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Mitochondrial Free Radicals, Antioxidants, Nutrient Substances, and Chronic Hepatitis C

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1. Introduction

Clinical evidence shows that oxidative stress plays vital roles in a wide variety of pathological processes. Oxidative stress can arise as result of the production of free radicals, highly reactive molecules containing one or more unpaired electrons, which overwhelms the body's endogenous antioxidant defense capacity. In general, free radical molecules are representative of both reactive oxygen species (ROS) and reactive nitrogen species (RNS). The term ROS refers to several products that result from the partial reduction of oxygen, including oxygen free radicals (superoxide $[O_2^{\cdot-}]$, hydroxyl $[OH^{\cdot}]$, peroxy $[RO_2^{\cdot}]$, and alkoxy $[RO^{\cdot}]$), and some non-radical derivatives of oxygen such as hydrogen peroxide (H_2O_2), singlet oxygen (1O_2), and hypochlorous acid (HOCl). ROS can be further converted to RNS such as nitric oxide (NO^{\cdot}), peroxynitrite ($ONOO^-$), nitrogen dioxide (NO_2^{\cdot}), and other oxides of nitrogen (Wiseman and Halliwell, 1996). The excessive generation of ROS and/or RNS can be attributable to the action of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, p450 monooxygenase, xanthine oxidase, monoamine oxidase, mitochondrial oxidative phosphorylation, lipoxygenase, cyclooxygenase, endothelial NOS (eNOS) uncoupling, and myeloperoxidase (Muller and Morawietz, 2009).

Mitochondrial oxidative phosphorylation is regarded as the main source of free radicals (Naoi and Maruyama, 2009). Once generated, free radicals can directly impair mitochondrial structure and function. A decline in mitochondrial respiratory function along with an insufficient supply of energy can significantly increase mitochondrial free radical production (Van Houten et al., 2006; Lee et al., 2007). Increased oxidative damage may enhance inflammatory responses and alter immune function and appear to be involved in the pathologic mechanisms of many diseases.

This review article focuses on the production of free radicals from the mitochondria, as well as oxidative stress and antioxidant defense in patients with chronic viral hepatitis C. In addition, this article discusses recent advances in the antioxidant therapeutic intervention.

2. Chronic Hepatitis C

Hepatitis C virus (HCV) infection is a major cause of chronic liver disease. HCV infection frequently does not resolve, leading to chronic hepatitis with increasing risk of developing hepatic fibrosis, steatosis, liver cirrhosis, hepatocellular carcinoma, and extrahepatic diseases (Choi and Ou, 2006). The combination of pegylated interferon (IFN)- α and ribavirin is the only treatment for chronic HCV infections with proven efficacy. Unfortunately, this therapeutic strategy results in a low sustained virologic response (SVR), defined as an absence of detectable serum HCV-RNA at six months after completion of antiviral therapy; SVR is achieved in less than 50% of treated patients that have HCV genotype 1 and a high viral load (Ghany et al., 2009).

There is evidence indicating that SVR is associated with long-term clearance of HCV infection and lower HCV-related complications (Ghany et al., 2011; Pearlman and Traub, 2011). However, IFN- α in combination with ribavirin is generally not well tolerated, and the adverse side effects may lead to interruption or cessation of therapy. The major adverse effects are anemia, fatigue, hair loss, depression, insomnia, vertigo, anorexia, nausea, nasal congestion, cough, dyspnea, pruritus, and growth delay (Ko et al., 2005a). Thus, further advances in effective antiviral treatments against chronic hepatitis C are necessary.

