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Pathophysiological Basis for Antioxidant Therapy in Chronic Liver Disease

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Abstract

Oxidative stress is a common pathogenetic mechanism contributing to initiation and progression of hepatic damage in a variety of liver disorders. Cell damage occurs when there is an excess of reactive species derived from oxygen and nitrogen, or a defect of antioxidant molecules. Experimental research on the delicately regulated molecular strategies whereby cells control the balance between oxidant and antioxidant molecules has progressed in recent years. On the basis of this evidence, antioxidants represent a logical therapeutic strategy for the treatment of chronic liver disease. Clinical studies with large numbers of patients have not yet been performed. However, results from several pilot trials support this concept and indicate that it may be worth performing multicentre studies, particularly combining antioxidants with anti-inflammatory and/or antiviral therapy. Oxidative stress plays a pathogenetic role in liver diseases such as alcoholic liver disease, chronic viral hepatitis, autoimmune liver diseases and non-alcoholic steatohepatitis. The use of antioxidants (e.g. S-adenosylmethionine [SAMe;

ademetionine], tocopherol [vitamin E], polyenylphosphatidylcholine or silymarin) has already shown promising results in some of these pathologies.

Chronic inflammatory liver diseases share many of the pathogenetic processes that contribute to liver injury, independent of the aetiological factors causing the pathology (viruses, autoimmune reactions, alcohol consumption, etc.). In most cases, initial liver injury leads to chronic hepatitis, fibrosis, cirrhosis and eventually hepatocellular carcinoma. A common feature of pathologies such as chronic viral hepatitis, alcoholic liver disease (ALD), autoimmune liver diseases (AILDs) and non-alcoholic hepatitis is extensive evidence of oxidative stress, which might be responsible, at least in part, for the dysfunction or death of hepatocytes and other liver cell types that contributes to disease pathogenesis.

Oxidative stress is defined as an imbalance between the oxidant and antioxidant systems in the cell, potentially leading to tissue damage.[1] Activation of molecular oxygen as reactive oxygen species (ROS) is a natural mechanism of most life forms, required for its utilisation during normal metabolism. This occurs through interaction of O_2 with the redox-active metals copper and iron, leading to the formation of ROS. Specialised families of metalloenzymes exist which make it possible for the organism to take advantage of this process. However, ROS are also toxic. Under certain circumstances, this property of ROS is used by cells as a defence mechanism for destroying pathogens. Several means of regulating their generation to prevent unwanted cellular damage have been developed by organisms. As a consequence, a tightly regulated, delicate cellular balance exists, aimed at controlling the production of ROS when required, or to facilitate their elimination.

Direct measurement of specific oxidants is hampered by their inherent reactivity. Therefore, indicators of the reaction of these molecules with endogenous or exogenous targets are generally determined as markers of the generation of ROS and reactive nitrogen species (RNS).[2,3]

1. Generation and Effects of Reactive Oxygen Species

Several ROS may be involved in the pathogenesis of chronic liver disease (figure 1). Superoxide anion ($O_2^{\bullet-}$), although not a potent oxidant *per se*, appears to be a key starting point of oxidative stress.[4] $O_2^{\bullet-}$ is produced readily by multiple processes *in vivo* and leads to the generation of many other oxidants. This species is catalytically reduced by the enzyme superoxide dismutase (SOD) to hydrogen peroxide (H_2O_2), which may react with redox-active metals to form hydroxyl radicals, probably the most potent and dangerous cellular oxidants: this is called the Fenton reaction. H_2O_2 may also form hypochlorous acid following reaction with Cl^- via myeloperoxidase in neutrophils. In addition, $O_2^{\bullet-}$ reacts with nitric oxide ($NO\bullet$) to form peroxynitrite, a strong oxidising and nitrating species that influences some SH-related enzyme activities and cooperates to induce membrane lipid peroxidation. Finally, although not directly derived from $O_2^{\bullet-}$,

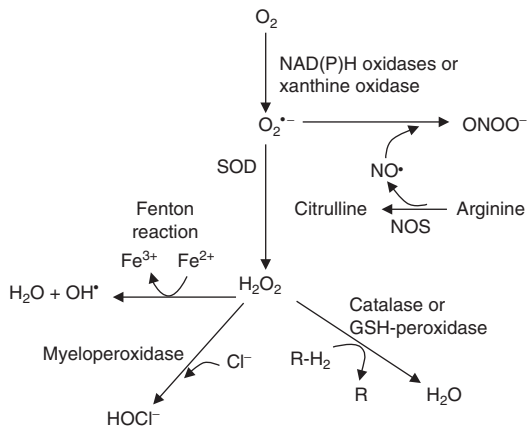

Fig. 1. Pathways of generation of the main reactive oxygen species. **Cl^-** = chloride anion; **H_2O_2** = hydrogen peroxide; **GSH** = glutathione; **$HOCl^-$** = hipochlorous anion; **NAD(P)H** = nicotinamide adenine dinucleotide phosphate (reduced form); **$NO\bullet$** = nitric oxide; **NOS** = nitric oxide synthase; **$O_2^{\bullet-}$** = superoxide anion; **$OH^\bullet$** = hydroxyl radical; **$ONOO^-$** = peroxynitrite; **SOD** = superoxide dismutase.

