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Nitric Oxide and Redox Regulation in the Liver: Part II Redox biology in Pathologic Hepatocytes and Implications for intervention

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Abstract

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are created in normal hepatocytes and are critical for normal physiological processes including oxidative respiration, growth, regeneration, apoptosis, and microsomal defense. When the levels of oxidation products exceed the capacity of normal antioxidant systems, oxidative stress occurs. This type of stress, in the form of ROS and RNS, can be damaging to all liver cells, including hepatocytes, Kupffer cells, stellate cells, and endothelial cells, through induction of inflammation, ischemia, fibrosis, necrosis, apoptosis, or through malignant transformation by damaging lipids, proteins, and/or DNA. In part I of this review, we will discuss basic redox biology in the liver, including a review of ROS, RNS, and antioxidants, with a focus on nitric oxide as a common source of RNS. We will then review the evidence for oxidative stress as a mechanism of liver injury in hepatitis (alcoholic, viral, non-alcoholic). In part II of this review, we will review oxidative stress in common pathophysiological conditions including ischemia/reperfusion injury, fibrosis, hepatocellular carcinoma, iron overload, Wilson's disease, sepsis and acetaminophen overdose. Finally, biomarkers, proteomic, and antioxidant therapies will be discussed as areas for future therapeutic interventions.

Keywords

nitric oxide; hepatocytes; oxidative stress; reactive oxygen species; hepatitis; ethanol induced hepatitis

Introduction

Part I of this review discussed the role of reactive oxygen species (ROS) and reactive nitrogen species (RNS) in normal physiological function of hepatocytes including oxidative respiration, cell signaling, and protein modification required for normal cellular growth, regeneration, apoptosis, and microsomal defense. However, ROS and RNS can damage any cells in the liver causing inflammation, ischemia, fibrosis, necrosis, apoptosis, or malignant transformation. Previously, we discussed the pathology of hepatitis as it relates to redox biology in the liver. In Part II of this review, we will discuss the pathology of ischemia/reperfusion injury, fibrosis, iron overload, Wilson's disease, sepsis, and acetaminophen

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overdose as it relates to redox biology. We will also discuss redox proteomics and the potential of antioxidant therapy in the attenuation of disease progression.

Redox in Pathologic Hepatocytes

Ischemia/Reperfusion in Transplantation

Hepatic ischemia/reperfusion injury occurs in two main settings: after liver resection or transplantation due to anoxia or ischemia of the liver itself or due to systemic hypoxia or low flow states associated with sepsis or shock. Warm ischemia/reperfusion is associated with increased oxidative stress and mitochondrial dysfunction resulting in liver failure and/or multi-system organ failure and the extent of injury is related to preexisting conditions of the liver and the duration of insult. Cold ischemia, seen only in transplantation, is associated with reduced oxidative phosphorylation, decreased ATP, and increased glycolysis [19]. Interventions such as preoperative chemotherapy or embolization may make the liver more susceptible to ischemic stress, [137] while preexisting condition such as cirrhosis and steatosis predispose to poor liver function. In the area of liver transplantation, ischemia reperfusion injury is a common cause of primary graft dysfunction and increased mortality and morbidity [33].

The injury seen with ischemia and reperfusion can, at least in part, be attributed to ROS and RNS because there is an increased level of ROS and RNS produced and a consumption of antioxidants with apoptosis and cell death is noted. (Figure 1) During periods of hypoxia, ROS and RNS species are generated, which then cause increased cellular damage [100]. Initially, the mitochondria also become reduced because of alterations in the respiratory chain, as a secondary reaction to hypoxia. This results in reduction of adenosine triphosphate (ATP), causing membrane ion disturbances, including sodium influx due to the inhibition of the ATP-dependent sodium/potassium ATPase. The subsequent influx of sodium can then cause the cell to swell and rupture [10]. Accumulation of intracellular calcium causes activation of cell membrane phospholipase, which causes phospholipid degradation and membrane damage [24,32]. Mitochondria become more permeable, lysosomes are disrupted, membranes are disrupted causing cell leakage, and cells themselves swell [77,180].

During reperfusion, there are two phases of injury: the early/initial phase (usually the first two hours) and late phase. Initially, damage appears to be related to free radical formation by Kupffer cells and is activated by complement and CD4+T lymphocytes followed by cytokine production and PMN infiltration [31,70]. Specifically, xanthine dehydrogenase is metabolized to xanthine oxidase during hypoxia. Upon reperfusion, oxygen reacts with xanthine oxidase to produce ROS [29,100]. This ischemia/reperfusion injury involves APE/Ref-1 and NF-κB, specifically overexpression of APE/Ref-1 that results in decreased oxidative stress, as seen in mice [129]. Kupffer cells also produce cytokines, including interleukin (IL-1) and tumor necrosis factor–α(TNF-α). These cytokines, along with ROS and RNS, are already deleterious to the hepatocytes. TNF-α is of particular importance in ischemia reperfusion injury as it induces expression of adhesion molecules on vascular endothelial cells, stimulates chemokines, and thus recruits neutrophils, which release further ROS and proteases and create further injury [176].

The late phase of ischemia/reperfusion injury is caused by neutrophil activation, ROS, TNF-α, and IL-1B and results in more substantial injury than initially caused by the Kupffer cells. CD4 T-lymphocytes, mediated by TNF-α and IL-1, adhere to hepatic sinusoids and can increase Kupffer cell activation and general cellular recruitment via granulocyte colony-stimulating factor and interferon gamma (INFγ) [183]. There is also upregulation and expression of iNOS, creating large quantities of NO that results in further creation of RNS [66].

Nitric oxide is an important mediator of inflammation, specifically in the hepatocytes themselves during warm ischemia/reperfusion injury [68,69]. iNOS RNA expression is increased approximately one hour after reperfusion, with increased activity noted at five hours after reperfusion [66]. Whether iNOS is protective or deleterious in the setting of ischemia/reperfusion is controversial. For example, iNOS knockout mice develop less warm ischemia/reperfusion injury [91], but the use of non-specific NOS inhibitors, including L-NNA and L-NAME actually increases liver damage [81,174]. It is proposed that ONOO−, the product of superoxide anion and NO by iNOS, may be the cause of the damage [103]. Peroxynitrite can cause lipid peroxidation, inhibition of the mitochondrial respiratory chain, inhibition of Na^+/K^+ ATPase, and/or oxidative protein modifications [25,159]. This difference between the protective and destructive influences may be dependent on the amount and duration of NO exposure, as well as the pre-insult condition of the liver and the level of antioxidants available to consume the ROS and RNS.

eNOS appears to play a role in liver ischemia/reperfusion injury. Basally, eNOS induces vasodilation at the level of the pre-sinusoids and sinusoids [101,112] and also prevents platelet adhesion, thrombosis, and PMN accumulation [37,84,114,141]. During the initial phase of ischemia/reperfusion injury, there is a decreased release of NO from eNOS and a relative vasoconstriction of the microvascular bed. Animal studies have demonstrated that inhibition of NO production from eNOS may contribute to ischemia/reperfusion injury [74]. eNOS knockout mice have increased hepatic damage in ischemia/reperfusion, suggesting a protective role of eNOS in this injury [60]. Genetic overexpression of eNOS also attenuates hepatic ischemia/reperfusion injury as compared to control mice [26,72]. This effect was noted to be ODQ dependent, thus cGC dependent, and HO-1 independent [26]. On the other hand, eNOS knockout mice and eNOS adenovirus treatment resulted in increased ischemia-reperfusion injury in diabetic mice, suggesting that hepatic eNOS is dysfunctional in the setting of diabetes and may lead to increased production of peroxynitrite [28].

