functions in cell survival and in anti-apoptotic activities to guard cells; however, this also induces uncontrolled cell survival resulting in malignant transformation^[89]. High levels of serum adiponectin are associated with reduced risk for several cancers such that prostate, breast, colorectal and pancreatic cancers^[90,91]. However, higher levels of low- and medium-molecular weight

adiponectin are also associated with a higher risk of HCC through a relationship with the inflammatory response^[92]. Increased levels of adiponectin might be induced *via* a compensatory mechanism to dampen inflammation. However, several studies have also found that adiponectin has direct proinflammatory activities. More evidence is needed to confirm this.

The relationship between hepatic iron deposition and disease progression in NASH remains controversial^[93]. Iron accumulation in hepatocytes correlates with more severe damage^[94]. The iron metabolic pathway is implicated in the insulin resistance and hepatic cholesterol synthesis pathways. Hepatic lipid-induced ER stress results in an unfolded protein response and hepatic iron accumulation^[95]. Several reports describe the risk of iron overload in NASH^[96].

TREATMENTS FOR HCC THAT TARGET OXIDATIVE STRESS

Oxidative stress in HCC

Oxidative stress closely correlates with HCV- and NASH-related hepatocarcinogenesis, but relatively weakly with HBV-related HCC. Thus, anti-oxidant therapy would seem reasonable for controlling HCV- and NASH-related hepatocarcinogenesis. Liver inflammation is definitely associated because hepatocarcinogenesis arises mostly in patients with chronic hepatitis. The major inflammatory cytokine TNF- α alters mitochondrial integrity by mimicking a mild uncoupling effect in liver cells, as indicated by a reduction in membrane potential and ATP depletion^[97]. The TNF- α induced ROS activation of NF- κ B and downstream target genes such as *CXCL1*, *IkB α* and *A20* results in enhanced migration activity of hepatoma cell lines. Reduced hepatic inflammation *via* nucleos(t)ide analogues in hepatitis B and interferon in hepatitis C correlates with reduced oxidative stress. However, preventing or controlling HCC using antioxidants is a matter of debate.

Oxidative stress is increased through the generation of ROS and defects in redox defense mechanisms with glutathione (GSH), catalase or superoxide dismutase (SOD)^[98]. Mitochondria comprise the most important and abundant source of intracellular ROS. Mitochondrial dysfunction therefore plays a central role in the pathological mechanisms of chronic hepatic inflammation and subsequent hepatocarcinogenesis (Figure 1). An imbalance in the mitochondrial respiratory chain is the main source of ROS, O₂⁻, H₂O₂ and hydroxyl radicals (\cdot OH). The transport of high-energy electrons through the mitochondrial electron transport chain (ETC; complexes I, III and IV) is an important step for ATP production. This energy-producing pathway also produces ROS. High-energy electrons in ETC complexes I–III react with O₂ and produce superoxide (O₂⁻) which accounts for up to 4%–5% of the consumed O₂. The amount of resulting O₂⁻ increases in damaged mitochondria. The tricarboxylic acid cycle and the β -oxidation of fatty acids generate reduced