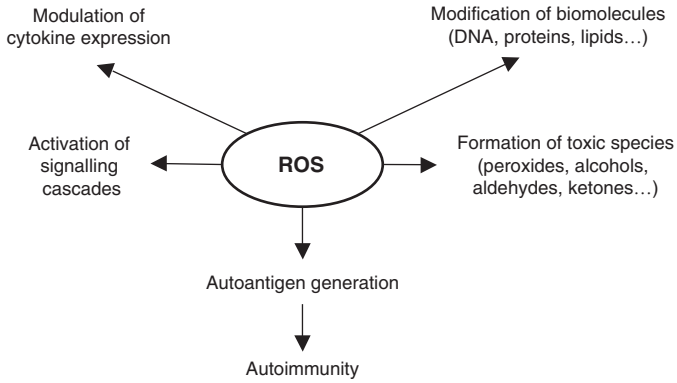

Fig. 2. Types of effects elicited by reactive oxygen species (ROS).

generation of other oxidants may be triggered by it (e.g. peroxyl radicals).[5]

ROS are highly reactive and lead to the oxidation of lipids, proteins and DNA, the deleterious consequence of oxidative stress. ROS can cause cell damage in several ways (figure 2). First, formation of modified biomolecules leads to a suboptimal, impaired cellular function. Secondly, toxic species may be formed, such as peroxides, alcohols, aldehydes and ketones. Thirdly, altered biological molecules may stimulate the host immune response and cause autoimmune-like diseases. Fourthly, oxidants can modify signalling cascades within the cell, which may lead to self-amplification of their effects. Several oxidant-sensitive signalling cascades have been identified: stress-activated protein kinases (c-Jun N-terminal kinase, extracellular-signal-regulated kinases 1 and 2, p38, etc.),[6] intracellular calcium,[7] transcription factors (activator protein-1, hypoxia inducible factor-1, nuclear factor-κB [NF-κB]),[8] and modulators of apoptosis signalling (caspases, Bad, Bcl-2), etc. Additionally, there is accumulating evidence that cells use redox modification of thiols as a post-translational mechanism to regulate the levels of certain proteins.[9] Fifthly, oxidants also influence intercellular signalling through activation/modulation of cytokine expression.[6] For instance, oxidant-induced activation of NF-κB in Kupffer cells stimulates production of proinflammatory tumour necrosis factor (TNF)-α by these cells.

2. Generation and Effects of Reactive Nitrogen Species

Several isoforms of nitric oxide synthase (NOS) are expressed in the liver: neuronal NOS (peribiliary plexus), endothelial NOS (endothelial cells) and inducible NOS (iNOS) [hepatocytes, cholangiocytes, Kupffer and stellate cells].[10] The latter is regulated at the transcriptional level by cytokines, ROS and other factors. All isoforms of NOS produce NO•, which acts as an RNS. Hepatic NO• may also derive from cytokine-activated neutrophils and/or lymphocytes during inflammation, as well as from regeneration by the reaction of glutathione with peroxynitrite. NO• binds reversibly to free thiol groups, either directly or through the action of glutathione-S-transferase on glutathione and organic nitrites. The resulting adducts are used as an endogenous cellular source, storage and transport of NO•.

NO• may exert opposing actions that may translate into a protective role in certain circumstances or into deleterious effects in others (table I).[11] For example, the vasodilating effects of NO• help maintain perfusion and inhibit thrombosis. There is also substantial experimental evidence indicating a protective function of NO• in liver inflammation, probably related to its antiapoptotic capacity.[12-18] NO• also functions as a chain-breaking antioxidant by reaction with lipid peroxyl radicals, thus hampering propagation of lipid peroxidation and providing enhanced oxidant protection.[19] On the other hand, NO• contributes to liver necrosis through the forma-

Table I. The dual nature of nitric oxide (NO•) effects: NO• may play a protective or damaging role in liver disease

Beneficial/protective effects
Vasodilatory factor
Antiapoptotic in liver inflammation
Chain-breaking antioxidant (scavenges lipid peroxyl radicals)
Participates in liver regeneration
Deleterious effects
Forms nitrotyrosine → cell dysfunction and death
Stimulates immune mechanisms
Inhibits mitochondrial function → depletion of cellular pyridine nucleotides → DNA damage

tion of reactive intermediates that can cause nitration (nitrotyrosine formation)[5] and nitrosation (nitrosothiol formation) reactions.[20] NO•-induced liver damage can also derive from immunomediated mechanisms and from DNA fragmentation as a result of the inhibition of mitochondrial function and depletion of cellular pyridine nucleotides.[21,22]

3. Oxidative Stress in Liver Disease

The degree of liver damage caused by oxidative stress depends on two factors: (i) the type and amount of oxidant stimuli, and their persistence in time and space; and (ii) the availability of suitable cellular defence mechanisms. It is the balance between these two opposing processes that ultimately determines the contribution of oxidative stress to the pathogenesis of a particular liver disease. The available experimental or clinical evidence of the involvement of oxidative stress as a pathophysiological mechanism in ALD, viral hepatitis, AILDs, nonalcoholic steatohepatitis (NASH) and cirrhosis, is reviewed, as is the available data with respect to antioxidant therapy in liver disease. There are increasing numbers of reports on this topic, most of which are case reports, case series and pilot studies, with only a few randomised controlled trials that included a placebo control or comparator group. We performed literature searches using the MEDLINE database (via PubMed) [from its inception to March 2005]. The search terms included antioxidant and oxidative stress in combination with liver disease, viral hepatitis, AILDs, NASH and cirrhosis.