In the clinical setting, decreased hepatic eNOS production after orthotopic liver transplantation (OLT) was associated with ischemia/reperfusion injury [169]. Use of inhaled NO in patients undergoing OLT was associated with decreased hospital stay and improved liver function lab tests. Although no changes in inflammatory markers were noted, there was decreased hepatic apoptosis [87]. Use of a NO donor has shown some promise in animal models. Administration of L-arginine, a NO precursor, prior to ischemia/reperfusion injury, was associated with reduced injury and intact microcirculation [166,167]. In mice, intravenous delivery of NO in the form of polyethylene glycol-conjugated bovine serum albumin (PEG-poly SNO-BSA) during ischemia/reperfusion injury resulted in decreased injury and decreased neutrophil infiltration [75].

In organ transplantation, cold ischemia/reperfusion leads to damage of the endothelial cells [68]. In this setting, the use of L-NAME (a nonspecific NOS inhibitor) demonstrated increased hepatic damage while L-NIL (an iNOS inhibitor) did not [38]. Inhibition of iNOS actually leads to increased apoptosis while subsequent infusion of L-arginine led to decreased injury [38]. Use of NO donors has also been associated decreased portal venous pressure and decrease hepatic injury [145]. All of these studies suggest that, in periods of cold ischemia, both iNOS and eNOS may be protective.

Hemorrhagic Shock

Hemorrhagic shock is similar to ischemia/reperfusion injury. In hemorrhagic shock, there is an initial shock phase followed by a reperfusion phase. Initially, due to shock, there is an activation of JNK and an upregulation of cyclooxygenase (COX)-2, CD14, and HIF-1 [53,55]. COX-2 is upregulated by oxidative stress and produces large amounts of prostaglandins. HIF-1 and iNOS are activated by hypoxia and regulate cellular adaptation to

hypoxia by binding to erythropoietin (EPO), vascular endothelial growth factor (VEGF), heme oxygenase (HO-1) and iNOS, resulting in increased erythropoiesis, angiogenesis, vasodilation, and anaerobic metabolism [44]. There is also systemic upregulation of AP-1, NF-κB, p53, release of prostaglandins, and the release of gut-derived products in response to hypoxia [44].

This shock phase is then followed by the reperfusion phase after resuscitation; this phase is similar to that seen in ischemia/reperfusion injury in organ transplantation. During resuscitative, but not unresuscitative, shock, upregulation of G-CSF was noted in rat lungs and liver [58]. Granulocyte colony-stimulating factor (G-CSF) is the cytokine involved in PMN production and activation. Increased product of the cytokines IL-6 and activation of Stat3 was also noted in resuscitative, but not unresuscitated, rats [57,58]. This activation of IL-6 may then lead to increased PMN infiltration, and thus, further release of ROS. The activation of these cytokines during the resuscitative phase, as opposed to the shock phase, suggests one possible area of therapeutic intervention during resuscitations [57].

Animal models of shock have results similar to those of warm ischemia/reperfusion injury, where eNOS appears to be protective while iNOS appear to be detrimental, but the studies available are contradictory. Within 1–2 hours of hemorrhagic shock, mice have increased levels of iNOS [54]. This increase in iNOS is associated with increased cytokine mRNA levels and increased NF-κB and Stat3 activation [56]. This increase in oxidative stress is also with a decrease in the levels of antioxidants, including vitamin E, ascorbic acid, and α-tocopherol and also with an impairment of antioxidant capacity, as noted by decreases in superoxide dismutase, catalase, and glutathione peroxidase [53].

In animal models, mice given iNOS inhibitors (L-NIL) or iNOS knockout mice had decreased NF-κB, cytokine production, and infiltration of PMN after resuscitation, [56] possibly due to the decreased production of $ONOO^-$ [53]. Rats given NOS inhibitors had improved survival, which decreased with the addition of L-arginine [64,102,107]. On the other hand, other studies demonstrated that the administration of L-arginine during hemorrhagic shock was associated with a survival benefit [22]. Rodents given the NO scavenger, NOX, had decreased pulmonary and hepatic injury, decreased inflammation as measured by PMN infiltration, and improved survival [55,59,106]. However, the use of an NO donor has also been shown to improve hepatic microcirculatory perfusion and decreased hepatic inflammation in a hemorrhagic shock model [7]. These contradictory results may be due to different experimental designs with different concentration, routes, and timing of pharmacological interventions and various outcome measures. Even though the results of these studies do not clearly elucidate the mechanism by which NO is related to hemorrhagic shock and resuscitation, it is clear that NO does play a role of some sort in the oxidative stress created and thus serves as a potential therapeutic target.

Sepsis

Sepsis is a clinical syndrome characterized by fever, tachycardia, tachypnea, and leucocytes increases as a result of a systemic inflammatory response secondary to a blood stream infection. Infectious organisms including bacteria, viruses, and parasites are known to induce ROS/RNS including NO. Sepsis involves a diffuse inflammatory response with effects on various organ systems, depending on the virulence organism and the inflammatory response of the host. This discussion will focus on the role of NO on hepatocytes in sepsis.

NO in the liver is protective against these microbial invaders in many ways. In sepsis, NO is able to invade these organisms and cause damage to lipids, proteins, enzymes, and DNA [161]. Nitric oxide ameliorates the oxidative stress from xenobiotics such as hypochlorite

(ClO−) and carbon tetrachloride (CCL_4) by decreasing lipid peroxidation and collagen deposition [118]. Treatment with L-arginine was found to attenuate collagen formation, increase in liver enzymes, and glycogen depletion, but had no effect on lipid peroxidation [118]. IFN-γ is able to decrease intracellular plasmodial parasites in malaria-infected hepatocytes [105], while the addition of arginase and L-NMMA ameliorates this effect [105,124].

In septic shock, NO has been shown to be protective. Specifically, nonspecific NOS inhibition (L-NMMA and L-NAME) results in increased hepatic damage, while NO donors ameliorate this damage [8,47,48,65,73]. Although the results are not exactly clear, inhibition of iNOS has been noted to increase hepatic apoptosis [128] and limit PMN accumulation [52]. iNOS expression is necessary for immune-mediated liver injury in mice [148] and NO scavengers (NOX) reduced LPS-induced liver damage [119]. It is, again, likely to be related to the source, amount, and duration of NO exposure, as are the beneficial versus detrimental effects of NO in sepsis. Endothelial NO appears important to the maintenance of vascular homeostasis and the prevention of WBC and platelet aggregation. NO from Kupffer cells, on the other hand, is associated with the increased production of TNF-α. Specifically TNF-α/D-Gal itself can lead to hepatic damage [148].

