

The Contemporary Role of Antioxidant Therapy in Attenuating Liver Ischemia-Reperfusion Injury: A Review

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Oxidative stress is an important factor in many pathological conditions such as inflammation, cancer, ageing and organ response to ischemia-reperfusion. Humans have developed a complex antioxidant system to eliminate or attenuate oxidative stress. Liver ischemia-reperfusion injury occurs in a number of clinical settings, including liver surgery, transplantation, and hemorrhagic shock with subsequent fluid resuscitation, leading to significant morbidity and mortality. It is characterized by significant oxidative stress but accompanied with depletion of endogenous antioxidants. This review has 2 aims: firstly, to highlight the clinical significance of liver ischemia-reperfusion injury, the underlying mechanisms and the main pathways by which the antioxidants function, and secondly, to describe the new developments that are ongoing in antioxidant therapy and to present the experimental and clinical evidence about the role of antioxidants in modulating hepatic ischemia-reperfusion injury. (*Liver Transpl* 2005;11:1031-1047.)

Liver ischemia-reperfusion (I/R) injury is well recognized as a significant cause of morbidity and mortality in 2 principal settings. Firstly, it occurs in major liver resections¹ and transplantation^{2,3} where anoxic or ischemic liver injury takes place. Secondly, it happens as a consequence of systemic hypoxia or with conditions that cause low blood flow to the liver resulting in insufficient perfusion. The latter occurs in hemorrhagic, cardiogenic, or septic shock with subsequent fluid resuscitation,⁴ in cardiovascular surgery with extracorporeal circulation,⁵ in laparoscopic surgery,^{6,7} and in abdominal compartment syndromes.⁸

Severe warm liver I/R can lead to liver or even to multiple organ failure. The extent of hepatic injury caused by I/R depends primarily on the condition of the liver prior to the ischemic insults and its duration.⁹

In liver surgery, new techniques have been applied to increase the resectability rate of liver tumors such as downstaging chemotherapy and portal vein embolization. However, these techniques can make the liver more susceptible to ischemic insults.¹⁰ Furthermore, the commonest primary liver cancer, hepatocellular carcinoma usually develops on the background of liver

cirrhosis, which increases the risk of liver failure during any subsequent surgery.¹¹

In the field of liver transplantation, I/R injury is closely related to the development of primary graft non-function (occurs in <5 % of grafts) and primary graft dysfunction (occurs in 10-30 % of grafts).¹² Both conditions are associated with high rates of morbidity and mortality. I/R injury increases the incidence of subsequent graft rejection.¹³

Another field where I/R injury affects outcome is hepatic resection or transplantation with steatotic livers. It is reported that 25% of the western population has some degree of hepatic steatosis,¹⁴ which is the result of the abnormal accumulation of triacylglycerol within the cytoplasm of hepatocytes, attributed to the effects of alcohol excess, obesity, diabetes, or drugs. Furthermore, hepatic steatosis is associated with an impaired microcirculation,^{15,16} increased postoperative morbidity and mortality, and poor graft function.¹⁴

It is obvious that liver I/R occurs in many diverse clinical settings and has a major impact on clinical out-

Abbreviations: I/R, ischemia-reperfusion; ROS, reactive oxygen species; RNS, reactive nitrogen species; ATP, adenosine triphosphate; XOR, xanthine oxidoreductase; TNF- α , tumor necrosis factor- α ; O₂, oxygen; OH, hydroxyl radical; NO, nitric oxide; H₂O₂, hydrogen peroxide; ONOO⁻, peroxynitrite; HO, haem oxygenase; NOS, NO synthetase; iNOS, inducible NOS; GSH, glutathione; SOD, superoxide dismutase; NAC, N-acetylcysteine.

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come. Reoxygenation of the ischemic liver causes the generation of numerous reactive oxygen species (ROS) and reactive nitrogen species (RNS). Although ROS and RNS in low concentrations have an important role as mediators in normal cellular metabolism and signal transduction,^{17,18} in higher concentrations they can be damaging. In light of this, a complex defense network, called the antioxidant system, has been developed in mammals, to prevent or reduce the injury caused by high concentrations of ROS or RNS.

Free radical scavenging or administration of agents that enhance the endogenous antioxidant system could reduce postischemic tissue injury and so be useful in clinical settings against hepatic I/R damage.

The aim of this review is to present the main mechanisms by which antioxidants function and then the potential use of exogenous antioxidants as a therapeutic strategy in conditions associated with liver I/R injury. For information on other therapeutic strategies against liver I/R injury, the reader is referred to recent reviews.¹⁹⁻²³

Search Strategy Methods

All the studies were identified by PubMed, Web of Science, and Embase searches between years 1966 and 2004 with the following keywords: liver, hepatic, ischaemia or ischemia, reperfusion, injury, antioxidant, pharmaceutical preconditioning. In vitro, experimental and clinical studies included focused on the effect of antioxidant therapy on liver I/R injury.

Ischemic Injury

When oxygen supply to cells becomes insufficient as a direct result of reduced blood flow or hypoxia, the mitochondrial respiratory chain function alters and the reduction-oxidation (redox) state of the mitochondrial enzymes becomes reduced. This results in the inhibition of the oxidative phosphorylation process with a subsequent reduction in adenosine triphosphate (ATP) synthesis.²⁴ Reduction of cellular ATP causes disturbances in membrane ion translocation by inhibition of the ATP-dependent sodium (Na^+)/potassium (K^+) ATPase, resulting in sodium influx and intracellular sodium accumulation with corresponding cell swelling and death.²⁵

Intracellular calcium accumulation is also strongly implicated in the development of ischemic injury and thought to be a crucial step in the transition to irreversible damage.²⁶ The increased cytosolic calcium level causes activation of cell membrane phospholipases,

resulting in phospholipids degradation and cell membrane disruption.²⁷ Prior to cell death, hepatocytes and other cells develop a state characterized by mitochondrial permeability transition,²⁸ lysosomal disruption, bleb formation and growth, cell swelling, and leakage of small molecular mass solutes.^{29,30} Calcium also activates xanthine oxidoreductase (XOR) which has a role in oxygen free radical production following reperfusion³¹.