3.1 Alcoholic Liver Disease (ALD)

The liver is the organ most severely affected by chronic alcoholism. Three different enzyme systems oxidise ethanol (alcohol) in the liver: alcohol dehydrogenase, cytochrome P450 (CYP) 2E1 and acetaldehyde dehydrogenase. There is a significant body of evidence indicating that oxidative stress and mitochondrial dysfunction are major factors in the pathogenesis of liver injury during ALD.[23] Ethanol is converted into acetaldehyde, a highly reactive and potentially toxic compound. Acetaldehyde is further oxidised to acetate by mitochondrial aldehyde dehydrogenase. Apart from a direct toxic effect of ethanol and disturbances in the intermediary metabolism, increased acetaldehyde production is directly involved in the pathogenesis of ALD. Acetaldehyde promotes glutathione depletion, resulting in free radical-mediated cytotoxicity and lipid peroxidation. Glutathione is selectively depleted in mitochondria as a result of inhibition of its transport into the organelle from the cytosol.[24] This is the basis of some therapeutic approaches aimed at replenishing mitochondrial glutathione stores in order to attenuate or correct functional alterations. The impairment of the antioxidant defence system in the liver, including a reduction of tocopherol (vitamin E), selenium and glutathione, reinforces the use of antioxidants in the treatment of ALD.[23] Additionally, in this multifactorial process, evidence also exists that specific nutrients may be depleted in patients with ALD, for instance S-adenosylmethionine (SAMe; ademetionine), an amino acid essential for transmethylation and transsulfuration reactions and for membrane integrity,[25] as well as polyenylphosphatidylcholine (PPC), which may decrease alcohol-induced oxidative stress and enhance collagenase activity.[26] Finally, local hypoxic situations may also contribute to alcohol-induced oxidative stress in the liver through several mechanisms.[27,28]

As a consequence of oxidative stress, membrane lipids are peroxidised and lipid derivatives (malonyldialdehyde, 4-hydroxynonenal) bind to various enzymes, such as cytochrome oxidase, thus altering their activity.[29] These adducts, as well as those of acetaldehyde, serve as antigens for eliciting

an immune response. Consequently, therapeutic strategies against oxidative stress may be directed both at decreasing the production of ROS and at providing antioxidant and membrane-protective capacity; that is, to restore mitochondrial glutathione content and to stabilise membrane fluidity, preventing lipid peroxidation.

Activation of signalling cascades by oxidative stress represents an additional mechanism whereby alcohol causes liver damage in the context of ALD.[23] Some of the pathways activated by ethanol have been mentioned previously. Furthermore, alcohol seems to prime inflammatory cells, making them more robust for eliciting a cell-killing response (see later in this section). In parallel, hepatocytes become more sensitive after alcohol exposure.[30] The simultaneous occurrence of these two processes results in a situation of chronic exacerbation of injury to the liver.

Sources of oxidant enzymes or enzyme systems in the liver in ALD include inflammatory cells (Kupffer cells and neutrophils) and hepatocytes. Non-parenchymal cells (e.g. stellate cells) may also contribute, although the information available is more scarce.[23]

Regarding inflammatory cells, alcohol activates Kupffer cells and monocytes, and leads to production of ROS and RNS by nicotinamide adenine dinucleotide phosphate (reduced form) [NAD(P)H] oxidase and iNOS, respectively. There is evidence that NAD(P)H oxidase-derived $O_2^{\bullet -}$ is crucial for the initiation of oxidative stress in experimental ALD.[31] Both iNOS knockout mice and NAD(P)H oxidase-deficient mice are protected completely against alcohol-induced oxidative stress,[31,32] indicating that $O_2^{\bullet -}$ and NO• are involved in liver damage caused by alcohol. Additional sources of oxidants in inflammatory cells include xanthine oxidase and myeloperoxidases.[23,33]

In hepatocytes, CYP2E1 and mitochondria are the two main sources of oxidants. CYP2E1 colocalises with regions of hepatic lesion in alcohol-induced liver injury and its inhibition leads to partial prevention of damage caused by enteral ethanol in the rat.[34] However, CYP2E1 knockout mice are not fully protected against oxidative stress caused by alcohol in the liver, suggesting the involvement of alternative cytochromes or other sources of oxidants, at least in mice, during the early phases of alcohol-induced liver injury.[35] Mitochondria produce $O_2^{\bullet -}$ during the metabolism of O_2, a process that is increased by exposure to alcohol.[36] As a result, more oxidant species are produced and mitochondrial-mediated apoptotic pathways are stimulated. Furthermore, mitochondrial glutathione is depleted by alcohol and hepatocytes are sensitised to undergo apoptosis.[37,38]

The role played by oxidative stress in hepatic injury as a result of excessive alcohol consumption is also associated with different proinflammatory cytokine effects, TNFα being the best studied. This is demonstrated by the fact that mice deficient in TNFα receptor TNFR1 are completely resistant to alcohol-induced liver injury.[39] However, these animals show normal formation of the α-hydroxyethyl radical, indicating that inflammatory cells do not directly damage hepatocytes during alcohol-induced liver injury.[40] Rather, they enhance the activation of signalling cascades/molecules, which in turn mediate their effects. Therefore, TNFα is a critical link between oxidant production in inflammatory cells and hepatocytes.

There is increasing evidence indicating that iron overload may play a significant role in the pathogenesis of ALD by exacerbating oxidative stress.[41] As mentioned earlier, iron catalyses the conversion of superoxide and H_2O_2 radicals to more potent oxidants, thereby enhancing tissue injury. Several mechanisms have been described: iron-induced oxidative stress leads to NF-κB activation and increased transcription of proinflammatory cytokines in Kupffer cells;[42] it exacerbates CYP2E1-induced oxidative stress, particularly in hepatocytes;[43] and it also stimulates extracellular matrix production by hepatic stellate cells, thus favouring fibrogenesis.[43] Consequently, the severity of ALD may be exacerbated in clinical situations characterised by excessive iron levels (hereditary haemochromatosis, transfusional iron loading, uncontrolled dietary iron

supplements, etc.). The efficacy of antioxidants in this setting may, therefore, be compromised.