Initially it was thought that inhibitors of iNOS may improve outcomes in shock, specifically in septic shock, by decreasing the activation of downstream cytokines. Unfortunately, the results were not as expected. While smaller studies had suggested a potential benefit of NOS inhibitors in cardiogenic shock, [20] these results were not seen in larger studies. In a study of septic shock, L-NMMA was noted to cause an increase in vascular tone and blood pressure; however, it also caused a reduction in cardiac output, which is of concern for overall tissue perfusion [133]. Nonselective NOS inhibition was examined again in septic patients and was associated with an increase in mortality [95]. Most recently, L-NMMA was used to treat patients with refractory cardiogenic shock, but the study was terminated because of futility [1]. Therefore, while iNOS is associated with increased inflammatory response, inhibition of NO in the clinical setting does not seem to improve outcomes. Better understanding of the roles of NO and the signaling pathways involved in shock are necessary in order to develop more effective treatment options.

Fibrosis

Hepatic fibrosis is a condition associated with increased extracellular matrix (ECM) proteins, including collagen type I and III, proteoglycans, fibronectin, and laminin. Under normal conditions, matrix metalloproteinases (MMPs), produced by stellate cells, regulate the production of the ECM. In pathologic conditions with noted fibrosis, the deposition of collagen exceeds the ability of MMPs to absorb it and thus, extra collagen deposits and scars form. Fibrosis has been well documented in many chronic liver diseases, including hepatitis, cholestatic disease, hemochromatosis, steatohepatitis, and chronic venous outflow obstructions. These conditions usually begin with an inflammatory phase, which progresses to fibrosis after chronic oxidative stress [85].

During the inflammatory phase, cytokines, chemokines, and ROS are then followed by PMN infiltration and iNOS upregulation. Lymphocytes are also recruited via the portal tract, sinusoids, and hepatic vein. Leukocytes and Kupffer cells are able to produce large amounts of NO and cytokines, especially TGF-β and TNF-α. The influx of Kupffer cells coincides with the activation of stellate cells. Activation leads to increased proliferation, motility, contractility, and synthesis of ECM.

Oxidative stress can activate stellate cells, which are collagen-producing cells in the liver. Specifically, 4-hydroxynonenal is a profibrotic stimulus, which upregulates procollagen and

tissue inhibitor metalloproteinase-1 (TIMP1) gene expression [181]. Stellate cells have also been implicated in the regulation of blood flow and tone in the liver. Capillarization of the sinusoids is a condition in which ECM proteins deposit in the sinusoids of the liver. This deposition is a result of stellate cells and has been is associated with portal hypertension and fibrosis [27]. In fibrosis, it appears this loss of parenchyma precedes the onset of venous obstruction, so therapeutic interventions aimed at the prevention or treatment of fibrosis requires an understanding of the activation and action of stellate cells.

The exact mechanism of stellate cell activation is unclear, but associated proteins have been identified. It appears that a zinc finger molecule, KLF6, is involved in activation of many cells, including stellate cells [142]. DDR2 has also been implicated in the upregulation of MMP expression and downstream activation of stellate cells [127]. Stellate cells themselves are also able to express immune proteins (HLA-II), to internalize macromolecules, and to affect T cell activation. In cirrhosis patients, in particular, HLA-II and CD40 expression is noted [172]. Stellate cells can process antigens and are implicated in antigen presentation, internalization of macromolecules, and modulation of T-lymphocyte proliferation [172].

Cytokines play a critical role in the formation of fibrosis. TGFβ is produced by the Kupffer and stellate cells. TGFβ mediates the further activation of stellate cells and the influx of PMS, both of which are necessary for fibrosis formation. TGFβ then upregulates collagen gene expression, expression of TIMP-1, and further expression of TGFβ. Activation of TGF receptors lead to phosphorylation of SMAD proteins. TGF β may be stimulated by IL-6 and ROS in cirrhotic patients, which suggests that therapeutic intervention involving the production of TGFβ or the stimuli of TGFβ may prove beneficial in patients with hepatic fibrosis.

The role of NO in fibrosis formation is not as clearly understood. In patients with cirrhosis, increased resistance of portal blood flow has been associated with impaired NO bioavailability. In animal models, shear-induced NO production from eNOS was attenuated compared to that of control mice. Decreased NOS activity, with increased eNOS-caveolin binding, was also noted although eNOS protein levels were the same as controls [154]. This suggests a decrease in the function of eNOS, resulting in a relative state of vasoconstriction. ROS are also implicated in reduced NO bioavailability in cirrhotic livers [43]. Koreuk et al. noted a decreased SOD level and increased serum NO level in decompensated cirrhosis patients, compared to compensated cirrhosis patients and healthy controls, indicating an antioxidant dysregulation [83]. This implies that reduction of ROS by inhibition of oxidative stress may be clinically beneficial in the prevention of further fibrosis. Continued research is needed to thoroughly understand the process of fibrosis; the roles played by cytokines and NO need to be established before meaningful therapeutic targets can be identified.

Iron overload

Excessive accumulation of iron can result from multiple conditions, including hemochromatosis, hereditary sideroblastic anemias, severe α and beta thalassemia, myelodysplastic syndrome (MDS), and chronic liver diseases. The excess accumulation of iron disturbs the redox equilibrium in the liver and results in oxidative stress. Specifically, accumulation of iron can cause increased oxidation of membrane lipids and subcellular organelles, such as the mitochondria, leading to structural and/or functional impairment and cellular injury [5]. Iron is a transition metal that, when it reacts with oxygen via the Haber-Weiss reaction, is able to create reactive oxygen species. (Table 1) The catalytic properties of iron may also lead to exacerbation of the cytotoxic effects of other ROS-generators, such as chemicals including alcohol, viruses, or other toxins. For example, Tsukamoto et al. noted that animals with alcohol infusions and iron supplementation had increased hepatocyte damage, liver fibrogenesis, and cirrhosis, suggesting a significant interaction between iron

and iron-catalyzed oxidant stress noted from lipid peroxidation, increases in procollagen α1, and TGFβ1 [165].

The reactive oxygen species created from the Haber-Weiss reaction can then cause lipid peroxidation and the formation of lipid hydroperoxides forms peroxyl-($ROO^{\cdot}$) and alkoxyl-($RO^{\cdot}$) free radicals. In cases of iron overload, relatively high levels of malonaldehyde and low levels of other toxic hydroxyalkenals are noted [123,138]. High level of etheno-DNA adducts, a measure of lipid peroxidation, are also noted in these patients. Some patients with hemochromatosis also have increased iNOS expression in the liver, suggesting another source of RNS, although there are limited data on the role of NO in the hemochromatosis [67]. In patients with hemochromatosis, high HNE-protein and MDA-proteins levels are also noted [126]. Chronic accumulation of iron is associated with low cytolysis, progressive fibrosclerosis, cirrhosis, and hepatocellular carcinoma. In animal models, increased iron deposition is associated with increased lipid peroxidation and collagen type 1 deposition [6]. This pattern of iron deposition, occurring mainly in the periportal hepatocytes in rats, closely approximates that of hereditary hemochromatosis seen in humans [131,136].

Hemochromatosis is associated not only with increased markers of lipid peroxidation but also with decreased levels of antioxidants, including ascorbate, retinol, and α-tocopherol [179]. This iron-mediated toxicity and fibrosis seen in the animal model may be attenuated by antioxidant treatments, including vitamin E or flavonoid silibin [135]. In animal models of iron overload, enhanced TGF-β1 expression was also attenuated by antioxidant treatment [63,135].