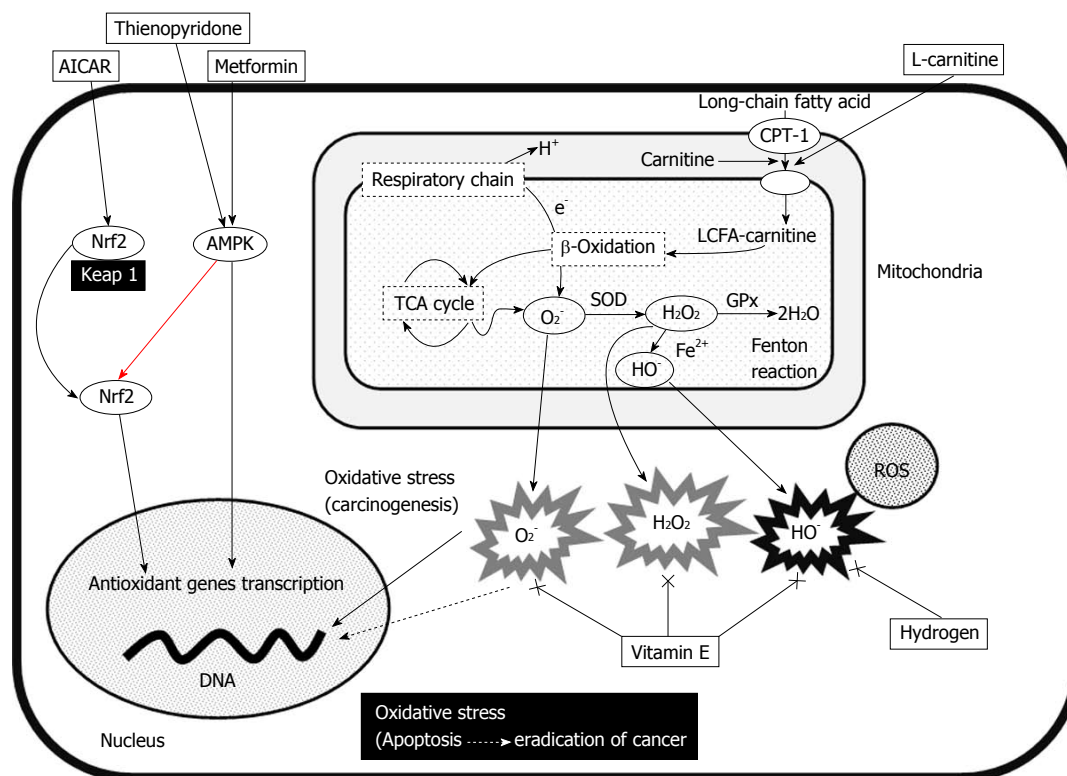


Figure 1 Oxidative stress production and treatment targets in hepatocytes. High levels of plasma free fatty acids increase levels of hepatic free fatty acids. Long-chain fatty acids taken up by mitochondria as complexes with L-carnitine are subsequently metabolized in β -oxidation pathway. Under oxidative stress, oxidative reactions convert oxidized cofactors (NAD^+ and FAD) into reduced cofactors (NADH and FADH₂) and deliver electrons to respiratory chain. Imbalance between increased delivery of electrons to, and decreased outflow from respiratory chain causes electrons and ROS products to accumulate. Antioxidant defenses, such as superoxide dismutase (SOD), glutathione peroxidase (GPx) or catalase can metabolize O_2^- and H_2O_2 to non-toxic H_2O . However, Fenton and/or Haber-Weiss reactions generate highly reactive, toxic, hydroxyl radicals ($\cdot\text{OH}$). Vitamin E and hydrogen as general and selective cytotoxic ROS scavengers erase oxidative stress. L-carnitine supports mitochondrial function to increase long-chain fatty acid uptake. Metformin or thienopyridone activates AMPK and induces antioxidant gene transcription and AICAR activates Nrf2, possibly like metformin. AICAR: 5-aminoimidazole-4-carboxamide-1- β -D-ribofuranoside; AMPK: AMP-activated protein kinase; Keap1: Kelch-like ECH associating protein; Nrf2: Nuclear factor erythroid 2-related factor.

(NADH and FADH₂) cofactors from oxidized (NAD^+ and FAD) cofactors. The NADH uses two electrons from the oxidation pathway in the inner membrane to supply the rest of the ETC for the reduction of O_2 to water. As the substrates and cofactors of complex I have the lowest reduction potential in the mitochondrial respiratory chain, side reactions could occur such as nonspecific electron transfer to species in solution. Under physiological conditions, reactive and incompletely reduced forms of oxygen such as superoxide (O_2^-) are detoxified into water by anti-oxidant defenses and repair enzymes to maintain a low steady state of toxic oxidants^[14].

The mitochondrial capacity to control the oxidative balance would be destroyed under conditions of continuous oxidative stress. Excess O_2^- could be produced within damaged mitochondria through electron leakage and SOD would convert the resulting excess O_2^- into H_2O_2 . Glutathione plays an important role in the balance between pro- and anti-oxidants correlating with the detoxification of H_2O_2 and other ROS. Glutathione-dependent enzymes as glutathione peroxidase, glutathione reductase (GR), and glutathione S-transferase (GST) play important roles in the stimulation of glutathione function. Glutathione peroxidase catalyzes H_2O_2 to non-toxic H_2O , GR regenerates the pool of

reduced glutathione and GST catalyzes glutathione reactions. Despite such anti-oxidant mechanisms, the Fenton and/or Haber-Weiss reactions generate the highly reactive toxic ROS, $\cdot\text{OH}$ from H_2O_2 and oxidative stress produced by Fenton reactions is mediated by iron.