3.1.1 Antioxidant Therapy in ALD

Several antioxidants, such as SAMe, PPC, silymarin (milk thistle), tocopherol and metadoxine, have been tested in patients with ALD.

SAMe is the activated form of methionine. The rationale for using SAMe rests on a sound theoretical basis: it is the main methyl donor in biological reactions and is a precursor in the synthesis of key metabolites such as glutathione and polyamines. It plays a key role in the phospholipid metabolism in membranes. Long-term ethanol consumption in experimental animals is associated with hepatic SAMe depletion;[25] patients with ALD have elevated plasma methionine levels, delayed methionine clearance and decreased availability of SAMe synthase.[44] This enzyme is also decreased in cirrhotic liver.[45] In experimental animals, SAMe administration restores mitochondrial glutathione content and mitochondrial membrane fluidity,[46] and attenuates endotoxin-induced liver damage associated with SAMe depletion and mitochondrial injury in long-term ethanol feeding.[47]

With regard to clinical data, a study in which an oral SAMe dose of 1200 mg/day was given for 6 months resulted in a significant increase in hepatic glutathione in patients with alcoholic and non-ALD.[48] These results suggest that the pharmacological actions of SAMe may rely on its ability to restore glutathione content in the liver. More important, results of a long-term randomised, placebo-controlled, double-blind multicentre clinical trial of SAMe in 123 patients with alcoholic cirrhosis demonstrated that oral SAMe 1200 mg/day improved survival compared with placebo. The effect was particularly marked when patients in Child C class were excluded from the analysis. Similarly, the drug delayed the time to death or to liver transplantation compared with the placebo group. SAMe was well tolerated with no important adverse effects being reported.[49] The encouraging data of this relative modest series of patients clearly point out that more extensive, long-term studies (biochemical as well as histological) of this agent could show a great potential in patients with different stages of ALD.

The use of PPC in ALD is based on its ability to increase resorption of fibrous tissue by enhanced collagenase activity,[50] and to decrease oxidative stress through reduction or normalisation of levels of 4-hydroxinonenal, F_2-isoprostanes and glutathione content,[26,51] as shown in experimental studies. In a randomised, prospective, double-blind, placebo-controlled clinical trial involving 789 ALD patients, a trend for biochemical (transaminases and bilirubin) improvement was observed in treated patients, but without statistically significant differences (the effects were more pronounced in the subgroups of hepatitis C virus [HCV]-positive drinkers or heavy drinkers). However, fibrosis progression, which was the main outcome of this particular trial, was not affected by PPC with respect to placebo. No serious adverse effects were observed.[52] Interpretation of the results of this trial was complicated by the fact that alcohol consumption was diminished in both the treatment and placebo groups during the course of the study. Thus, any potential effect of PPC may have been masked by the benefits of decreased alcohol consumption. Nevertheless, the observations were encouraging. Further studies are required to obtain conclusive data with regard to the use of PPC in patients with ALD.

Silymarin, a widely used form of alternative medicine, has antioxidant activities, protects against lipid peroxidation, and has anti-inflammatory and anti-fibrotic effects. It is a well tolerated drug. In a double-blind, prospective, randomised study of 170 cirrhotic patients, administration of silymarin 140mg three times daily for a mean duration of 41 months significantly improved the 4-year survival rate.[53] Silymarin was significantly effective in the subgroup of patients with alcoholic cirrhosis and in those with milder disease. The treatment was well tolerated. However, the drug did not show beneficial effects versus placebo in a study of 200 patients with alcoholic cirrhosis, some of whom also had hepatitis C.[54] It has been suggested that the lack of effects in this study could have been associated with poor compliance.[55] In summary, data obtained about the

efficiency of silymarin in ALD have been controversial so far.

Vitamin E, which is often deficient in ALD patients, has well documented antioxidant properties.[56] Moreover, it exerts its beneficial effects by stabilising membranes, reducing NF-κB activation and TNFα production,[57] and inhibiting hepatic stellate cell activation and collagen production.[58] A randomised study that used tocopherol 500mg showed no benefit to ALD in terms of hepatic laboratory parameters, mortality or hospitalisation rates.[59] However, whether or not the lack of effects was due to insufficient dose levels remains unknown. Similarly, a dose of tocopherol 1000 IU/day given to 25 patients with mild-to-moderate alcoholic hepatitis did not improve liver function markers in a recent double-blind, placebo-controlled randomised trial.[60] However, a significant decrease in serum levels of the fibrogenesis marker hyaluronic acid was observed in treated patients, a result that deserves further investigations in larger cohorts of patients.

Metadoxine, a drug that restores hepatic glutathione concentrations and acts as an antifibrogenic agent, proved efficacious in a double-blind, randomised multicentre trial involving 136 patients with chronic active alcoholism.[61] A dosage of metadoxine 1500 mg/day administered for 3 months accelerated the normalisation of liver function tests and the ultrasonographic changes indicative of steatosis. This suggests that the drug could be useful in the treatment of the early stages of ALD.