The excess iron may also result in DNA damage from oxidative stress leading to an increased incidence of cancer. The incidence of hepatocellular carcinoma is increased 200-fold in patients with hemochromatosis [122]. Increased p53 mutations in hepatic tissue from hemochromatosis patients strongly suggest a correlation between oxidative damage from iron overload and cancer formation, but further research is necessary to fully understand the mechanism by which this occurs [67,96]. Morrogi et al. [67] noted various p53 mutations such as G:C to T:A transversions at codon 249 and C:G to A:T and C:G to T:A changes at codon 250 in patients with hemochromatosis or Wilson's disease. While the relationship between p53 mutation and oxidative stress is compelling, a more complete understanding of the role of oxidative stress and carcinogenesis is needed.

Wilson's Disease

Wilson's disease is an autosomal recessive condition characterized by impaired biliary excretion, and thus, excessive hepatic deposition of copper. It is believed that this accumulation of copper results in excessive oxidation and free radical generation, resulting in hepatic damage. As the hepatocytes are damaged, copper spills into the blood and deposits in other organs including the brain, heart and kidney, resulting in subsequent end-organ impairment. As copper enters the blood, ceruloplasmin levels decrease as copper is bound. It is believed that excess copper leads to increased free radical generation, but this is still only theory. Sixty percent of patients with Wilson's disease were also noted to have increased iNOS expression, another possible source of oxidative stress other than the copper itself [67]. These patients also had increased levels of allantoin, an oxidation product of uric acid, and decreased levels of antioxidants including ascorbate and urate [125]. Increased lipid peroxidation and copper content have been documented and the mitochondrial copper content in these samples correlated with the severity of mitochondrial lipid peroxidation. Hepatic α-tocopherol, an antioxidant, was decreased significantly in hepatocytes of Wilson's disease patients [155]. Hussain et al. also noted increased p53 mutation in nontumorous hepatocytes in Wilson's patients [67]. Nair et al. showed a correlation between etheno adducts, DNA damage, and copper content in patients with Wilson's disease. Limited data

are available on this small group of patients, but it appears that, due to excess copper accumulation, they are under increased oxidative stress, as seen by measures of lipid peroxidation and DNA damage; this stress may then lead to increased p53 mutation and possibly cancer formation.

Acetaminophen-induced Liver Damage

Acetaminophen (paracetamol) overdose is an uncommon, but potentially devastating, condition characterized by centrilobular hepatic necrosis after excessive consumption of acetaminophen [9,45]. Acetaminophen is metabolized by sulfation and glucuronidation and is a substrate for CYP2E1. Acetaminophen reacts with CYP2E1 creating quinoneimine, which binds to −SH groups, especially those on glutathione. As glutathione levels decrease, quinoneimine then binds other plasma membrane proteins, mitochondrial proteins, and other protein complexes and this leads to increased intracellular calcium and cell death [46,140].

NO produced from macrophages, cytokines, superoxide, and peroxynitrite are all implicated in this damage [61,62,88,89]. The destruction of Kupffer cells by gadolinium chloride ameliorates this damage [109]. By the depletion of GSH, the disruption of the mitochondria, and the formation of $ONOO^-$, acetaminophen is able to cause quite an oxidative stress on hepatocytes [80]. iNOS knockout mice and aminoguanidine treated rats showed decreased hepatic damage, [35,36] although complete inhibition of NOS by L-NMMA demonstrated increased liver damage. Treatment with a liver–selective NO donor (V-PYRRO/NO) also demonstrated decreased lipid peroxidation and hepatic damage [94], which leaves the exact role of NO in acetaminophen induced liver damage still in question.

Acetaminophen overdose is treated with N-acetylcysteine (NAC), a precursor of glutathione. NAC increases glutathione stores, limits the formation of NAPQI by direct binding and increases sulfate conjugations [12,92,111]. NAC also functions as an anti-inflammatory and antioxidant by modulating cytokines and scavenging free radicals [49,76]. NAC is also able to improve hemodynamics and oxygen transport in patients with fulminant hepatic failure [50]. The key to treatment is prompt diagnosis and treatment with NAC. Without immediate intervention, substantial hepatic necrosis may develop, necessitating a liver transplant.

Implication for future interventions

Biomarkers/Proteomics

In order to identify and treat hepatic conditions associated with increased oxidative stress, it is important to identify the genes/proteins whose expression/function serves as markers of oxidative stress. The lack of thorough understanding of the mechanism by which oxidative stress results in hepatic injury limits the identification of unique therapeutic targets. Nonetheless, current proteomic strategies, especially in the emerging area of redox proteomics, promise further understanding of possible molecular markers of disease and future therapeutic targets.

Biomarkers of oxidative stress (lipid peroxidation, DNA damage, certain protein isolation/expression) are helpful in identifying a physiologic state over time. The measurement of biomarkers allows elucidation of the presence of oxidative stress but not necessarily the etiology of that stress. The presence of oxidative stress may be suggestive of causation but, by using biomarkers alone, only a correlation, not causation, can be drawn. Many of the studies previously mentioned in this review of liver pathology noted only the correlation between markers of oxidative stress and liver pathology so, while causation can be inferred, it cannot be accepted as proven fact.

In addition to biomarkers, one can also measure gene expression in relation to oxidative stress. Current research is focusing on the specific gene modifications that are known to be associated with redox biology, such as c-jun [21] and NF-κB [160]. This approach allows close examination of one gene during various periods of stress, but limits the researcher to the gene already identified as being of interest [116]. Rigorous examination of individual genes is necessary for systematic knowledge of specific interactions, but the complexity of convergent signaling cascades is lost. Identification of new genes not previously known to be associated with oxidative stress is also difficult.

In order to obtain a more complete understanding of gene regulation as it relates to oxidative stress, one may utilize DNA microarrays or proteomics in order to see the effects of a particular oxidative stress on the expression of thousands of genes or proteins. The use of proteomics for the study of molecular modifications secondary to oxidative stress may be termed redox proteomics. One of the first studies that focused on redox proteomics examined the effects of oxidative stress on human epithelial lens cells and noted that several cytoskeletal proteins and enzymes that showed differential expression profiles as a result of H_2O_2 oxidation [132]. Subsequently, Vascotto et al. performed a proteomic analysis of liver tissue subjected to early ischemia/reperfusion injury during human liver transplantation and found 36 proteins that were significantly altered upon injury [171]. These proteins tended to be those involved in lipid and energy metabolism, in different metabolic pathways, in redox signaling, and in oxidative-stress response [171] (Table 2). Some of these proteins were previously known to have been associated with ischemia/reperfusion injury while others were not. Identification of these proteins allows for further understanding of the early response of the liver to reperfusion and identification of future therapeutic targets of early damage from oxidative stress.

Avellini et al. also examined the effects of oxidative stress on protein profiles of hepatic tissue and noted modification in multiple peroxiredoxin proteins including PrxI, PrxII, PrxVI [4]. These proteins function as hydrogen peroxide scavengers, so it is not surprising to see modifications after hydrogen peroxide treatment. Further studies that more closely approximate warm and cold ischemia are needed to more closely simulate the actual oxidative stress experienced by the cells and to make further inferences as to the implications for protein and gene modifications.

Further studies are needed to elucidate the role of the above noted proteins in ischemia/reperfusion injury and the interaction between these proteins and the known signaling pathways involved in oxidative stress. Future studies in redox proteomics are also needed in other liver pathologies, such as alcohol induce liver injury, viral hepatitis, and hepatocellular carcinoma.