3. Oxidative stress and related risk factors in chronic Hepatitis C

Recent studies indicate that oxidative stress not only accelerates the progression of liver damage (Vidali et al., 2008), but also affects the immune response to HCV infection and decreases SVR (Onoda et al., 2004; Polyak et al., 2007). Altered innate immunity (i.e., NK cells, neutrophils, dendritic cells, monocytes, and macrophages) and adaptive immunity (T- and B-lymphocytes) have influences in the development and progression of HCV infection. Although innate immunity can regulate adaptive immune response, HCV may escapes innate immune sensing by Toll-like receptors and exacerbates HCV infection and replication (Zhang et al., 2006; Montero Vega and de Andrés Martín, 2008). Thus, this sometimes makes it difficult for the immune response to suppress or eliminate HCV. The imbalance between cell-mediated and humoral immunity in chronic HCV-infected patients was also observed. Insufficient helper (CD4) and cytotoxic (CD8) T-lymphocytes have been shown significantly linked to HCV persistence (Grüngreiff and Reinhold, 2010). Recent evidence has shown that damaging ROS and mitochondrial injury play a vital role in immune responses (Kohchi et al., 2009; West et al., 2011).

Further, potential risk factors associated with SVR in HCV-infected patients include baseline HCV-RNA and aminotransferase levels, obesity, alcohol, insulin resistance (IR), non-alcoholic fatty liver disease (NAFLD), and fibrosis stage (Yamada et al., 2008; Pillai et al., 2010). In particular, NAFLD is not only strongly associated with IR and metabolic syndrome, but also with chronic HCV infection. The presence of hepatic steatosis correlates directly with serum and intra-hepatic titers of HCV-RNA (Younossi et al., 2004; Hübscher, 2006). Hepatic stellate cells can be activated by pro-inflammatory cytokines thus contributed

to liver fibrosis. Evidence have shown that the involvement of oxidative stress and inflammation in the progression of NAFLD and IR (Reiman et al., 2006; Narasimhan et al., 2010).

HCV-infected patients have significantly higher oxidative stress status, including increased hepatic, erythrocyte, lymphocyte, plasma, and serum malondialdehyde (MDA)(Farinati et al., 1995; De Maria et al., 1996; Barbaro et al., 1999a; Farinati et al., 1999; Mahmood et al., 2004; Guo et al., 2012), hepatic and serum 4-hydroxy-2-nonenal (4-HNE)(Kageyama et al., 2000; Mahmood et al., 2004), plasma F₂-iso- prostanes levels (Konishi et al., 2006), serum protein carbonyl (De Maria et al., 1996), plasma, hepatic and leukocyte 8-oxo-7-hydrodeoxy-guanosine (8-oxo-dG)(Farinati et al., 1999; Cardin et al., 2001; Mahmood et al., 2004; Chuma et al., 2008; Lin and Yin, 2009), as well as hepatic inducible nitric oxide synthase (iNOS) expression and nitrotyrosine production (i.e., nitration on the ortho- position of aromatic amino acids)(Garcia-Monzón et al., 2000).

Recent evidence has demonstrated that this oxidative stress induced during HCV infection via mitochondrial dysfunction generates ROS (Choi and Ou, 2006). High serum, plasma, erythrocyte and PBMC concentrations of MDA, 4-HNE, and F₂-isoprostanes, in combination with decreased levels of the antioxidant enzymes catalase, superoxide dismutase (SOD) and glutathione peroxidase (GPx), and decreased glutathione (GSH) and ascorbic acid (vitamin C) levels could reflect mitochondrial dysfunction (Wiswedel et al., 2002; Wen et al., 2006; Gomez-Cabrera et al., 2008; Sahach et al., 2008). The determination of serum, plasma, erythrocyte, urine, and PBMC concentrations of oxidative stress markers serves as an indirect index of mitochondrial oxidative stress in pathologic conditions (Modica-Napolitano et al., 2007). However, few studies have elucidated clinical importance of mitochondrial oxidative damage in chronic hepatitis C.