Iron loading of the liver causes further hepatic oxidative stress that is a main cause of HCC^[99]. Iron overload can induce hepatocarcinogenesis in animals and is associated with the high incidence of HCC among African and Taiwanese individuals^[100,101]. Iron depletion in HCV- and NASH-related hepatocarcinogenesis should play a more important role in the prevention of HCC.

Preferential antioxidative drugs to treat HCC

Several antioxidant agents and foods are widely available and the effects of several of them *in vitro* and *in vivo* are under investigation. The optimal choice of agents to control chronic hepatitis or related hepatocarcinogenesis without suppressing the physiological roles of oxidative stress is difficult to determine.

Phlebotomy is a method of reducing iron overload that has been effective against chronic hepatitis C and NASH in selected patients^[102,103]. Iron depletion improves not only iron overload but also insulin resistance, suggesting that iron toxicity is involved in several metabolic pathways^[104].

Iron depletion for NASH remains a matter of debate but it could nevertheless function as an antioxidant treatment strategy.

Candidate antioxidant treatments for HCC include antioxidant genes, inducible transcriptional factors such as AMP-activated protein kinase (AMPK) or nuclear factor erythroid 2-related factor (Nrf2) activators, ROS scavengers and agents that support mitochondrial uptake. The following are representative drugs that are associated with reducing oxidative stress.

Metformin: Metformin is an anti-diabetic drug, but it also has an anti-oxidative function. Metformin increases intracellular levels of AMP after activating AMPK, which is a highly conserved heterodimeric serine-threonine kinase that serves as an energy sensor in eukaryotic cells and bridges metabolism to carcinogenesis^[105]. It is activated by an increase in the cellular AMP/ATP ratio under hypoglycemia, hypoxia, ischemia and heat shock^[106]. The activation of AMPK suppresses cell proliferation in non-malignant and malignant cells *via* regulation of the cell cycle, apoptosis, autophagy and the inhibition of fatty acid synthesis^[107]. Phospho (p)-AMPK is down-regulated in HCC tissues from patients and low levels of p-AMPK expression correlates with a poor prognosis, indicating the importance of AMPK signaling in HCC^[108]. Adding metformin to hepatoma cell lines results in AMPK activation as well as dose- and time-dependent growth inhibition. Metformin also induces cell-cycle block, apoptosis, STAT3-induced IL-6 production^[109] and the antioxidant enzyme heme oxygenase-1 (HO-1) in human endothelial cells *via* the Nrf2 signaling pathway^[110]. The recently-discovered direct AMPK activator thienopyridone also activates AMPK through distinct mechanisms with metformin^[111]. This might be a future approach to activate AMPK.

A meta-analysis of anti-diabetic drugs found that metformin, sulfonylurea and insulin induce a 50% reduction, and 62% and 161% increases, respectively in HCC incidence^[112]. However, randomized controlled trials have not shown significant effects. Metformin reduced the occurrence of HCC and liver-related death, and increased survival rates in patients with diabetes and HCC who underwent radiofrequency ablation without any severe side effects^[113]. The standard treatment for NASH according to the guidelines of the American Association for the Study of Liver Disease (AASLD) is vitamin E. This was derived from the findings of a clinical trial that has shown improvements in the clinical profile and histological findings of NASH activity within two years^[114]. Metformin improves histological activity in the livers of mouse models of non-diabetic NASH, but not in human NASH^[115]. Thus, metformin is preferential for treating NASH-related HCC in mouse models^[116], but clinical trials are needed to confirm the effects of metformin on HCC management.

Nrf2-acting agents: Under basal conditions, Nrf2 binds to Kelch-like ECH associating protein 1 (Keap 1)

which exists in the cytoplasm in an inactive form^[117]. The AMPK activator 5-aminoimidazole-4-carboxamide-1- β -D-ribofuranoside (AICAR) induces an increase in Nrf2 protein and antioxidant enzyme expression in endothelial cells, whereas AICAR activates Nrf2 in hepatoma cell lines resulting in antioxidant enzyme expression^[118]. A combination of metformin and AICAR might activate AMPK and Nrf2 to control HCC. A more detailed analysis in a basic approach with clinical trials might be needed to determine the value of this new type of drug.