Antioxidants

While endogenous antioxidants are critical for the regulation of hepatic homeostasis, exogenous antioxidants have been of limited usefulness in the treatment of hepatic injury. Studies, as outlined below, have used antioxidants to prevent and/or treat liver injury related to ROS but have had mixed results. [40] The data are difficult to interpret due to mixed and/or varied patient populations, different types and amounts of antioxidant therapy, and differing outcome measures, but some of these studies show the promise of antioxidants for amelioration of hepatic injury arising from oxidative stress.

Alcohol-Induced Liver Disease

To treat alcohol-induced liver disease, some researchers have focused on the administration of a single antioxidant, while others have advocated for combination antioxidant therapy.

The type of antioxidant is usually driven by previous studies, indicating a relative antioxidant deficiency in patients with this condition.

In a promising study, Wenzel et al. treated patients suffering from acute alcohol-induced hepatitis with D-α tocopherol, selenium, and zinc. Compared to controls, the patients receiving adjuvant antioxidant therapy had decreased mortality from 40% to 6.5% [177]. Results from other studies investigating the potential of combined antioxidant therapy on progression of alcohol-induced liver disease were not as promising. Treatment of severe alcoholic hepatitis with a combination of antioxidants versus corticosteroids noted a decreased short term (1 month) but not a longer term (1 year) mortality and sepsis in the corticosteroid treated patients [134]. This study did not have a steroid and antioxidant group; thus, an appropriate control group was lacking, until Stewart et al. examined the effects of antioxidant therapy alone or with corticosteroids in severe alcoholic hepatitis [157]. The antioxidant therapy included N-acetylcysteine, vitamin A-E, biotin, selenium, zinc, manganese, copper, magnesium, folic acid, and coenzyme Q, none of which improved 6-month survival when given alone or in combination with corticosteroids.

Vitamin E supplementation for alcohol-induced liver disease does not look promising as a therapeutic intervention. de la Maza conducted a study treating decompensated ambulatoryalcoholic cirrhotic with vitamin E supplementation and found no change in hepatic laboratory values, mortality, or hospitalization rates in these patients [23]. Treatment of mild to moderate alcoholic hepatitis with vitamin E was found to decrease hyaluronic acid level but showed no other improvement in liver function test [108]. A meta-analyses by Miller et al. noted an increase in all-cause mortality after high-dose vitamin E supplementation in patients with a variety of medical conditions, but the significance of this finding is unclear with such a large heterogeneity of patients [110].

S-adenosylmethionine (SAMe) is an antioxidant that functions as a methylating agent, a precursor for glutathione, and a modulator of cytokine metabolism. Patients with alcohol-induced liver disease treated with SAMe were noted to have improved mortality and decreased need for transplantation [99]. Specifically, when patients with severe liver disease (Child class C) were excluded, overall mortality/liver transplantation was significantly greater in the placebo group (29%) verses the SAMe group (12%) and the differences lasted through 2 year follow-up [99].

These studies, taken in combination, suggest that treatment of alcoholic hepatitis is probably not improved with vitamin E therapy but may be improved with S-adenosylmethionine (SAMe) or a combination of antioxidant therapy but, due to the variability of the studies, firm conclusions cannot be drawn as yet.

NAFLD

In patients with NAFLD, antioxidant therapy has also been used in varying degrees to improve biochemical or clinical evidence of hepatitis. Pamuk & Sonsoz treated NAFLD patientswith N-acetylcysteine and noted an equivocal biochemical responses [130]. Other studies noted that patients treated vitamin E were able to normalized their serum aminotransferase [90], while vitamin E and C in combination were able to decrease fibrosis scores at 6 months [51]. Sanyal et al. evaluated Vitamin E versus vitamin E plus pioglitazone for NAFLD and noted decreased steatosis in both groups, but noted an improved pericellular fibrosis, Mallory's hyalin, and glucose clearance only in the combination group [147]. Vitamin E and/or adherence to a low-calorie diet was also associated with normalization of transaminases in children with obesity-related NAFLD [168], although a study examining metformin versus Vitamin E or a prescriptive diet in NAFLD patients noted an improvement in the metformin arm, with no benefits noted with

vitamin E supplementation. A Cochrane review noted that, due to the small number of trials and to the variability in the methods, assessments, treatments and outcomes, no conclusions could be drawn on the use of antioxidants for the treatment of non-alcoholic fatty liver disease [93].

Viral Hepatitis

For patients with Hepatitis C, ROS/RNS have been strongly implicated in the onset and progression of disease and its malignant transformation, but trials of antioxidants in animals and patients to attenuate HCV infection have had mixed results. von Herbay noted that vitamin E improved aminotransferase status of patients, although no long term follow-up is available [173]. Look et al. found that treatment of HCV infected patient with IFN-α plus vitamin E versus IFN-α alone resulted in a trend toward more complete recovery and decreased viral load in the IFNα plus vitamin E patients. These studies are limited by their size and significance but suggest interesting areas for further study.

The most substantive human trials of antioxidants for chronic HCV infection used a combination of oral antioxidants (glycyrrhizin, schisandra, silymarin, ascorbic acid, lipoic acid, L-glutathione, α-tocopherol) in chronic HCV patients and noted improvement in liver enzymes (44%), decreased viral load (25%), histologic improvement (36%), and improved quality of life [104]. Due to the success of this trial, a phase 2 randomized, double-blinded placebo controlled trial examined the use of the IV and/or oral antioxidants versus placebo in chronic HCV patients. An improvement in liver enzymes, improvement in histology, and a decrease in viral load was found in patients receiving combined oral and intravenous antioxidant therapy but not in patients receiving oral therapy alone [34]. While the route of administration is at issue between these studies, these results do suggest a beneficial effect of antioxidant therapy. Further studies are needed to properly elucidate the types, amounts, and routes of antioxidant drug administration that are most appropriate.

Ischemia/Reperfusion Injury

There have been many antioxidants that have been shown beneficial effects in ischemia reperfusion injury in animals, although few of these have been properly studied in humans. Vitamin E, often used interchangeably with α-tocopherol, is a nutritional term referring to eight different substances with similar fat-soluble properties and biochemical properties. The most effective form of vitamin E is RRR-α-tocopherol (alpha-tocopherol), which is the most biologically active form. Alpha-tocopherol is an antioxidant that functions by scavenging free radicals, increasing GSH, inhibiting protein kinase C, and attenuating lipid peroxidation [11,14,98,143]. Alpha-tocopherol is decreased after reperfusion and pretreatment with α-tocopherol improves ATP levels, prevents lipid peroxidation product formation, attenuates glutathione loss, and increases survival [42,97,156]. Alpha-tocopherol analogs also show beneficial effects, as does the combination of α-tocopherol with pentoxyphylline [30,170] (Table 3).

Vitamin C supplementation has had mixed results in the prevention of ischemia/reperfusion injury. Vitamin C, also known as ascorbic acid, is synthesized from l-gulono-γ-lactone and oxygen thus creating l-ascorbate and hydrogen peroxide. This reaction occurs in plants and some animals, but humans are not able to synthesize ascorbate so intake is diet-dependent. In addition to regular dietary intake, low doses of vitamin C supplementation have been shown to decrease liver peroxidase activity while high dose vitamin C has been shown to increase ischemia/reperfusion injury, perhaps from the excess reduction of iron [153]. One clinical study demonstrated improved PT time and decreased complication in patients supplemented with an antioxidant vitamin infusion containing α-tocopherol acetate and ascorbate after liver resection [15] (Table 3). Currently, studies on vitamin C

supplementation are preliminary with mixed results. Further systemic evaluation is necessary before conclusions can be drawn on the potential benefits of supplementation.