4. Mitochondria-driven free radical propagation

Not only are mitochondria the source of adenosine triphosphate (ATP) through oxidative phosphorylation on the inner mitochondrial membrane, but also the target of potentially damaging free radicals (Orrenius et al., 2007). Mitochondrial energy generation is first accomplished by tricarboxylic acid (TCA) cycle and represented in the form of ATP, nicotinamide adenine dinucleotide (NADH) and reduced flavin adenine dinucleotide (FADH₂). Furthermore, oxidative phosphorylation is the primary energy process by which the oxidoreduction energy of mitochondrial electron transport is converted to the high-energy phosphate bond of ATP. Oxygen (O₂) serves as the terminal electron acceptor for cytochrome c oxidase of complex IV in the mitochondrial electron transport chain (ETC) that catalyzes the four electrons reduction of O₂ to H₂O (Thannickal and Fanburg, 2000).

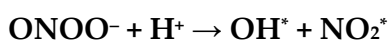
Coenzyme Q (CoQ, ubiquinone) behaves as an electron pool and a mediator of the electron transport between complex II (succinate dehydrogenase; also referred to as FADH₂: succinate CoQ reductase) and complex III (ubiquinone-cytochrome c reductase) with complex I (NADH dehydrogenase; also referred to as NADH: ubiquinone oxidoreductase).

A decrease in CoQ concentrations, activated reverse electron transfer, decline in the electron transport rate, or inhibition of electron flow can result in high-energy electrons leaking from the ETC at complexes I, II, III, and IV to produce $O_2^{\cdot-}$ (Lenaz et al., 2007). The major production site of $O_2^{\cdot-}$ is reportedly complexes I and III. Complex I produces $O_2^{\cdot-}$ predominantly on the matrix side of the inner membrane, whereas complex III-derived $O_2^{\cdot-}$ is produced both towards the inner-membrane space and the matrix (Matsuzaki et al., 2009). In particular, the matrix contains the components of the TCA cycle and fatty acid β -oxidation pathway, as well as mitochondrial deoxyribonucleic acid (mtDNA). The mtDNA is also a critical target for oxidative damage. Once damaged, mtDNA can amplify the secondary ROS generation (Van Houten et al., 2006). It appears that mitochondria are the organelle responsible for the majority of ROS production.

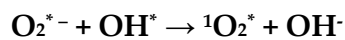
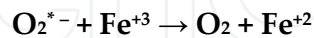
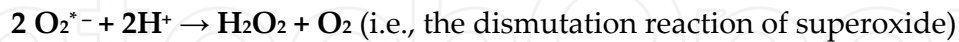
It is also noteworthy that self-amplification of the mitochondrial ROS generation can occur following ROS activation of mitochondrial permeability transition pore (MPTP). Once MPTP opening is triggered, ROS can induce the simultaneous collapse of the mitochondrial membrane potential ($\Delta\psi$, Dym) and a further increase in ROS generation by the ETC (Andreyev et al., 2005). In addition, damaged mitochondria produce increasingly more ROS in a process known as ROS-induced ROS release (RIRR) activation. In turn, cytosolic ROS released from the mitochondria could potentially function as second messengers to activate RIRR in neighboring mitochondria (Zorov et al., 2006).

4.1. Mitochondrial oxidant production

$O_2^{\cdot-}$ is the initial ROS generated in mitochondria during oxidative phosphorylation. Leakage of electrons from the mitochondrial ETC can result in incomplete reduction of molecular oxygen to produce $O_2^{\cdot-}$. The $O_2^{\cdot-}$ itself is not particularly reactive in biological systems; however, $O_2^{\cdot-}$ anions can damage heme moieties or enzymes with iron-sulfur centers such as aconitase ($[4Fe-4S] \rightarrow [3Fe-4S]^+$) to release ferrous ion (Fe^{+2}) (Ott et al., 2007). The Fe^{+2} can subsequently react with H_2O_2 to generate hydroxyl radicals (i.e., a Fenton process). Those superoxide radical anions can also react with NO^* to form the damaging oxidant $ONOO^-$, which is more reactive than either precursor (Barber et al., 2006). In turn, hydroxyl radical and nitric dioxide can be produced from $ONOO^-$, and membrane lipid peroxidation and nitration of proteins on tyrosine residues are promoted (Beckman and Crow, 1993). $ONOO^-$ further damages complex I, II, and V as well as mitochondrial SOD, GPx, and aconitase (Holley et al., 2011). A growing body of evidence demonstrates that NO diffuses easily along its gradient into mitochondria and that NO is also produced by mitochondria (Alvarez et al., 2003). The above-described reactions are summarized in the following equations:



As illustrated in the equations below, $O_2^{\cdot-}$ can either spontaneously dismutate to H_2O_2 by reacting with itself or $O_2^{\cdot-}$ can be catalyzed by antioxidant enzymes. Because the mitochondrial membrane is permeable to H_2O_2 , hydrogen peroxide can diffuse into the cytoplasm. H_2O_2 also decomposes to form the highly reactive hydroxyl radical, and this decomposition is accelerated in the presence of either ferrous or cuprous ions (Cu^+). Moreover, superoxide can react with the radical OH^{\cdot} to form highly reactive single oxygen.



4.2. Consequences of mitochondrial oxidative stress

Increased free radicals generated by damaged mitochondria can cause oxidative damage and a significant decline in metabolic processes; increase the mitochondrial membrane potential; impair the flow of electrons along the ETC; decrease mitochondrial membrane fluidity; decrease respiratory control ratios and cellular oxygen consumption; oxidate cardiolipin (a phospholipid and located at both the inner and outer membranes); deplete cytochrome c; induce cellular calcium (Ca^{+2}) dyshomeostasis; and produce high levels of unwanted oxidants (Mecocci et al., 1997; Petrosillo et al., 2003; Mei et al., 2012; Nowak et al., 2012). The inevitable by-products of oxidative phosphorylation can modify and damage mtDNA, proteins, lipid, and matrix components in the mitochondria, as well as deplete cellular antioxidants, which all lead to cell death (Marchi et al., 2012).

Mitochondrial membranes are primarily composed of protein and phospholipids, whose interdependence is crucial for mitochondrial function (Gohil and Greenberg, 2009). In particular, fatty acids of the inner membrane are highly unsaturated (Berdanier, 1988). ROS attack to the mitochondrial membrane lipid components result in lipid peroxidation, which alter the membrane potential (Paradies et al., 2004). Therefore, ROS-induced mitochondrial damage that is considered an important mechanism involved in the onset and development of a diverse series of pathologies.

5. Enzymatic antioxidants in mitochondria

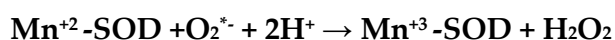
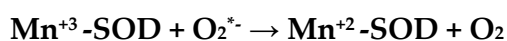
A network of specific non-enzymatic and enzymatic antioxidants can counteract mitochondrial ROS generation. Among these antioxidants, the non-enzymatic antioxidant systems are the second line of defense against free radical damage. It has been known that non-enzymatic antioxidants can act synergistically with enzymatic antioxidants. In animal models, administration of antioxidant vitamins increases mitochondrial SOD, GPx and catalase activity and significantly decreases MDA and carbonyl group levels, and thus

prevents rupture of mitochondrial membrane (Siler-Marsiglio et al., 2005; Zang et al., 2007; Rosa et al., 2009). The GSH, CoQ, lipoic acid, vitamin C and E are the non-enzymatic components of the antioxidant defense system in mitochondria (Ott et al., 2007; Liu, 2009).

The enzymatic antioxidant systems in mitochondria involve SOD, GPx, glutathione reductase (GR), catalase, glutaredoxin, thioredoxin, thioredoxin reductase (TrxR), and peroxiredoxin (PRx). Decreased activity of mitochondrial SOD and GPx were associated with mitochondrial oxidative stress (Zang et al., 2007). In this review, we discuss the characteristics and functions of SOD, GPx, and catalase.

5.1. Manganese-dependent superoxide dismutase

Mn-SOD is highly restricted and located in the mitochondrial matrix. This enzyme is a nuclear- encoded primary antioxidant and plays a vital role in the modulation of redox states. Although the dismutation reaction of $O_2^{\bullet-}$ can take place spontaneously, Mn-SOD can accelerate the reaction and rapidly convert $O_2^{\bullet-}$ to H_2O_2 . In the equations that follow, both the +2 and +3 states of manganese (Mn) are involved in the course of Mn-SOD turnover and the dismutation cycle.