3.2 Viral Hepatitis

Oxidative stress is an important contributor to the development of liver damage during chronic viral hepatitis and the progression to carcinogenesis.[62] There is abundant evidence that both the virus itself and the host-immune response can cause oxidative stress.[63] For instance, the HCV core protein has been shown to alter the redox status in the liver in the absence of inflammation.[64] Similarly, both the HCV and hepatitis B virus (HBV) induce hepatic iNOS.[65,66] We have recently shown that the antioxidant N-acetyl-cysteine inhibits cytokine-induced iNOS messenger RNA induction and nitrite production in 2.2.15 cells (human hepatocytes transfected with the HBV genome), indicating a potential relevance in the context of chronic hepatitis B-associated liver damage.[67] In HCV-infected patients, hepatic glutathione levels are depleted, mitochondria undergo morphological changes,[68] and lipid peroxide-protein adducts, nitrotyrosine and markers of DNA damage become detectable.[69-71] Plasma levels of lipid peroxidation products are increased in these patients[2] and their peripheral blood mononuclear cells present elevated SOD activity.[72] In a recent manuscript by Otani et al.,[73] it has been demonstrated that HCV and ethanol synergistically induce cell damage of hepatocytes through a mechanism involving oxidative stress. In particular, hepatoma cells overexpressing both HCV core protein and CYP2E1 showed increased oxidant production and mitochondrial permeability transition in response to ethanol, with respect to cells expressing HCV core or CYP2E1 alone. Interestingly, toxic effects were inhibited by antioxidants. The extent to which oxidative stress may contribute to the progression of severe liver disease[74] remains to be investigated.

3.2.1 Antioxidant Therapy in Viral Hepatitis

The available clinical data further support the pathogenetic role of oxidative stress in chronic hepatitis C (CHC). Antioxidant therapy with tocopherol in CHC has shown beneficial effects both alone and in combination with interferon: administration of 1200 IU/day for 8 weeks in six CHC patients resistant to interferon therapy resulted in biochemical changes indicative of a delayed progression of fibrogenesis and of reduced oxidative stress.[75] However, serum ALT levels, HCV titres, or histological degree of hepatocellular inflammation or fibrosis were not affected by tocopherol. In a pilot study with 24 treatment-naive CHC patients, the group treated with tocopherol 544 IU/day plus interferon for 24 weeks showed a 2.4 greater chance of obtaining a complete response and had significantly greater reduction in viral load than patients without tocopherol.[76] Further studies with higher numbers of patients are required to confirm the potential of tocopherol as an efficacious adjuvant to interferon

for the treatment of CHC. Another effect observed after tocopherol therapy in CHC patients was a reduction of serum thioredoxin (a stress-inducible thiol-containing protein that is increased in the serum of CHC patients with the progression of fibrosis) and ALT levels.[77] Finally, a study was conducted to determine the effect of tocopherol supplementation on ribavirin-associated haemolysis in CHC patients treated with standard interferon-α and ribavirin.[78] A dose of tocopherol 800IU twice daily together with the antiviral therapy for 24 weeks did not significantly improve the sustained viral response rate with respect to patients with combination therapy alone. Ribavirin-associated haemolysis was also not diminished by tocopherol.

3.3 Autoimmune Liver Diseases (AILDs)

Primary biliary cirrhosis (PBC), autoimmune hepatitis (AIH) and primary sclerosing cholangitis (PSC) are chronic liver diseases, the major pathogenetic mechanism of which is an autoimmune reaction. Cellular and humoral-mediated immune reactions against self antigens participate in the development of liver pathology.[79,80] However, some experimental and clinical evidence indicates that additional factors may play a role in the progression of liver lesions in AILD. Among these factors are ROS and RNS.[81] We have recently reported an increased intrahepatic iNOS expression and nitrotyrosine accumulation in patients with PBC and AIH.[82] Overexpression of iNOS correlated with the histological severity of liver disease, suggesting that NO•-mediated nitration of hepatocellular proteins might directly contribute to liver damage in both diseases. However, the initial trigger for iNOS induction in PBC and AIH remains unidentified. Given the differences between these two liver diseases, it could be suggested that this nitration process represents a nonspecific pathogenic mechanism, common to different autoimmune chronic inflammatory diseases and secondary to the elicitation of inflammation, rather than its cause. This is supported by the observation of increased nitrotyrosine localisation in tissues affected by other chronic inflammatory disorders, such as rheumatoid arthritis[83] and ulcerative colitis.[84]

Patients with PBC might be particularly sensitive to ROS and/or RNS-mediated damage because a predictable consequence of cholestasis is malabsorption of fat-soluble vitamins and other free radical scavengers, such as carotenoids, leading to an impaired antioxidant capacity.[85] Therefore, therapeutic approaches that potentiate the generation of free radical scavengers may be efficacious in this disease. For example, in experimental models of cholestatic disease, ursodeoxycholic acid has been shown to partially prevent hepatic and mitochondrial glutathione depletion and oxidation, suggesting that the efficacy of the drug in human patients might also be associated, at least in part, with its antioxidant actions.[86]

An additional mechanism whereby oxidative stress contributes to liver damage in AILDs has been described: in patients with PSC, catalase has been characterised as one of the possible autoantigens eliciting an autoimmune reaction.[87] Because catalase is an important antioxidant enzyme that prevents cell damage induced by highly reactive oxygen-derived free radicals, an impairment of the redox status of cells by catalase autoantibodies has been suggested as a possible pathogenic mechanism in PSC.

3.3.1 Antioxidant Therapy in AILD

Prince et al.[88] tested the effects of 12 weeks oral antioxidant supplementation (a combination of vitamins A, C and E, selenium, methionine and ubiquinone) on fatigue or other liver-related symptoms in PBC patients in a double-blind, placebo-controlled crossover trial. Although the drug was well tolerated, no disease improvement was seen after treatment. Angulo et al.[89] treated PBC patients unresponsive to ursodeoxycholic acid with oral silymarin 140mg three times daily for 1 year, in the hope that it could be beneficial based on its antioxidant, immunomodulatory and antifibrotic properties. Unfortunately, the medication did not provide a benefit to patients. Whether or not the absence of effects in these studies could be related to insuffi-

cient dosages, or to impaired absorption of liposoluble vitamins, remains to be elucidated.