Although the basic mechanisms of ischemic injury after warm and cold liver ischemia are similar, there are also significant differences. In liver transplantation the liver undergoes cold ischemic storage followed by rewarming ischemia and reperfusion.²³ Cold ischemia is associated with reduced oxidative phosphorylation, lower cellular ATP levels, and increased glycolysis,³² while warm ischemia leads to greater oxidative stress and mitochondrial dysfunction.^{33,34} The main site of injury in cold ischemia are nonparenchymal cells (Kupffer, sinusoidal endothelial cells, Ito cells and biliary epithelium) whereas in warm ischemia are hepatocytes.³⁵

Reperfusion Injury

Although ischemia causes significant injury to tissue and cells, the injury during reperfusion is more severe. A complex network of hepatic and extrahepatic mechanisms is involved in the pathophysiology of hepatic I/R injury.

Experimental evidence shows that there are two distinct phases of liver reperfusion injury. The early phase covers the first 2 hours after reperfusion. During this phase the main event is the activation of Kupffer cells.³⁶ Complement activation and the recruitment and activation of CD4^+ T lymphocytes are factors that enable the activation of the Kupffer cells.^{20,37}

Kupffer cell activation leads to structural changes, formation of vascular ROS and production of cytokines such as tumor necrosis factor- α (TNF- α) and interleukin 1.^{22,38} These ROS and cytokines have a direct cytotoxic effect on endothelial cells, and hepatocytes can induce changes in cell membrane receptors in hepatocytes and release of cytokines. TNF- α acts as the central mediator in the hepatic response to I/R. The production of TNF- α induces the expression of adhesion molecules on vascular endothelial cells and stimulates the production and release of neutrophil-attracting chemokines. The final result is the recruitment of neutrophils. These activated neutrophils release ROS and proteases that are responsible for the induced oxidative stress during the late phase of reperfusion injury.^{39,40}

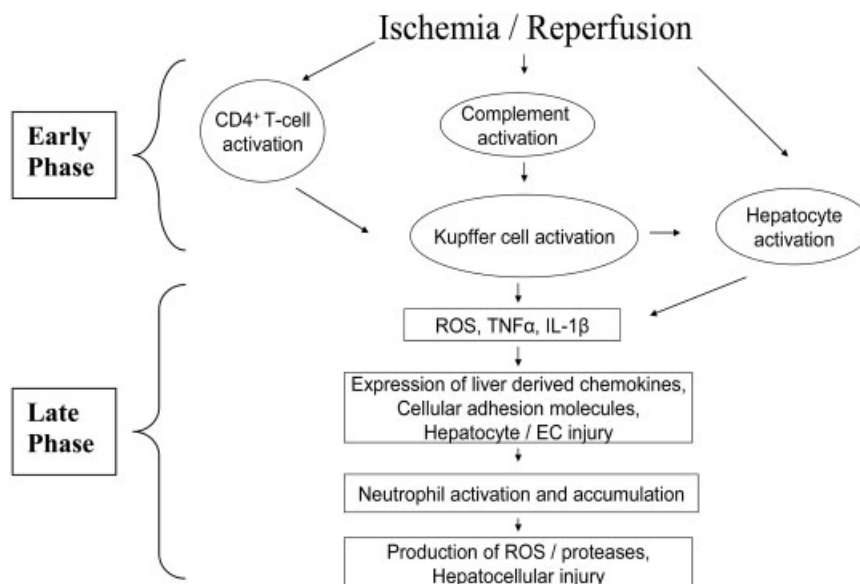


Figure 1. Schematic presentation of pathophysiology of liver ischemia / reperfusion injury. IL-1 β , interleukin 1 β ; EC, endothelial cells.

which is much more severe compared to that during the early phase.

Recent evidence suggests that the T-lymphocytes can also be important mediators in short- and long-term liver I/R injury. Their role seems to be a multifactorial one. There is evidence that systemic immunosuppression attenuates hepatocellular injury following I/R.⁴¹⁻⁴³ The adherence of CD4⁺ T-lymphocytes in hepatic sinusoids occurs during the early phase of reperfusion and is mediated by TNF- α and interleukin 1.⁴⁴ These T-lymphocytes can increase Kupffer cell activation and can act as cellular mediators in polymorphonuclear cell recruitment through the release of substances such as granulocyte colony stimulating factor and interferon γ .⁴⁵ The basic pathophysiological mechanisms in hepatic I/R injury are summarized in Figure 1.

Reactive Oxygen Species and I/R Injury

A "radical" is defined as any atom or biomolecule that contains unpaired electrons.⁴⁶ These unpaired electrons influence the chemical reactivity, making the radical more reactive than the corresponding nonradical. Although oxygen (O_2) is the most important biological molecule for sustaining life, it is also the main source for free radical formation due to its high availability.

The biologically relevant radicals are the superoxide anion ($O_2^{\bullet-}$), the hydroxyl radical ($\cdot OH$) and nitric oxide (NO). Under normal conditions around 1 to 3%

of the oxygen that is metabolized in the mitochondria is converted to the radical $O_2^{\bullet-}$.⁴⁷ Some other species are intermediate in the metabolism of O_2 or NO but are not radicals, as they do not contain unpaired electrons. These intermediate species along with the radical species are called ROS and RNS, respectively. The most representative examples of nonradical ROS are hydrogen peroxide (H_2O_2) and hypochlorous acid. The most representative of nonradical RNS is peroxynitrite ($ONOO^-$)⁴⁸. Peroxynitrite is formed when there is simultaneous production of nitric oxide with superoxide anion (equation 1):



Tissue toxicity from superoxide generation is based on its direct reactivity with numerous types of biological molecules (typically lipids, DNA, RNA, catecholamines, and steroids) and from its dismutation to form H_2O_2 .⁴⁹ Trace amounts of metals ions (principally iron or copper) react with H_2O_2 in what is known as the Fenton reaction to produce the toxic radical $\cdot OH$.⁵⁰ This radical can cleave covalent bonds in proteins and carbohydrates and destroy cell membranes.