Vitamin E: Controversy surrounds the value of ROS-scavenging agents because ROS have essential functions for life. Scavengers of ROS consistently exert effective chemical activities *in vitro*, but not often *in vivo*^[18]. Scavenging ROS is considered effective in preventing the development of cancer and cancer stemness, yet ROS also contribute to the prevention of cancer^[119]. Stem cell-like cancer cells that express the CD44 variant have an antioxidative phenotype. The CD44 variant protects cancer stem-like cells from oxidative stress and prevents their apoptosis^[120]. Oxidative stress upon normal cells might induce transition to a cancer cell phenotype that is highly resistant to further oxidative stress. Several clinical trials are presently investigating the induction of oxidative stress under these conditions as a cancer treatment^[121]. The AASLD recommends treating NASH with vitamin E at a dose of 800 IU/d, which is higher than that usually administered to treat NASH^[17]. This recommendation is based on a two-year randomized study of NASH that demonstrated improved alanine transaminase and histological activity^[114]. However, that trial did not find an improvement in liver fibrosis^[122]. Further investigations of longer duration are required to determine the effects of vitamin E on hepatocarcinogenesis.

Peroxisome proliferator-activator- γ agonist (pioglitazone):

The transcription factor peroxisome proliferator-activator (PPAR) γ regulates lipid metabolism and its activation suppresses hepatic lipoperoxide and reduces the production of the hepatic pro-inflammatory cytokines, TNF- α and IL-6^[123]. The effects of PPAR γ on a model of diethylnitrosamine (DEN)-induced HCC *via* cyclin-dependent kinase inhibitor p27 expression are favorable^[124]. Pioglitazone serve as an anti-oxidant to treat NASH. A large clinical study has shown fair results of using pioglitazone to treat NASH with respect to serum alanine aminotransferase levels^[114]. Although an improvement in histological activity was not proven, this drug will be assessed for patients with NASH-related diabetes. A long observational assessment of hepatocarcinogenesis will be needed later.

Hydrogen: Molecular hydrogen (H₂) has powerful and selective antioxidant effects with unique features^[125]. Hydrogen scavenges toxic hydroxyl radicals, but not O₂⁻, H₂O₂ or nitric oxide in cultured cells. This selective reduction of ROS has been explained by the strong

oxidative activity of hydroxyl radicals reacting with mild anti-oxidative function of hydrogen. The easy distribution of hydrogen is one characteristic of the effects. Most hydrophilic compounds are retained at membranes and cannot pass into the cytoplasm, whereas hydrophobic compounds such as vitamin E need specific carriers or receptors to penetrate biomembranes. However, H₂ can penetrate biomembranes, diffuse into the cytosol and easily reach the nucleus where it can protect nuclear DNA and prevent mitochondrial damage. Hydrogen is effective against DEN-induced HCC and HCC associated with type 1 diabetes and NASH^[126,127]. Data from patients with NASH are not yet available and thus clinical trials are required to further evaluate the effects of molecular hydrogen on HCC.

L-carnitine: L-carnitine is an essential nutrient that converts fat into energy in mitochondria and ameliorates liver damage. It acts as a fatty acid carrier across the mitochondrial membrane and it also exists as free or acyl forms in plasma^[128]. L-carnitine plays an important role in lipid metabolism as it is an essential cofactor for the β -oxidation of fatty acids through facilitating the transport of long-chain fatty acids and its ability to activate carnitine palmitoyltransferase, the key enzyme in fatty acid oxidation^[129]. L-carnitine has recently been proposed as treatment for various diseases, including liver damage. Several studies have shown that L-carnitine can ameliorate or prevent liver damage with various etiologies. Animal studies have shown that dietary supplementation with L-carnitine prevents chemically induced hepatitis and subsequent HCC, as well as NASH-related HCC^[130,131]. L-carnitine supplementation greatly improves plasma glucose levels in patients with NASH, lipid profiles and histological manifestations^[132]. The results of these clinical trials were fair, and more clinical trials of larger populations are required to further evaluate the effects of L-carnitine on hepatocarcinogenesis.

CONCLUSION

Because oxidative stress is an essential survival mechanism, erasing it is not a feasible approach to disease control. Rather, controlling oxidative stress should be effective because hepatocarcinogenesis is closely associated with increased oxidative stress *via* viral proteins or chronic inflammation and lipids. Agents that can control oxidative stress such as the AMPK activator metformin or the mitochondrial support agent L-carnitine probably comprise a more effective approach than ROS scavengers such as vitamin E.

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