3.4 Non-Alcoholic Steatohepatitis (NASH)

According to Day[90] and Day and James,[91] the pathogenesis of NASH comprises two steps. First, the healthy liver becomes steatotic through a variety of mechanisms. Fatty liver is particularly sensitive to further insults that constitute a second hit, such as oxidative stress and proinflammatory cytokines (basically, TNFα). As a consequence, an exacerbation of insulin resistance occurs, along with further oxidative stress and organelle dysfunction within liver cells, resulting in an inflammatory process, hepatocellular degeneration and fibrosis.[92]

Peripheral resistance to insulin and high levels of leptin allow entrance to the mitochondria of free fatty acids (FFA) reaching the liver as a consequence of previously inhibited oxidation.[90] Although oxidation of long-chain and very-long-chain fatty acids is partly extramitochondrial (in microsomes and peroxisomes), free oxygen radical production occurs mainly in mitochondria.[93,94] Massive FFA hepatic upload, and particularly acetylcoenzyme A, lead to peroxisome proliferator-activated receptor (PPAR)-α -mediated activation of the synthesis of enzymes responsible for oxidation, thereby increasing peroxide levels.[90,95] Formation of free oxygen radicals in a fat-rich medium induces lipidic peroxidation. Increased oxidative stress and lipidic peroxidation induce damage in plasmatic membranes, intracellular organelles, mitochondrial DNA and respiratory chain-related proteins.[96] Additionally, the end-products of oxidative stress activate NF-κB-mediated NO• synthesis, leading to the formation of peroxynitrates.[97] FFA also increase the expression of microsome oxidases CYP4A and CYP2E1, responsible for the production of hydroxyethyl radicals.[98-100] Since CYP2E1 is inhibited by insulin, its expression levels are higher in case of peripheral resistance to this hormone.[101]

Another consequence of the increased production of free oxygen radicals is the induction of Fas ligand expression in hepatocellular membranes (not expressed under normal conditions), since its promoter contains a binding site for NF-κB. Interaction of Fas ligand with Fas-expressing hepatocytes leads to their death through a process termed fratricidal apoptosis.[94]

Mitochondrial dysfunction seems to be a critical event in the second hit of NASH. Whether induced by lipidic peroxidation products secondary to oxidative stress or directly by TNFα, it leads to alterations in electron transfer along the respiratory chain, thus generating more free oxygen radicals.[94] The expression of mitochondrial oxidative phosphorylation uncoupling protein (UCP)-2 is increased. UCP-2 reduces free oxygen radical synthesis but also decreases ATP levels, thus making the cell more sensitive to insults[102-104] and facilitating hepatocellular apoptosis and necrosis. Decreased ATP synthesis has been reported in NASH patients following perfusion with fructose.[105]

3.4.1 Antioxidant Therapy in NASH

Given the pathogenetic role of oxidative stress in NASH, several antioxidant agents could be useful as potential therapies for the disease, including tocopherol, betaine and pentoxyphylline.

In several studies, tocopherol has been shown to improve liver biochemistry and histological lesions of NASH as a result of its actions as an antioxidant agent and an inhibitor of transforming growth factor (TGF)-β, a cytokine involved in liver fibrogenesis. In a study with 12 NASH patients given tocopherol 300 mg/day for 1 year, serum TGFβ levels were decreased, and biochemical markers and hepatic pathological findings were improved.[106] Furthermore, inflammation and fibrosis were improved in five of nine NASH patients in whom liver biopsy was performed after tocopherol treatment. Long-term treatment with tocopherol has also been evaluated in obese children with presumed diagnosis of NASH and a beneficial effect of this vitamin has been suggested.[107] Kawanaka et al.[108] treated ten NASH patients with tocopherol 300 mg/day for 6 months. Significant reductions in serum levels of ALT and gamma glutamyl transferase (γGT) were attained and the oxidative stress markers thioredoxin and thiobarbituric acid reactive substance were also decreased. However, tocopherol was not effec-

tive in a pilot clinical trial with 16 patients with biopsy-proven NASH after administration of an oral dose of 800IU for 12 weeks.[109] Tocopherol did not independently influence the biochemical data and plasma cytokine levels in patients with NASH. It has been suggested that this lack of observable effects could be related to the shorter treatment period, concurrent lifestyle modifications and/or poor adherence, but no conclusive data clarifying this issue are available.

Betaine treatment has shown beneficial biochemical and histological effects in a pilot study involving ten patients with NASH.[110] The drug normalised or significantly reduced the serum levels of transaminases of nine of ten patients, and induced a marked improvement in the degree of steatosis, necroinflammatory grade and stage of fibrosis. The treatment was well tolerated in all patients.

An additional drug currently under evaluation in patients with NASH is pentoxifylline.[111] In an experimental animal model of steatohepatitis, pentoxifylline has been shown to decrease serum ALT levels and hepatic inflammation, probably by increasing glutathione levels or reducing TNFα expression.[112] In a 12-month pilot trial conducted among 20 patients with NASH, administration of pentoxifylline 400mg four times daily significantly reduced aminotransferase levels, although several patients withdrew from the study, primarily because of nausea.[113]

Ursodeoxycholic acid has also been tested as a therapy in NASH, based, among other factors, on its antioxidant properties. Although well tolerated, the drug did not induce any significant changes in the degree of steatosis, necroinflammation or fibrosis in a placebo-controlled randomised trial of 166 patients.[114]

Because HMG-CoA reductase inhibitors (statins) have also been shown to have antioxidant effects,[115] their potential in the treatment of NASH has been explored. In a pilot study including 27 hyperlipidaemic NASH patients, administration of atorvastatin 10 mg/day significantly reduced serum cholesterol, AST, ALT, alkaline phosphatase and γGT levels.[116] The drug was well tolerated. Although these results are promising, further studies with larger cohorts of patients are required.