Liver injury induced by I/R is caused, at least partially, by ROS and RNS. There is evidence that during hepatic I/R there is generation and release of ROS and RNS with concomitant consumption of endogenous antioxidants and apoptotic or necrotic cell death.^{39,51-54}

Although the exact sources of ROS generation in

Table 1. Role of ROS in I/R Injury

Function	References
Enhance proinflammatory gene expression (TNF- α ; IL-1, IL-8, cellular adhesion molecules)	Lentch, ²² 2000 Liu, ³⁸ 2001 Zwacka, ⁵⁸ 1998
Induce expression of the transcription factors NF- κ B and activator protein-1	Harada, ⁵⁹ 2003
Direct cellular damage through protein oxidation and degradation, lipid peroxidation, and DNA damage	Jaeschke, ³⁹ 2000 Rauen, ⁵³ 1999
Direct induction and regulation of apoptotic and necrotic cell death	Rudiger, ⁵⁴ 2002 Weiss, ⁴⁰ 1989
Inactivation of antiproteases	Jaeschke, ³⁹ 2000
Induction of protective stress genes in hepatocytes	Bauer, ⁶³ 2002 Nakatani, ⁶¹ 1997
Formation of mediators involved in regulating sinusoidal blood flow and liver regeneration	Paxian, ⁶⁰ 2001
Abbreviations: IL-1, interleukin-1; IL-8, interleukin-8; NF- κ B, nuclear factor kappa B.	

liver I/R are still under investigation, the nicotinamide adenine dinucleotide phosphate oxidase, the xanthine/XOR system, and the mitochondria have been suggested to play key roles.⁵⁵ Although XOR was regarded as the principal source of postischemic oxidant stress in the liver, recent evidence suggests that XOR plays a minor role as compared to the mitochondria.⁵⁶ Mitochondria are the site of the production of large amounts of superoxide, under conditions of oxidative stress. It is this stress that finally leads to the formation of membrane permeability transition pores and the breakdown of the mitochondrial membrane potential that can cause cellular death.⁵⁷

ROS and RNS can have an important role in signal transduction pathways coordinating the body's inflammatory response after liver I/R injury.^{20,39,58,59} They are involved as mediators in the production of substances regulating liver blood flow⁶⁰ and regeneration.^{61,62} Transgenic mice with overexpression of antioxidant enzymes have decreased cellular ploidy during liver regeneration, suggesting a role for ROS in cell cycle control.⁶¹ ROS also induce stress genes such as haem oxygenase (HO)-1.⁶³ The induction of HO-1 leads to formation of the antioxidant biliverdin, the vasodilator carbon monoxide, and iron. The multiple roles of ROS in liver I/R are summarized in Table 1.

Role of Nitric Oxide in Liver I/R Injury

NO is a radical synthesized via the oxidation of L-arginine by NO synthetase (NOS).⁶⁴ There are two major isoforms of NOS in the liver, endothelial NOS and inducible NOS (iNOS). Endothelial NOS is expressed

constitutively, and its activity is dependent on Ca²⁺ and calmodulin.⁶⁵ iNOS is synthesized by endothelial cells, hepatocytes and Kupffer cells and its activity is Ca²⁺ independent. NO is a lipophilic biomolecule that diffuses to adjacent cells and enters the cytosol, where it activates soluble guanylyl cyclase by binding to the iron in the heme center, resulting in an intracellular increase of cyclic guanosine monophosphate levels.⁶⁶ Many of the biological actions of NO are mediated through the guanyl cyclase/cyclic guanosine monophosphate system.

Under physiologic conditions only constitutive endothelial NOS is present in the liver and the low level of NO produced regulates hepatic perfusion, prevents platelet adhesion, thrombosis, polymorphonuclear cell accumulation and secretion of inflammatory mediators.^{67,68} NO also induces vasodilatation at the level of the sinusoid and at pre-sinusoid sites^{69,70} to keep a balance with vasoconstrictors such as endothelin.⁶²

Induction of iNOS may have either toxic or protective effects. The effects are dependent on the type of insult, the level and duration of iNOS expression and the simultaneous production of superoxide anion.⁴⁹

In liver I/R iNOS messenger RNA expression starts 1 hour postreperfusion with increased iNOS activity at 5 hours postreperfusion.⁷¹ The literature concerning the effect of iNOS in liver I/R injury is still ambivalent. Some studies suggest that iNOS expression has detrimental effects⁷²⁻⁷⁴ to liver function, while others suggest that it is beneficial^{75,76} or has no effect at all.^{77,78} One study with mice deficient in iNOS showed a moderate reduction in reperfusion injury.⁷⁹

The toxic effects of NO are linked with the produc-

tion of peroxynitrite, which is the product of $O_2^{\cdot-}$ and NO. Peroxynitrite can cause cell injury through multiple pathways: initiation of lipid peroxidation, direct inhibition of mitochondrial respiratory chain enzymes, inhibition of membrane Na^+/K^+ ATPase activity, or oxidative protein modification such as formation of nitrotyrosine.⁸⁰⁻⁸²

However whether NO will act as a cytoprotective or cytotoxic agent depends on an number of factors, such as NO-superoxide radical ratios, hepatic stores of reduced glutathione, and the duration of ischemia.

The Antioxidant System

The body has developed major antioxidant defense mechanisms to protect it from damage from free radicals. An antioxidant is any substance that when present at low concentrations, compared with those of an oxidizable substrate, significantly delays or prevents oxidation of the substrate.⁸³ The endogenous antioxidants mainly are small molecular weight substances that are able to prevent initiation of oxidative damage or to limit its propagation and enzymes that convert and detoxify ROS and RNS. Cellular redox balance is in normal circumstances under tight control. However, when ROS and RNS are produced at levels that cannot be counteracted by endogenous antioxidant systems, an imbalance takes place, called oxidative stress.⁸⁴ This condition can lead to the damage of lipids, proteins, carbohydrates, and nucleic acids. Hepatocytes tend to be resistant to injury by ROS and RNS, since they contain high intracellular concentrations of glutathione (GSH), superoxide dismutase (SOD), catalase, and lipid soluble antioxidants.