Melatonin is another antioxidant that is normally produced in the pineal gland and is known to regulate circadian rhythms. The precursor is serotonin, which, once methylated and acetylated, becomes melatonin. It has mild antioxidant effects through inhibitory action on lipid peroxidation. Melatonin was noted to inhibit iNOS, reduce TNF-α, and preserve functional status in mice [144]. When melatonin was given to a small cohort of patients after liver resection, there was enhanced neutrophil apoptosis and neutrophil responsiveness[17]. Again, these studies are small and need to be followed in larger clinical trials with measures of clinical outcomes beforeconclusions can be drawn.

Glutathione is a ubiquitous tripeptide important in many metabolic processes, including ascorbate metabolism, gap junction communication, and antioxidant protection of thiol-protein groups. Glutathione can react with hydroxyl radicals, hypochlorous acid, peroxynitrate, and carbon radicals, but not with superoxide radicals. Because glutathione reacts with so many reactive oxygen species and reactive nitrogen species, it is an effective antioxidant and its measurement can be a useful assessment of redox biology. Glutathione peroxidase is a major antioxidant enzyme in the cell and thus the administration of GSH would be expected to produce beneficial antioxidant effects. Unfortunately, initial studies have not shown any protective effects in the setting of warm ischemia[18], which may be due to limited cellular uptake of the large molecule. On the other hand, recent studies have noted a protective effect of intravenous large doses of glutathione in the prevention of warm and cold ischemic injury [149,150]. N-acetylcysteine is the precursor to glutathione and can be administered with effective uptake, but the effects on ischemia-reperfusion injury in the liver are unclear [16,82]. NAC acts by allowing GSH synthesis, cysteine metabolism, and the scavenging of free radicals [115]; thus, it is used to treat a variety of conditions including cystic fibrosis and acetaminophen overdose. In rabbit models, intravenous NAC administration improved tissue oxygenation, microcirculation, alanine aminotransferase activity, and indocyanine green clearance [41]. Clinical trials of intravenous NAC have also had equivocal results, but three studies did document improved liver function, decreased liver injury, less graft dysfunction, less reject, and/or decreased hospital stay [13,162,175] (Table 3).

SOD treatment has also shown mixed results, probably again due to the difficulty of getting SOD into the cells. SOD is not known to cross cell membranes but in order for it to consume superoxide, it would have to enter the cell or superoxide would have to leave the cell. Of the four animal studies conducted, two showed beneficial effects [3,120] while two showed no effect [86,113].

Inhibitors of iNOS have shown promising results in animal models of ischemia reperfusion injury [69,78]. In a pig model of warm ischemia, iNOS inhibition decreased aspartate aminotransferase levels after reperfusion, inhibited nitrotyrosine expression, and attenuated hepatic damage [69]. In a pig model of cold ischemia, iNOS inhibition resulted in decreased serum nitrate/nitrite and AST. There were also decreased hepatic damage, thrombi, and iNOS staining compared with control animals [78]. The theory is that increased expression of iNOS leads to increased peroxynitrite ($ONOO^-$) production that is out of proportion to the antioxidant capacity of the liver and that leads to damaging peroxynitrite production and cellular damage. Targeting peroxynitrite production through superoxide dismutase, iNOS, or peroxynitrite scavengers may prove beneficial.

Other antioxidant agents that have been shown to have beneficial effects in liver ischemia reperfusion injury include coenzyme Q/pentoxyfylline [139], idebenone [151], lipoic acid

[117], desferrioxamine [131], trimetaxidine [163], quercetin [158], cyanidin [164], plant extracts [164,182], bucillamine [2], SOD derivatives [121], CAT derivatives [178], allopurinol [71], and aminoguanidine [78]. (Table 3) Allopurinol at high [71], but not low doses [70], have shown some beneficial effects in preventing liver damage, but again, the results are preliminary, with more systematic in depth evaluation needed.

Conclusion

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are clearly important for normal physiological function of hepatocytes and are involved in physiological metabolism including oxidative respiration, cell signaling, and protein modification required for normal cellular growth, regeneration, apoptosis, and microsomal defense. However, when production of ROS/RNS overwhelms consumption, oxidative stress develops. In the form of ROS and RNS, this stress can damage all of the cells present in the liver, including hepatocytes, Kupffer cells, stellate cells, and endothelial cells, by induction of inflammation, ischemia, fibrosis, necrosis, apoptosis, or malignant transformation through damage to lipids, proteins, and/or DNA. This is seen in the setting of hepatitis, ischemia/reperfusion injury, fibrosis, iron overload, Wilson's disease, sepsis, and acetaminophen overdose, although the exact mechanism(s) by which ROS/RNS results in the clinical syndromes seen is unclear. Increased protein modification/degradation is seen, but the functional roles of these modifications and their role in signal transduction is poorly understood. Upregulation of cytokines and chemokines both induce and are induced by ROS/RNS, although again the mechanisms are unclear. Further thoughtful, systematic investigation is needed to better understand the role of ROS/RNS in liver disease. In order to better appreciate the role of redox biology in protein and gene expression, redox proteomic and micoarray gene analysis may be able to clarify the complex role of oxidative stress. Continued research is also needed in the areas of antioxidant therapy, in order to appreciate the impact of oxidative stress on disease, and thus, the potential of antioxidant therapy in the attenuation of disease progression.

Abbreviations

AP	apurinic/apyrimidinic
AP-1	activator protein-1
APE/Ref-1	apurinic/apyrimidinic endonuclease/redox factor 1
ATP	adenosine triphosphate
ALT	aminotransferase
Bcl-2	B-cell lymphoma-2
BER	base excision repair
BH_4	tetrahydrobiopterin
Bid	A BH3 domain-only death agonist protein
CaM	calmodulin
CAT	catalase
cGMP	cyclic guanosine monophosphate
COX-2	cyclooxygenase-2
CSF	colony stimulating factor

DNA	deoxyribonucleic acid
ECM	extracellular matrix
EPO	erythropoietin
eNOS	endothelial nitric oxide synthase
ESR	electron spin resonance
FAD	flavin adenine dinucleotide
FMN	flavin mononucleotide
γ-IRE	γ-interferon response element
G-CSF	granulocyte colony stimulating factor
GSH	glutathione
GSSG	glutathione disulfide
GST	glutathione S-transferase
HBV	hepatitis B virus
HCC	hepatocellular carcinoma
HCV	hepatitis C virus
HIF	hypoxia-inducible factor
HO-1	heme oxygenase
HNE	4-hydroxynonenal
HSP70	heat shock protein 70
IL	interleukin
INFγ	interferon gamma
im	intramuscular
iNOS	inducible nitric oxide synthase
iv	intravenous
JNK	c-Jun NH2-terminal kinase
KLF6	a zinc finger molecule
L-NIL	N-iminoethyl-L-lysine
L-NMMA	L-N(G)-monomethyl arginine citrate
L-NNA	L-N^{ω}-nitro-L-arginine
LPS	lipopolysaccharides
MAP kinase	mitogen-activated protein kinase
MAT	methionine adenosyltransferase
MDA	malondialdehyde
MDS	myelodysplastic syndrome
MEOS	microsomal ethanol-oxidizing system
MMP	matrix metalloproteinases