3.5 Cirrhosis

Cirrhosis, the advanced stage of liver fibrosis, may be reversible at least in part and, therefore, therapies that can prevent or reverse cirrhosis are eagerly awaited. Oxidative stress is pathogenetically associated with the development and progression of fibrosis: among other factors, ROS generated by CYP2E1 in hepatocytes, Kuppfer and inflammatory cells, along with glutathione depletion and dysregulated hepatic microcirculation through NO•-mediated pathways, directly and indirectly lead to increased matrix production and determine the shift to cirrhosis. Therefore, antioxidants represent a potential therapeutic strategy for the treatment of cirrhosis.

In experimental models of cirrhosis, several compounds have produced interesting results. For example, PPC restores phospholipids of the damaged membranes and reactivates enzymes involved in phospholipid regeneration, such as phosphatidylethanolamine methyltransferase. In baboons, PPC prevented cirrhosis by stimulating collagenase and opposing lipid peroxidation, by inhibiting the production of fibrogenic hydroxynonenal.[117] PPARα ligands showed an antifibrotic action in a rat model of liver cirrhosis, probably as a result of an antioxidant effect of enhanced catalase expression and activity in the liver.[118] In rats with secondary biliary cirrhosis, ursodeoxycholic acid treatment enhanced the antioxidant defence mediated by glutathione, leading to prevention of cardiolipin depletion and cell injury.[86]

3.5.1 Antioxidant Therapy in Cirrhosis

In clinical studies, SAMe has already shown benefits in the treatment of alcoholic liver cirrhosis. These patients present with elevated serum methionine levels and have abnormal methionine clearance after an oral load of this amino acid, as well as delayed sulfate excretion. These alterations are the consequence of a reduced liver methionine adenosyltransferase (MAT I/III) activity in these pa-

tients. In fact, the gene *MAT1A* is expressed at reduced and, in some cases, undetectable levels in human liver cirrhosis, whereas the expression of *MAT2A* remains low.[119] The reduced enzymatic activity has an impact on many essential metabolic pathways in the liver and influences the development of the disease. The inhibition of MAT I/III is caused by the oxidant conditions in the cirrhotic liver.[120] This mechanism explains the efficacy of SAMe in the treatment of patients with alcoholic liver cirrhosis discussed in section 3.1.1.[49] However, it must be mentioned that other mechanisms of MAT1A impairment have been reported, such as through TNF-induced downregulation of the enzyme.[121]

Silymarin has also shown promise for the treatment of patients with alcoholic cirrhosis, although controversial data have been obtained in different studies.[53,54] Theoretically, other antioxidants such as tocopherol might also be useful in cirrhotic patients because they have diminished hepatic tocopherol levels.[122] However, as mentioned in sections 3.1.1 and 3.3.1, therapeutic results have, to date, been disappointing.[59]

3.6 Hepatocellular Carcinoma

Hepatocellular carcinoma (HCC) is a very common cancer worldwide and has a poor prognosis. Risk factors include chronic hepatitis B or C infection and liver cirrhosis. Exposure to aflatoxin B1, alcohol consumption, haemochromatosis and α-antitrypsin deficiency are the other major causes of HCC.[123] Oxidative stress may contribute to cancer initiation through ROS-induced DNA damage.[124] A typical example is 8-oxo-2′-deoxyguanosine, one of the major and most deleterious DNA base lesions, which is induced by the attack of either hydroxyl radical or singlet oxygen on deoxyguanosine.[125] This specific lesion has been recently reported to occur in inflammatory regions adjacent to HCC.[126] Oxidative stress has been described in many of the mentioned liver diseases that precede HCC, but the molecular mechanisms of ROS-mediated hepatocarcinogenesis remain poorly understood. In a study using a radical-probe magnetic resonance technique in patients with chronic hepatitis at different stages of malignant transformation, evidence was obtained that ROS participate in the development of HCC.[127] There are also studies indicating that antioxidant SOD levels are decreased in transformed hepatocytes and in HCC patients, in close correlation with disease severity.[128,129] ROS may also contribute to HCC initiation and progression by inducing the activation of telomerase. In a recent study, the dysfunction of the antioxidant systems has been shown to correlate with the progression from cirrhosis to HCC.[130] As in ALD, iron excess has been shown to be associated with the development of HCC.[131] The mechanisms through which iron may act in carcinogenesis are not fully known but potentiation of oxidative stress seems a plausible hypothesis, in addition to facilitation of tumour cell growth and modification of the immune response. Therefore, careful control of iron levels seems critical when considering the use of antioxidants in HCC.

Although all this evidence suggests that antioxidants, in combination with other treatments, might be of use in the prevention or treatment of HCC, this hypothesis remains to be investigated in appropriate clinical trials.