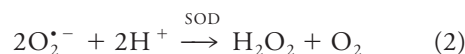
The antioxidant system is very important for the living species and has allowed them to use O_2 for energy production, without being exposed to the deleterious effects of O_2 . The composition of antioxidant defences differs from tissue to tissue and from cell to cell in a given tissue. Also, different organs contain different concentrations of antioxidants, and for this reason there is variability in organ resistance to I/R. However, there is evidence that the antioxidants operate as a balanced and coordinated system and each relies on the action of the others.^{85,86}

Antioxidants are a heterogenous family of molecules. Several classifications have been used in the past taking into account the origin (natural or synthetic), nature (enzymatic or nonenzymatic), properties (hydrophilic or lipophilic), mechanism (catalytical removal of ROS, metal chelation, scavenging of ROS), and site of action (intracellular; membrane, and extra-

cellular). This review is presented according to the site of action of the antioxidants.

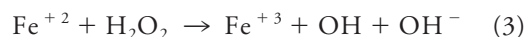
Intracellular antioxidant defenses include the superoxide dismutase; catalase; glutathione peroxidase and reductase enzymes, the tripeptide glutathione, the polypeptide thioredoxine, the enzyme HO, and peroxidases of the peroxiredoxin family.

SOD catalyses the dismutation of superoxide to hydrogen peroxide and oxygen (equation 2):

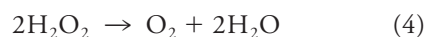


Three forms of SOD exist with different subcellular localizations. Those containing copper and zinc are located in the cytosol,⁸⁷ manganese in the mitochondria,⁸⁸ and the extracellular form usually located on the outside of plasma membrane interacting with matrix components.

The product of reaction 2, H_2O_2 , is a weak oxidant and is relatively stable. However, unlike superoxide, H_2O_2 can rapidly diffuse across cell membranes, and in the presence of transition metal ions it can be converted to toxic hydroxyl radicals via Fenton chemistry (equation 3):



Three main systems can break down H_2O_2 . One of them is the hemoprotein catalase. Catalase is present in all major body organs, especially concentrated in liver. It catalyzes the breakdown of hydrogen peroxide to oxygen and water (equation 4):



The second system consists of the glutathione peroxidases. This group includes 4 different isoforms, such as cellular, gastrointestinal, extracellular, and phospholipids.⁸⁹ They have a major role in removing hydrogen peroxide generated by superoxide dismutase with the oxidation of GSH to its oxidized form, glutathione disulphide (GSSG) (equation 5):



Glutathione reductase is also an important enzyme in this system. It expresses its action through the regeneration of GSH from glutathione disulphide using nicotinamide adenine dinucleotide phosphate.⁹⁰

GSH is a tripeptide present in millimolar concentrations in virtually all cells. It is an important component of the endogenous antioxidant system. GSH's main function is to act as a cosubstrate of glutathione peroxidase to reduce intracellularly generated peroxides. GSH also scavenges directly ROS and RNS. GSH is

involved in many other metabolic processes including prevention of oxidation of protein sulfhydryl groups and chelation of copper ions. GSH is also present in the extracellular fluids in very small concentrations.^{51,91,92}

The third system consists of peroxidases of the peroxiredoxin family that reduce hydrogen peroxide and alkyl hydroperoxides to water and alcohol respectively using reducing equivalents. These equivalents are derived specifically from thiol-containing donor molecules, such as thioredoxin. They are located in the cytoplasm (peroxiredoxin I and II) and in mitochondria (peroxiredoxin III).^{93,94}

Thioredoxin is a polypeptide especially concentrated in the endoplasmic reticulum (thioredoxin 1), but it is also found in mitochondria (thioredoxin 2). Thioredoxin contains 2 adjacent sulfhydryl groups in its reduced form that are converted to a disulphide in the oxidized form thioredoxin. In addition, it can undergo redox reactions with multiple proteins.^{86,95}

HO is an enzyme found in the endoplasmic reticulum that catalyses the breakdown of haem to biliverdin with the release of iron ions and carbon monoxide. Three isoforms of HO have been characterized: HO-1, which is highly inducible in conditions of inflammation and oxidative stress, and HO-2 and HO-3, which are constitutively expressed.⁹⁶ Induction of HO-1 protects the cell against oxidative injury by controlling intracellular levels of free heme (a prooxidant), producing biliverdin (an antioxidant), and improving microcirculation via carbon monoxide release.⁶³

Major extracellular antioxidant defenses include the metal-binding proteins.⁹⁷ Free metals iron and copper can promote free radical damage, accelerating lipid peroxidation and catalyzing hydroxyl radical formation. The body is protected against these potentially adverse effects by metal-binding proteins that ensure that these metals are maintained in a nonreactive state.⁹⁸ Transferrin and lactoferrin bind iron, while ceruloplasmin and albumin bind copper. Haemoglobin and myoglobin are normally intracellular proteins. When these proteins are exposed to a large amount of oxidative stress such as H₂O₂, they are degraded, releasing both haem and iron ions that can then stimulate lipid peroxidation. Hemoglobin binding proteins known as haptoglobins and haem binding proteins such as hemopexin decrease the effectiveness of these substances in stimulating lipid peroxidation.⁹⁹

In addition to the major protective role of the metal-binding proteins, various low-molecular-weight molecules that are synthesized in vivo have antioxidant properties.¹⁰⁰⁻¹⁰² The most important of these substances are bilirubin, melatonin, lipoic acid, coenzyme Q, uric acid

and the melamins. Recent evidence suggests that uric acid has an important role in the endogenous antioxidant system^{103,104} and that exogenous administration of uric acid could have beneficial effects in situations associated with oxidative stress.^{105,106}