Mn-SOD not only suppresses ONOO⁻ production and tyrosine residue nitration, but also inhibits membrane lipid peroxidation and mtDNA damage (Stojanović et al., 2005). $O_2^{\bullet-}$ has a pro-inflammatory role and induces ONOO⁻ formation, lipid peroxidation, and recruitment of neutrophils to sites of inflammation. Mn-SOD can scavenge $O_2^{\bullet-}$ and therefore mimics anti-inflammatory agent. Altered Mn-SOD levels and chronic inflammation have been associated with neurodegenerative diseases (Li and Zhou, 2011), metabolic diseases, and liver diseases (Kitada et al., 2011). Additionally, Mn-SOD participates in the mitochondrial repair processes and has a role along with p53 in preventing mitochondrial DNA damage (Bakthavatchalu et al., 2012).

The essential trace element Mn principally supports Mn-SOD activity and is required for a variety of physiological processes. Mn-SOD activity is positively related to the nutritional status of Mn (Luk et al., 2005). Clinical Mn deficiency is not common; however, many patients have decreased Mn levels and marked impairments in insulin sensitivity, glucose tolerance, and lipoprotein metabolism, resulting in decreased Mn-SOD and GPx levels, higher oxidative stress, and high mitochondrial abnormalities (Han et al., 2005; Rodríguez-Rodríguez et al., 2011). Thus, Mn dys-homeostasis may be inactive or decrease Mn-SOD levels, leading to mitochondrial oxidative damage.

5.2. Copper, zinc-dependent superoxide dismutase

A SOD isozyme, similar to cytoplasmic Cu,Zn-SOD, that contains Cu and Zn, is also found localized in the mitochondrial inter-membrane space (Kira et al., 2002), nuclei, lysosomes,

and peroxisomes (Culotta et al., 2006). Thus, while some O_2^* escapes into the inter-membrane space from the matrix side of the inner mitochondrial membrane, it can be partially catalyzed to H_2O_2 by Cu,Zn-SOD. Both trace elements Cu and Zn participate in the SOD enzymatic mechanisms that play an important role in oxidative balance. Apparently, deficiencies of Cu and Zn can result in impairment of the oxidant defense system (i.e., lower Cu,Zn-SOD, catalase, GPx, and cytochrome c oxidase activities), DNA repair, alterations in immune regulation, and increased oxidative stress (Ho and Song, 2009; Song et al., 2009; Guo et al., 2011). Mutations in the mitochondrial Cu,Zn-SOD gene result in SOD that are highly susceptible to glycation and are linked to elevated ROS production (Takamiya et al., 2003). Significantly lower serum and erythrocyte Cu,Zn-SOD activity and higher lipid peroxidation compared to controls have also been observed in patients with mitochondria injury-related disease conditions (Pawlak et al., 2005; Russo, 2009, 2010; Sagdic et al., 2011).

The electron carrier cytochrome c, which is also located in the mitochondrial inter-membrane space, oxidizes O_2^* back to O_2 (Pereverzev et al., 2003). Cytochrome c also scavenges H_2O_2 and significantly decreases H_2O_2 production in vitro (Wang et al., 2003). Recent evidence has shown that transgenic mice with overexpressing mutant Cu,Zn-SOD, have significantly decreased levels of inner mitochondrial membrane-associated cytochrome c and increased mitochondrial lipid peroxidation (Kirkinetzos et al., 2005). Therefore, Cu,Zn-SOD deletion and the loss of cytochrome c from the mitochondrial inter-membrane space can lead to reduced ETC and increased O_2^* production in disease conditions.