3.7 Drug-Induced Liver Damage

Oxidative stress has also been reported to be involved in the development of liver damage secondary to the exposure to liver toxins, including drugs,[132,133] as well as environmental agents, food additives, pesticides and other chemicals. Large amounts of ROS are generated in the liver in response to exogenous agents, because it is: (i) the most frequent organ for xenobiotic metabolism; (ii) exposed to comparatively high levels of compounds arriving from the gastrointestinal system; and (iii) the major organ for iron transport and storage.[134,135] In spite of the availability of high hepatic levels of antioxidant enzymes, and the tremendous capacity of the liver to regenerate, cell damage may lead to hepatic dysfunction. The fact that antioxidants have shown efficacy as antidotes for acute intoxications produced by some liver toxins supports the notion that oxidative stress plays a key role in the toxico-

Table II. Summary of antioxidant therapies investigated in liver disease

Antioxidant	Number and type of patients	Results	Reference
Alcoholic liver disease (ALD)			
S-adenosylmethionine	9 ALD, 7 non-ALD, 8 placebo-ALD, 15 healthy subjects	Increased hepatic glutathione	48
	123 ALD cirrhosis (61 placebo-treated)	Improved survival. Delayed time to death or to liver transplantation. Well tolerated	49
PPC	789 ALD	Biochemical improvement in HCV-positive drinkers and heavy drinkers. No effect on fibrosis progression. Well tolerated	52
Silymarin (milk thistle)	170 cirrhosis, including ALD	Improvement of 4-year survival rate. Efficacy statistically significant in alcoholic cirrhosis. Drug was well tolerated	53
	200 ALD cirrhosis	No beneficial effect	54
Tocopherol	67 ALD cirrhosis (34 placebo-treated)	No biochemical improvement, no influence on mortality or hospitalisation rates	59
	51 mild-to-moderate ALD (26 placebo-treated)	No improvement in liver function markers. Significant decrease in serum hyaluronic acid levels (suggesting decreased fibrogenesis)	60
Metadoxine	136 ALD (67 placebo-treated)	Normalisation of liver function tests and ultrasonographic evidence of steatosis	61
Viral hepatitis			
Tocopherol	6 CHC resistant to interferon therapy	Improvement in biochemical markers of fibrogenesis and oxidative stress. No changes in ALT, HCV titres or histology	75
	24 CHC treatment naive	Increased chance to undergo a complete response, greater reduction in viral load	76
	47 (27 tocopherol/20 controls) CHC treatment naive; plus antiviral therapy	SVR rate not improved. Ribavirin-related haemolysis unchanged	78
Autoimmune liver diseases			
Vitamin A, C and E, selenium, methionine, ubiquinone	61 PBC	No effects on fatigue or liver-related symptoms. Well tolerated	88
Silymarin	27 PBC unresponsive to UDCA	No significant effects	89
Non-alcoholic steatohepatitis (NASH)			
Tocopherol	12 NASH	Decrease in serum TGFβ levels. Biochemical and histological improvement	106
	10 NASH	Decreased serum ALT, γGT, thioredoxin and TBARS levels	108
	16 NASH	No change in biochemical tests and plasma cytokines levels	109
Betaine	10 NASH	Normalisation or significant reduction of ALT, improvement of steatosis, inflammation and fibrosis. Well tolerated	110
Pentoxifylline	20 NASH	Reduction of ALT. Main adverse effect: nausea	113
UDCA	166 NASH	No improvement compared with placebo	114
Atorvastatin	27 NASH with hyperlipidaemia	Reduced liver enzymes levels and cholesterol	116

CHC = chronic hepatitis C; **HCV** = hepatitis C virus; **PPC** = polyenylphosphatidylcholine; **PBC** = primary biliary cirrhosis; **SVR** = sustained virological response; **TBARS** = thiobarbituric acid reactive substance; **TGF**β = transforming growth factor-β; **UDCA** = ursodeoxycholic acid; **γGT** = glutamyl transferase.

logical mechanism of action. A thoroughly described example is the use of N-acetyl-cysteine for the treatment of liver damage induced by paracetamol (acetaminophen) overdose.[136]

Given the large variety of hepatotoxic substances with mechanisms of action described as involving oxidative stress, this topic is not reviewed in this paper. The interested reader is referred to recently published reviews.[135,137]

4. Conclusions

In recent years, abundant evidence has accumulated on the role played by oxidative stress in liver injury during the course of chronic inflammatory liver diseases. ROS and RNS are formed either in response to aetiological factors (e.g. viruses, alcohol, etc.) or as a result of the inflammatory and/or immune processes that characterise the different pathologies. The subsequent alteration of the cellular redox status triggers different responses, ranging from activation of signalling cascades involved in respiratory, energetic and regenerative processes, to imbalance of proinflammatory/anti-inflammatory cytokine production, cytotoxicity and apoptosis.

On the basis of this knowledge, some efforts have been made to evaluate the efficacy of antioxidant treatments for preventing and slowing progression of liver disease (table II). Some of the clinical studies conducted so far have shown promise in terms of tolerability and efficacy; however, most of the available information derives from investigational pilot studies with limited numbers of patients. Further research with larger cohorts of patients, in the context of clinical trials specifically designed to thoroughly evaluate the therapeutic activity of a given antioxidant drug in a particular liver disease, is needed. As oxidative stress is, in most cases, just one of the pathogenetic mechanisms contributing to liver damage, and not the only aetiological factor, it may be sensible to study the efficacy of antioxidants as adjuvants to other drugs (antiviral, antifibrotic and anti-inflammatory agents) as it is probable that their efficacy would be maximal in the early phases of disease. Altogether, the information provided by clinical trials aimed at investigating these issues may aid to define optimal therapeutic conditions for each disease in study.

In our opinion, from a purely medical and scientific point of view, there is a solid background and rationale for conducting such large clinical studies. However, this will certainly require the investment of significant financial resources. The pharmaceutical industry has not yet fully committed to investigate the potential of antioxidants in liver disease in depth, probably because antioxidants are generally non-expensive, widely available drugs, with few chances to produce large financial benefits. Therefore, it would be desirable for public funding entities to respond to this challenge by dedicating the resources required to investigate these drugs.

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