A large number of dietary constituents exert antioxidant effects in vivo. The most important are hydrophilic ascorbic acid (vitamin C) and lipophilic α -tocopherol (the most active form of vitamin E) that are important components of the human antioxidant system. Ascorbate is required in vivo as a cofactor for many enzymes, of which the best known are proline hydroxylase and lysine hydroxylase, both involved in the biosynthesis of collagen. Its main chemical property is its ability to act as a reducing agent. It can scavenge most radicals such as O₂⁻, \cdot OH, peroxy, thiyl, oxysulphur radicals, and peroxyxynitrite.^{98,107} The lipophilic α -tocopherol is a highly effective antioxidant when incorporated in the lipid core of cell membranes. It has the ability to scavenge intermediate peroxy radicals and therefore interrupt the chain reactions of lipid peroxidation.¹⁰⁸

Carotenoids are a group of colored pigments that are widespread in plant tissues. They serve as a precursor of vitamin A and are the principal dietary source of vitamin A in humans. They exert their antioxidant action as free-radical scavengers.¹⁰⁹

Another group of antioxidants are the plant phenols. Plants contain a huge range of phenols, including tocopherols, tocotrienols, flavonoids, anthocyanidins, and phenylpropanoids. They inhibit peroxidation by acting as chain-breaking peroxy-radical scavengers. In addition, they scavenge ROS and RNS such as \cdot OH, ONOO⁻, and hypochlorous acid, and they act as metal chelators.^{110,111}

There is synergism among the different antioxidants and they are linked to each other in a systematic relationship as part of the antioxidant network.⁸⁶ Recent studies in myocardial I/R injury suggest that the hydrophilic agents (ascorbic acid, GSH) are consumed first followed by the lipophilic species (vitamin E).^{85,112} It has also been shown that ascorbate can recycle α -tocopherol.⁸⁶ Figure 2 summarizes the main antioxidant mechanisms.

Antioxidant Therapy

A large number of antioxidant agents have been shown clinically or experimentally to have benefit in the treatment of liver I/R injury. Table 2 summarizes the effects of these agents in liver I/R injury.

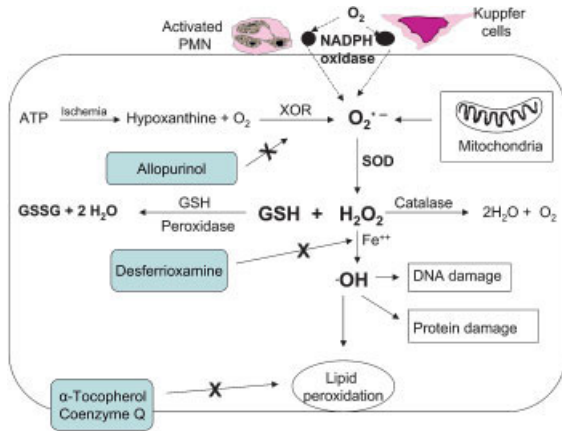


Figure 2. Basic mechanisms of endogenous antioxidant system. XOR and mitochondria are the main intracellular sources of production of ROS. Kupffer cells and activated neutrophils are the main extracellular sources for production of ROS during the early and late phase of reperfusion respectively. The main intracellular antioxidant enzymes are SOD, catalase and GSH peroxidase. A-tocopherol and coenzyme Q are the main antioxidants in cellular membranes. PMN, polymorphonuclear cell; NADPH, nicotinamide adenine dinucleotide phosphate; GSSG, glutathione disulphide.

Membrane and Extracellular Antioxidants

α -tocopherol is the most important inhibitor of the free radical chain reaction of lipid peroxidation. It acts as a direct free radical scavenger, increases GSH levels, and has other nonantioxidant properties, such as the inhibition of protein kinase C.¹¹³⁻¹¹⁶ A significant reduction in liver α -tocopherol levels was observed during the first hour of reperfusion in a rat model of liver ischemia.¹¹⁷ Pretreatment with high and very high doses of α -tocopherol (30 and 300 mg/kg of body weight intra-muscular, respectively) improved ATP levels, prevented the increase in lipid peroxidation products such as thiobarbituric acid reactive substances, and attenuated the loss of hepatic glutathione during the early phase of reperfusion after warm ischemia in rats.⁵² High doses of α -tocopherol (1,000 U/kg) also increased the survival of rats with steatotic liver that underwent warm liver ischemia.¹¹⁸ α -tocopherol has also shown beneficial effects in cold I/R injury.¹¹⁹ Trolox C, the hydrophilic analog of α -tocopherol, has shown beneficial effects in experimental liver I/R injury.¹²⁰ The combination of α -tocopherol and pentoxifylline, a drug that affects microcirculation as used in the treatment of peripheral vascular disease, reduces experimental warm liver I/R injury.¹²¹

Ascorbic acid in low doses (<100 mg/kg) has also shown protective effects in hepatic function in an experimental study of liver I/R injury. However, high doses of ascorbic acid (1,000 mg/kg) aggravated the injury. This harmful effect could be due to the increased reduction of ferric iron to the ferrous form under high ascorbic acid concentration.¹²² A prospective randomized clinical study in patients undergoing liver resection used an antioxidant multivitamin infusion containing 10 mg α -tocopherol acetate and 1 g ascorbate administered prior to reperfusion. In the treated group, less plasma lipid peroxidation occurred and less acute liver damage as assessed by measurement of the prothrombin time and aminotransferase levels. The treated group also had fewer postoperative complications (postoperative infections and bleeding disorders).¹²³

Melatonin is a hormone produced by the pineal gland that helps regulate circadian rhythms and exhibits antioxidant activity. In an experimental study with rats that underwent 60 minutes of total liver ischemia and 2 hours reperfusion, melatonin administration preserved functional and energetic status, reduced TNF- α production and inhibited expression of iNOS.¹²⁴ One recent clinical study in patients undergoing liver resections suggested that the protective effect of melatonin may be through the enhancement of neutrophil apoptosis.¹²⁵

Administration of lipoic acid in experimental models of warm and cold I/R injury showed protective effects through the enhancement of the phosphatidylinositol-kinase pathway.¹²⁶