MnSOD	manganese-containing superoxide dismutase
MS	methionine synthase
NAC	N-acetylcysteine
NADQI	*N*-acetyl-*p*-benzoquinone imine NAFLD, non-alcoholic fatty liver disease
NASH	nonalcoholic steatohepatitis
NOX	nitric oxide scavenger
nNOS	neuronal nitric oxide synthase
NADH	reduced nicotinamide adenine dinucleotide
NADPH	reduced nicotinamide adenine dinucleotide phosphate
NF-κB	nuclear factor-kappa B
NO	nitric oxide; reactive halogen species
NS5A	non-structural 5A protein
ODQ	1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one
OLT	orthotopic liver transplant
PEG-poly SNO-BSA	polyethylene glycol-conjugated bovine serum albumin
PMN	polymorphonuclear leukocytes
po	oral
PT	prothrombin time
RHS	reactive hydrogen species
RNA	ribonucleic acid
ROS	reactive oxygen species
RNS	reactive nitrogen species
SAMe	S-adenosylmethionine
sGC	soluble guanylate cyclase
SMAD	mothers against decapentaplegic
SOD	superoxide dismutase
TGFα/β	transforming growth factor α/β
TIMP1	tissue inhibitor metalloproteinase-1
TNFα/β	tumor necrosis factor α/β
TRAIL	TNF related apoptosis inducing ligand
UV	ultraviolet
VEGF	vascular endothelial growth factor
1400W	N-(3-aminomethyl)benzyl-acetamindine
8-OH-dG	8-hydroxydeoxyguanosine

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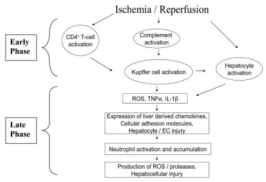

Figure 1.

During ischemic/reperfusion injury, there is initial complement and t-cell activation, which leads to reactive oxygen species, TNFα, and IL-1β production. During the late phase, there are chemokines, neutrophil activation, more reactive oxygen species, and hepatocellular injury. Reprinted by permission from Glantzounis GK, Salacinski HJ, Yang W, Davidson BR, Seifalian AM. The contemporary role of antioxidant therapy in attenuating liver ischemia-reperfusion injury: a review. Liver Transpl. 11:1031–1047,2005. [40] Copyright 2008 American Association for the Study of Liver Diseases. Reprinted with permission of Wiley-Liss, Inc., a subsidiary of John Wiley & Sons, Inc.

Table 1

Chemical equations relevant to reactive oxygen and reactive nitrogen species generation.

Reactive oxygen species generation
$O_2 + e^- \rightarrow O_2^{-\bullet}$ (superoxide anion)
$O_2^{-\bullet} + H_2O \rightarrow HO_2^{\bullet}$ (hydroperoxyl radical)
$HO_2^{\bullet} + e^- + H \rightarrow H_2O_2$ (hydrogen peroxide)
$H_2O_2 + e^- \rightarrow OH^- + {}^{\bullet}OH$ (hydroxyl radical)
Reactive nitrogen species generation
L-arginine + $O_2 \rightarrow {}^{\bullet}NO$ (nitric oxide) + L-citrulline
$O_2^{-\bullet} + {}^{\bullet}NO \rightarrow ONOO^-$ (peroxynitrite)
$ONOO^- + CO_2 \rightarrow ONOOCO_2^-$ (nitrosoperoxy carbonate)
$ONOOCO_2^- \rightarrow {}^{\bullet}NO_2$ (nitrogen dioxide) + $CO_3^{-\bullet}$ (carbonate anion radical)
Fenton reaction (catalyzed by transition metals)
$H_2O_2 + Fe^{2+} \rightarrow OH^- + {}^{\bullet}OH + Fe^{3+}$
Haber-Weiss Reaction (catalyzed by transition metals)
$H_2O_2 + O_2^{-\bullet} \rightarrow O_2 + {}^{\bullet}OH + OH^-$

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Table 2

Identification of various proteins upregulated by redox stress in hepatocytes via redox proteomics. Reprinted with permission from Vascotto C, Cesaratto L, D'Ambrosio C, Scaloni A, Avellini C, Paron I, Baccarani U, Adani GL, Tiribelli C, Quadrifoglio F, Tell G. Proteomic analysis of liver tissues subjected to early ischemia/reperfusion injury during human orthotopic liver transplantation. Proteomics. 6:3455–3465,2006. Copyright Wiley-VCH Verlag GmbH & Co. KGaA. [171]

Protein Number	Protein identity	Swiss-Prot entry	MW	P/	% Seguence coverage	T_2 *vs* T_1, ratio	#of patients/9	Function	Subcellular localization
a) Lipid metabolism									
1	3,2 *trans* enoyl CoA isomerase	P42126	35 (33)	5.8 (8.8)	39	4.79	2	Lipid metabolism	Mitochondrial
2	Apolipoprotein A1	P02647	30 (31)	5.0 (5.6)	38	14.45	2	Lipid metabolism	Plasma
3	ACADV very long chain	P49748	70 (70)	7.4 (8.9)	19	1.85	1	Lipid metabolism	Mitochondrial
4	HCD2	Q99714	30 (27)	6.9 (7.7)	34	2.09	1	Lipid metabolism	Membrane
5	Long-chain-fatty acid CoA ligase 1	P33121	80 (78)	6.4 (6.8)	32	0.42	1	Lipid metabolism	Membrane
b) Energy metabolism									
6	Fructose-biphosphate aldolase B	P05062	40 (39)	5.9 (8.1)	49	5.68	3	Glycolysis	Cytoplasmic
7	G3P2	P04406	16 (36)	5.9 (8.6)	29	Q	1	Glycolysis	Cytoplasmic
8	Methyl malonate semialdehyde dehydrogenase	Q02252	30 (58)	6.2 (8.7)	13	Q	1	Metabolic pathways	Mitochondrial
9	ODPB component beta subunit	P11177	40 (39)	5.3 (6.2)	26	4.37	1	Tricarboxylic acid cycle	Mitochondrial
10	KHK isoform C	P50053	35 (33)	5.6(5.6)	39	4.07	1	Dietary fructose metabolism	Cytoplasmic
11	Phosphoglucomutase	P36871	85 (61)	6.0 (6.3)	22	0.24	1	Glucose metabolism	Cytoplasmic
12	ETFB beta subunit	P38117	30 (28)	8.0 (8.2)	35	3.61	1	Electron transport	Mitochondrial
13	CYB5	P00167	16 (15)	4.5 (4.9)	49	Q	1	Electron transport	ER
c) Redox									
14	GST A1	P08263	18 (25)	7.4 (8.9)	35	9.88	3	Conjugation of reduced glutathione	Cytoplasmic
15	PRDX5	P30044	20 (22)	6.6 (8.8)	36	1.92	1	Peroxidase activity	Mitochondrial, peroxisomal an Cytoplasmic
16	GST A2	P09210	30 (26)	8.5 (8.5)	36	2.14	1	Conjugation of reduced glutathione	Cytoplasmic
17	BLVRB	P30043	25 (22)	7.1 (7.3)	62	1.87	1	Biliverdin reductase activity	Cytoplasmic
18	ADHA alpha chain	P07327	25 (40)	8.0 (8.6)	20	9.61	5	Alcohol oxidation	Cytoplasmic
19	ADH4	P08319	45 (42)	7.2 (8.2)	16	4.27	1	Alcohol oxidation	Cytoplasmic