Pretreatment with coenzyme Q prevented the post ischemic loss of hepatic α -tocopherol and glutathione¹¹⁷ and also attenuated the increase in lipid peroxidation and the decrease in mitochondrial respiration in rats submitted to partial hepatic ischemia.¹²⁷ Also, the administration of idebenone that is an analog of coenzyme Q-10, in isolated perfused pig livers significantly reduced the neutrophil mediated reperfusion injury.¹²⁸ The combination of coenzyme Q and pentoxifylline had a protective effect in warm I/R injury by maintaining GSH levels and by inhibiting lipid peroxidation.¹²⁹

As mentioned before, free iron in its ferrous state can catalyze the formation of hydroxyl radical from hydrogen peroxide (Fenton reaction). In this way it can initiate lipid peroxidation. Desferrioxamine is an iron chelator used for the treatment of iron loading diseases such as thalassemia. Pretreatment with desferrioxamine in vivo protected in experimental settings with warm and cold hepatic ischemia.¹³⁰⁻¹³²

A number of experimental studies using biomolecules principally trimetazidine¹³³ or trimetazidine ana-

Table 2. List of Antioxidant Agents With Beneficial Effects in Liver IR Injury

Antioxidant	Category	Species	Injury Type	Mode of Adm.	Dose	Main Protective Effect	Author
α -Tocopherol	Vitamin-diet	Rat	WI	im	30 and 300 mg/kg	Survival, histology	Giacoustidis ⁵²
α -Tocopherol	Vitamin-diet	Rat	CI/WI	iv	50 IU/Kg	Histology	Gondolesi ¹¹⁹
α -Tocopherol/ Ascorbate	Vitamins-diet	Clinical	WI	iv	2 mg/1,000 mg	Better PT ↓ Postop. complications	Cerwenka ¹²³
Ascorbate	Vitamin-diet	Rat	WI	iv	30 and 100 mg/kg	↓ Lipid peroxidation	Seo ¹²²
Coenzyme Q/ Pentoxifylline	In vivo LMM agent	Rat	WI	in/ip	10 mg/kg/50 mg/kg	↓ Lipid peroxidation	Portakal ¹²⁹
Idebenone	Coenzyme Q derivative	Pig	CI			↑ GSH levels	Schutz ¹²⁸
Lipoic acid	In vivo LMM agent	Rat	CI	iv	500 μ M	Histology	Muller ¹²⁶
Deferrioxamine	Iron chelator	Dog	CI/WI	iv	20 mg/kg	↓ AST activity	Park ¹³²
Trimetazidine	Metal chelator	Rat	WI	iv/ip	2.5 mg/kg	Histology	Tsimoyiannis ³
Quercetin	Plant phenol	Rat	WI	po	0.13 mmol/kg	↓ ALT, AST	Su ¹³⁶
Cyanidin	Plant phenol	Rat	WI	po	0.9 mmol/kg	↓ Lipid peroxidation	Tsuda ¹³⁷
Green Tea Extracts (catechines)	Plant extracts (catechines)	Rat	WI	po	0.1%	Histology	Zhong ¹⁴²
Magnifera indica	Plant extract	Rat	WI	po	250 mg/kg	↓ AST, ALT ↓ Lipid peroxidation	Sanchez ¹⁴³
GSH	In vivo LMM agent	Rat	WI	iv	100 μ M/h/kg	↓ ALT ↑ Survival	Schauer ¹⁵⁰
GSH	In vivo LMM agent	Rat	CI/WI	iv	100 μ M/h/kg	↓ ALT ↑ Bile flow	Schauer ¹⁴⁹
N-acetylcysteine	Thiol compound GSH precursor	Rabbit	WI	iv	150 mg/kg	↓ ALT ↑ Microcirculation	Glantzounis ¹⁵
N-acetylcysteine/ Melatonin	Thiol compound	Rat	WI	ip	150 mg/kg/ 10 mg/kg	↓ AST, ALT ↓ Lipid peroxidation	Sener ¹⁵⁸
N-acetylcysteine	Thiol compound	Clinical	CI/WI	iv		↓ ICAM, ↓ α -GST	Weigand ¹⁶²
Bucillamine	Thiol compound	Rat	CI/WI	iv		Survival	Amersi ¹⁶⁵
SOD derivatives	Intracellular enzyme	Rat	WI	iv	5,000 IU/Kg	↓ Lipid peroxidation	Nguyen ¹⁷⁴
CAT derivatives	Intracellular enzyme	Rat	WI	iv	0.1 mg/kg	↓ ALT, AST	Yabe ¹⁷⁷
Allopurinol	XO Inhibitor	Rat	WI	ip	50 mg/kg	↓ AST	Jeon ¹⁷⁹
Aminoguanidine	iNOS inhibitor	Pig	CI/WI	iv	10 mg/kg	Survival, histology	Kimura ⁷²

Abbreviations: Adm., administration; WI, warm ischemia; im, intra-muscular; CI, cold ischemia; iv, intravenous; PT, prothrombin time; in, intragastric; deriv, derivatives; LMM, low molecular molecule; AST, aspartate transaminase; po, per oral; ALT, alanine transaminase; ICAM-1, intercellular adhesion molecule-1; α -GST, α -glutathione S-transferase; CAT, catalase; XO, xanthine oxidase; GSH, glutathione.

logs¹³⁴ have shown beneficial effects after warm liver ischemia. The mode of action has been postulated to be through the chelation of metals, mainly copper.¹³⁵ In addition, plant phenols^{136,137} and herbal medicines¹³⁸⁻¹⁴³ have shown beneficial effects (Table 2).

From the above, it is clear that a lot of experimental evidence exists about the protective effects of extracellular antioxidants in hepatic I/R injury. However, the majority of these antioxidants have not as yet been tested in small or large clinical trials.