Protein Number	Protein identity	Swiss-Prot entry	MW	P/	% Seguence coverage	T_2 *vs* T_1, ratio	#of patients/9	Function	Subcellular localization
d) Protein and aminoacid metabolism									
20	SPYA	P21549	30 (43)	5.9 (8.6)	24	2.75	2	Aminotransferase activity	Peroxisomal
21	4-Hydroxyphenylpiruvate dioxygenase	P32754	50 (45)	6.1 (6.5)	32	0.46	2	Tyr, Phe catabolism	Cytoplasmic
22	Ornithine carbamoyl transferase	P00480	45 (40)	7.0 (8.8)	20	Q	1	Urea cycle, Arg biosynthesis	Mitochondrial
23	3-Phosphoglycerate dehydrogenase	Q43175	65 (57)	6.0 (6.3)	28	0.34	1	Ser biosynthesis	Cytoplasmic
e) Molecular chaperone									
24	Peptidyl-prolyl *cis-trans* iso- merase A (Cyclophilin A)	P05092	20 (18)	7.0 (7.8)	29	1.74	1	Protein folding	Cytoplasmic
25	Heat shock cognate 71 kDa protein	P11142	20 (71)	6.0 (5.4)	13	Q	1	Molecular chaperone	Cytoplasmic-nuclear
f) Metabolic pathway									
26	Liver carboxyl esterase	P23141	65 (63)	5.9 (6.1)	23	7.64	2	Metabolic pathways	EB
27	CAH1	P00915	35 (29)	6.5 (6.6)	43	3.47	2	Carbonate dehy dratase activity	Cytoplasmic
28	C-1- tetrahydrofolate synthase	P11586	115 (101)	6.9 (6.9)	20	2.00	1	Biosynthetic pathways	Cytoplasmic
29	G6PE	Q95479	100 (89)	6.9 (6.8)	25	1.71	1	Metabolic pathways	ER
30	Carbonyl reductase	Q9UHY9	30 (26)	7.8 (8.3)	39	1.99	1	Metabolic pathways	Membrane
31	CAH2	P00918	28 (29)	6.1 (6.9)	40	6.26	1	Carbonate dehydratase activity	Cytoplasmic
32	FTHFD	Q75891	115 (99)	5.6 (5.6)	41	0.41	1	Metabolic pathways	Cytoplasmic
33	Aldo-keto reductase family 1 member C1	Q04828	35:371	6.1 (8.0)	22	Q	1	Metabolic pathways	Cytoplasmic
g) Secreted proteins									
34	HSA	P02768	85 (69)	5.6 (5.9)	43	9.15	3	Transporter activity	Plasma
h) Inflammatory response									
35	S10AS	P05109	14 (11)	6.5 (6.5)	33	7.59	1	Calcium ion binding, inflammatory response	Cytoplasm of macrophages
i) unknown									
36	ES1 protein homologue	P30042	30 (28)	6.6 (8.5)	55	2.08	1	Unknown	Mitochondrial

Table 3

List of Antioxidants Treatments with beneficial effects in liver ischemia/reperfusion injury.

List of Antioxidant Treatments With Beneficial Effects in Liver Ischemia/Reperfusion Injury							
Antioxidant	**Category**	**Species**	**Injury Type**	**Mode of Adm [A]**	**Dose**	**Main Protective Effect**	**Author**
α-Tocopherol	Vitamin-diet	Rat	WI[B]	Im	30 and 300 mg/kg	Survival, histology	Giacoustidis[39]
α-Tocopherol	Vitamin-diet	Rat	CI[C]/WI[B]	iv	50 IU/Kg	Histology	Gondolesi[42]
α-Tocopherol/Ascorbate	Vitamins-diet	Clinical	WI[B]	iv	2 mg/1,000 mg	Better PT ↓Postop. complications	Cerwenka[15]
Ascorbate	Vitamin-diet	Rat	WI[B]	iv	30 and 100 mg/kg	↓Lipid peroxidation	Seo[153]
Coenzyme Q/Pentoxyfylline	*In vivo* LMM[D] agent	Rat	WI[B]	in/ip	10 mg/kg/50 mg/kg	↓Lipid peroxidation	Portakal[139]
Idebenone	Coenzyme Q derivative	Pig	CI[C]			↑GSH levels	Schutz[151]
Lipoic acid	*In vivo* LMM [D] agent	Rat	CI[C]	iv	500μM	Histology	Muller[117]
Deferrioxamine	Iron chelator	Dog	CI[C]/WI[B]	iv	20 mg/kg	↓AST activity	Park[131]
Trimetazidine	Metal chelator	Rat	WI[B]	iv/ip	2.5 mg/kg	Histology	Tsimoyiannis[163]
Quercetin	Plant phenol	Rat	WI[B]	po	0.13 mmol/kg	↓ALT, AST	Su[158]
Cyanidin	Plant phenol	Rat	WI[B]	po	0.9 mmol/kg	↓Lipid peroxidation	Tsuda[164]
Green Tea Extracts	Plant extracts (catechines)	Rat	WI[B]	po	0.1%	Histology	Zhong[182]
Magnifera indica	Plant extract	Rat	WI [B]	po	250 mg/kg	↓AST, ALT ↓ Lipid peroxidation	Sanchez[146]
GSH	*In vivo* LMM [D] agent	Rat	WI[B]	iv	100μM/h/kg	↓ALT↑ Survival	Schauer[149]
GSH	*In vivo* LMM [D] agent	Rat	CI[C]/WI[B]	iv	100μM/h/kg	↓ ALT↑ Bile flow	Schauer[150]
N-acetylcysteine	Thiol compound GSH precursor	Rabbit	WI[B]	iv	150 mg/kg	↓ ALT↑ Microcirculation	Glantzounis[40]
N-acetylcysteine/Melatonin	Thiol compound	Rat	WI[B]	ip	150 mg/kg/10 mg/kg	↓ AST, ALT↓ Lipid peroxidation	Sener[152]
N-acetylcysteine	Thiol compound	Clinical	CI[C]/WI[B]	iv		↓ ICAM,[E]↓ α-GST[F]	Weigand[175]
Bucillamine	Thiol compound	Rat	CI[C]/WI[B]	iv		Survival	Amersi[2]
SOD derivatives	Intracellular enzyme	Rat	WI[B]	iv	5,000 IU/Kg	↓Lipid peroxidation	Nguyen[121]
CAT derivatives	Intracellular enzyme	Rat	WI[B]	iv	0.1 mg/kg	↓ ALT, AST	Yabe[178]
Allopurinol	XO[G] Inhibitor	Rat	WI[B]	ip	50 mg/kg	↓ AST	Jeon[71]
Aminoguanidine	iNOS inhibitor	Pig	CI[C]/WI[B]	iv	10 mg/kg	Survival, histology	Kimura[78]

[A] Adm., administration;

[B] WI, warm ischemia;

[C] CI, cold ischemia;

[D] LMM, low molecular molecule;

[E] ICAM-1, intercellular adhesion molecule-1;

[F] α-GST, α-glutathione S-transferase;

[G] XO, xanthine oxidase;

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