Intracellular Antioxidants

Thiol-Containing Compounds

Sulphydryl groups exert their antioxidant action through the oxidation of the thiol (sulfhydryl) group of cysteine. Furthermore, they have a central role as mediators to the majority of redox-sensitive cell signaling mechanisms.^{86,144}

The main representative in this group is GSH. It serves as a substrate for glutathione peroxidase and also scavenges directly ROS.^{145,146} Glutathione peroxidase is the major defense system of the cell against ROS found in the cytosol and mitochondria and detoxifies H₂O₂ very effectively. The main problem is that its efficacy is dependent on the availability of intracellular GSH and the ability of the cell to rereduce the oxidized form, glutathione disulphide.¹⁴⁷

The administration of GSH or its precursors could be expected to be effective if the compounds are supplied at the time of declining tissue GSH levels. Although initial experimental studies with GSH administration in liver I/R have not shown a protective effect,¹⁴⁸ recent studies in rats have shown that intravenous administration of GSH in doses over 100 μ mol/h/kg offers significant protection from both warm and cold liver ischemia.^{149,150} Exogenous GSH administration has limited cellular uptake, due to its large molecular size. This may limit its value in situations associated with severe intracellular oxidative stress.¹⁴⁷ Glutathione precursors such as *N*-acetylcysteine (NAC) can enter cells more easily due to its smaller size. NAC is a biomolecule that is commercially available and was introduced for the treatment of congestive and obstructive lung diseases, primarily those associated with hypersecretion of mucus, such as chronic bronchitis and cystic fibrosis.¹⁵¹ NAC is currently the drug of choice in the treatment of fulminant liver failure due to paracetamol overdose.¹⁵² The diversity in the pharmacological uses of NAC is due to the multiple chemical properties of the cysteinyl thiol of the molecule. These include its nucleophilicity and redox reactions. The main mechanism of action of NAC is through the metabolism to cysteine *in vivo* and synthesis of GSH.¹⁵³ NAC can also act as a chemical antioxidant. *In vitro* studies show that the interaction with free radical species results in the intermediate formation of NAC thiyl radicals, with NAC disulphide as the major end product.¹⁵⁴

The results in the literature about the effect of NAC in liver warm I/R injury are still ambivalent.^{155,156} This probably relates to differences in the method of NAC administration and the duration of follow-up. Our

group has studied the effects of continuous intravenous NAC administration in both the early and late phases of I/R injury in a rabbit lobar liver I/R model. NAC significantly improved liver function, microcirculation, and hepatic tissue oxygenation during the late phase of reperfusion.¹⁵⁷ The combination of NAC and melatonin has more pronounced beneficial effect, during the early reperfusion period, than NAC or melatonin alone, in a rat model of 45 minutes' total warm liver ischemia.¹⁵⁸ Five small clinical trials have looked at the effect of intravenous NAC administration in patients undergoing liver transplantation. Two of these failed to show any clear protection on postoperative graft function.^{159,160} The third clinical study showed that NAC administration was associated with better liver function, less hepatocellular injury, and lower incidence of primary graft dysfunction.¹⁶¹ The fourth study showed that NAC attenuated the increase in α -glutathione *S*-transferase, circulating intracellular adhesion molecule-1 and vascular cell adhesion molecule-1 after liver transplantation, indicating cytoprotective effects.¹⁶² In the last study, NAC was used in combination with prostaglandin E₁ on pediatric liver transplant recipients. In this study, peak serum alanine aminotransferase was lower and median postoperative in-hospital stay was shorter in the treated group, while rejection was less severe.¹⁶³

Another related thiol compound is buccillamine, which contains 2 thiol groups and is more potent than other cysteine-derived agents that contain only 1 thiol.¹⁶⁴ In an experimental study with normal and steatotic rat livers, buccillamine administration significantly reduced hepatic I/R injury and improved outcomes after syngenic orthotopic liver transplantation.¹⁶⁵ Buccillamine in phase I human studies in normal volunteers, at doses within the therapeutic range (10-25 mg/kg/h), elicited no serious toxicity.¹⁶⁶

Superoxide Dismutase

The rationale behind using SOD is to accelerate the detoxification of the superoxide anion, thus preventing the generation of the highly reactive \cdot OH radical. *In vivo* experimental studies in hepatic I/R have reported protective effects with SOD pre-treatment.^{167,168} However, some other studies failed to show a protective effect with SOD administration.^{169,170}

To effectively detoxify intracellular ROS, the SOD molecule has to enter the cells intact, or superoxide has to leave the cell to be metabolized extracellularly. There is no evidence in the liver that superoxide moves through cell membranes.

The main problems with SOD administration are

the short half-life (about 6 min) and the lack of uptake of the intact protein into cells.¹⁴⁷ Inadequate delivery to target sites could be the cause for the mixed results reported to date in the literature.

To improve the intracellular availability of SOD, new derivatives have been developed. These include the conjugation of SOD with carbohydrate structures that facilitate the uptake into liver non-parenchymal cells. The techniques that have been developed are mannosylation, succinylation,¹⁷¹ and pegylation.¹⁷² Kupffer and sinusoidal endothelial cells have receptors that recognize and internalize ligands containing mannose, succinylated and pegylated proteins. Targeted SOD derivatives showed beneficial effects in preventing experimental warm liver I/R injury.^{173,174}

Catalase

The results in the literature are ambivalent concerning the role of catalase in liver I/R^{175,176} injury. The main problems are the same as with SOD, the short half-life in plasma, and the difficulty of protein uptake into cells. To bypass these difficulties, catalase-targeted derivatives have been developed from conjugation with carbohydrates. The initial results appear promising,¹⁷⁷ and the administration of a combination of both enzymes (catalase and SOD) has been tried and the results were found to be good.¹⁷⁸ Among the different combinations Man-SOD and Suc-catalase have shown the greatest efficacy in preventing liver injury. This combination reduced significantly inter-cellular adhesion molecule-1 expression and neutrophil infiltration.

Allopurinol

Allopurinol is an XO inhibitor. Low doses (5-10 mg/kg) are sufficient to inhibit hepatic activities of xanthine oxidase and xanthine dehydrogenase almost completely, but these doses were not protective of liver I/R.¹⁴⁷ On the other hand, an experimental study where allopurinol was administered intra-peritoneal, before ischemia, at high doses (50 mg/kg) showed a clear protective role in rats subjected to liver I/R injury.¹⁷⁹ There is experimental evidence that allopurinol has a protective role in acetaminophen-induced toxicity.¹⁸⁰ The most likely mechanism for this protective effect is the prevention of mitochondrial oxidative stress. An alternative mechanism is the action as a free radical scavenger and most probably as a scavenger of peroxynitrite.¹⁸⁰

Table 2 summarizes substances with extracellular and intracellular antioxidant action that have shown beneficial effects in liver I/R injury.

Antioxidants That Modulate NO Metabolism

NO can have either beneficial or detrimental effects in liver I/R injury. Recent evidence has shown that during the reperfusion period endothelial NOS is downregulated in both hepatocytes and inflammatory cells during the late phase.¹⁸¹ The expression of iNOS is associated with evidence of ONOO⁻ formation, although the exact role of peroxynitrite in liver I/R is not clear.²⁰ It has been postulated that when the endogenous amount of SOD or GSH is not enough to inhibit ONOO⁻ formation, cellular damage can occur. Pharmacological intervention to block ONOO⁻ formation could have a protective role against the toxic effects of massive ONOO⁻ production.⁴⁹ This intervention could act either at the level of the reactant (NO and O₂^{·-}) or the product (ONOO⁻). The blockage of O₂^{·-} can be done by the use of SOD or its derivatives. There are reports in the literature that showed beneficial effects with use of selective inhibitors of iNOS in both warm and cold liver I/R injury.^{72,182}

Strategies that aim at decreasing the intrinsic lifetime of ONOO⁻ could be either competitive stoichiometric trapping of ONOO⁻ or catalysis of ONOO⁻ decomposition to benign products (for example isomerization to nitrate). Such ONOO⁻ decomposers have been developed and have as their base iron porphyrin complexes.⁴⁹ The search for other redox-active complexes that will accomplish catalysis of the peroxynitrite isomerization to nitrate still continues.

Antioxidant Gene Therapy

Gene therapy has recently been applied as a therapeutic strategy against I/R injury. Two categories of vector systems have been used: viral and nonviral. The advantages of virus-based systems include higher infection efficiencies and the ability to encode multiple large genes.¹⁸³ The disadvantages include immunogenicity and vector production issues. For these reasons, nonviral vector systems using lysosomes and DNA-protein complexes have been developed.

In liver I/R injury predominantly, to date, viral vectors, typically recombinant virus, have been used. High doses of mitochondrial SOD administered via viral vector in rats prevented liver I/R injury via inactivation of the transcription factors nuclear factor kappa B and activator protein-1.¹⁸⁴ Experimental studies in a rat liver transplantation model with normal and steatotic livers have shown that cytosolic and mitochondrial SOD markedly improved survival, whereas extracellular SOD was not protective.^{185,186} In another experi-

mental study,¹⁸⁷ overexpression of the 3 SOD isoforms were all shown to protect against an increase in serum transaminases and hepatocellular necrosis following I/R injury. They also significantly reduced the production of lipid derived free radicals. The extracellular form was protective when administered in high doses.

A recent *in vitro* study has shown that the induction of human genes expressing the peroxidase peroxiredoxin can protect murine cells effectively from oxidative stress.¹⁸⁸ Gene therapy therefore has potential for amelioration of the effects of liver I/R. Although its value to cadaveric liver transplantation may be limited by the emergency nature of the procedure, it could have application to elective liver surgery, such as liver resection for tumors or living donor liver transplantation. However, the future success of gene therapies requires better understanding of the pathophysiology of I/R. Although ROS can cause cell injury during I/R, they may also have a significant role in the normal cell growth and proliferation. The targets should be ROS that are responsible for cell injury; in addition, the subcellular compartments that produce detrimental ROS should be identified. A recombinant adenoviral vector has been used to inhibit nuclear factor kappa B activation in a model of partial hepatectomy,¹⁸⁹ resulting in massive apoptosis and also delaying the regeneration process. This study showed that nuclear factor kappa B is important in preventing apoptosis and also in enabling liver regeneration.

Conclusions

Liver I/R injury occurs in a number of clinical settings in general surgery and is associated with increased morbidity and mortality. ROS and RNS play a major role in the pathophysiology of I/R injury. The antioxidant defense system is a complex one that includes intracellular enzymes, nonenzymatic substances that act as scavengers, and dietary components. It normally controls the production of ROS and RNS. Oxidative stress occurs when there is significant imbalance between production and removal of ROS and RNS. This occurs principally when antioxidants are depleted or oxidants are overproduced.

Antioxidant therapy is a promising therapeutic strategy to ameliorate liver I/R injury. Drug therapy has distinct advantages when compared to surgical strategies such as ischaemic preconditioning since it can be applied in conditions where the surgical strategies cannot be applied (typically transplantation or hemorrhagic shock) and at the same time avoid the detrimental effects that the surgical techniques pos-

sess. In recent years, new strategies have been developed using, for example, SOD and CAT derivatives, thiol compounds, selective NOS inhibitors, peroxynitrite decompositors, and gene therapy. Although the results are still not entirely clear; there is accumulative evidence, mainly from *in vitro* and *in vivo* experimental studies, that the administration of antioxidant substances can reduce the postreperfusion liver injury.

However, important parameters have to be taken in account before antioxidant therapy is applied: (1) Free radical pathways are very complex, and more studies are needed for an in depth understanding of the mechanisms involved. ROS and RNS at low concentrations have an important role as signal, trigger, and messenger molecules in cellular growth and metabolism.^{190,191} The understanding of the signal transduction mechanisms and the role of ROS and RNS in them enables us to develop therapeutic strategies that will then target detrimental oxidative stress but without affecting the pathways that are responsible for normal cell growth, repair and generation after liver I/R. (2) Free radicals are very reactive with extremely short half-lives. Timing of intervention is critical. (3) Certain antioxidants can also exert pro-oxidant effects under specific conditions.⁸⁶ (4) Differences between species, duration of ischemia, dose, timing and mode of drug administration, distribution problems, and different endpoints may account for the inconsistency found in the results.

The evaluation of antioxidants in large-scale randomized clinical trials in which treatment effects can be closely monitored is therefore a necessity before routine clinical application.

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