5.3. Glutathione peroxidase

Selenium (Se)-containing GPx is a selenocysteine-containing enzyme, of which multiple isoforms have been identified, including GPx-1, GPx-2, GPx-3, GPx-4, GPx-5, and GPx-6. GPx-1 is a major isoform localized in the cytoplasm and mitochondrial matrix (Orrenius et al., 2007) that metabolized H_2O_2 to O_2 and H_2O . However, GPx-1 levels in mitochondria are very low, compared with those in the cytoplasm. GPx-4 is membrane-associated that is found in the inter-membrane space of mitochondria, and is capable of reducing lipid hydroperoxides, alkyl peroxides, and fatty acid hydroperoxides with protect mitochondrial ATP generation. GPx-4 has also been shown to repair mitochondrial oxidative damage (Liang et al., 2009).

Se deficiency is associated with marked decreases in GPx activity and expression and the inhibition of ATP production. Patients with low GPx activity, have significant associations with increased levels of MDA, viral infection, and retroviral therapy (Stephensen et al., 2007). GPx can interfere with nuclear factor- κ B (NF- κ B) activation by IL-1 and TNF- α , inhibit cyclooxygenase-2 (COX-2) expression along with reduce production of arachidonic acid (AA) metabolites, prevent transport of lipid peroxides and oxidative damage, and maintain the mitochondrial oxidative-phosphorylation (Brigelius-Flohé, 2006; Cole-Ezea et al., 2012). Further, lipoproteins synthesis and secretions have been shown to decline by lipid peroxides (Murthy et al., 1998), indicating that activated GPx can attenuate hepatic triglyceride accumulation.

5.4. Catalase

Catalase is also an important antioxidant enzyme that catalyzes the conversion of H_2O_2 to H_2O . Catalase consists of four subunits, each of which contains a ferric (Fe^{+3}) heme group bound to its active site (Bras et al., 2005); however, Fe deficiency causes a significant decrease of catalase activity. The mitochondrial membrane is impermeable to catalase. Catalase is found primarily in peroxisomes and is also present in heart mitochondria (Bai and Cederbaum, 2001), but has not been found in mitochondria from other tissues (Phung et al., 1994). In fact, in the presence of large amounts of H_2O_2 and thereby diffusing to the cytosol from the mitochondria, catalase along with GPx becomes the most important scavenger in the cytosol. Various studies have reported lower plasma and erythrocyte catalase activity and increased oxidative stress in patients suffering from mitochondria-related diseases (Wang et al., 2005; Tinahones et al., 2009; Guo et al., 2011).

6. Mitochondrial injury in chronic Hepatitis C

Aberrant production of mitochondrial ROS and decrease GSH is thought to be caused by HCV core proteins and possibly contributes to oxidative stress in HCV-infected patients (Thorén et al., 2004; Choi and Ou, 2006; Simula and de Re, 2010). Decreased mtDNA levels have also been found in these patients (Barbaro et al., 1999; Bäuerle et al., 2005).

In infectious cell system, MPTP was shown to prevent a range of pathological changes included by HCV core proteins, including the following: induction of ROS, reduction of respiration, disruption of mitochondrial membrane potential, increased mitochondrial permeability transition in response to exogenous oxidants and TNF- α , loss of complex I activity, cleavage of DNA repair enzyme poly (ADP-ribose) polymerase, overproduction of mitochondrial ROS and 8-oxo-dG, Ca^{+2} overload, decreased GSH, incorporation of core proteins into the mitochondrial outer membranes and endoplasmic reticulum via its COOH-terminal region, and enhanced release of cytochrome c from the mitochondrial to the cytosolic fraction (Okuda et al., 2002; Korenaga et al., 2005a, b; Hara et al., 2006; Piccoli et al., 2007; Quarato et al., 2012). On the other hand, HCV core protein has been shown to induced IR (Cheng et al., 2005). HCV-induced ROS generation suppresses the expression of hepcidin (i.e., a peptide which regulate Fe metabolism by decreasing Fe absorption), facilitating the Fe overload; whereas hepcidin expression was restored by antioxidants (Miura et al., 2008). Fe overload in vitro were observed to cause further ROS augmentation and amplify the expression of catalase, Cu,Zn-SOD, and NADPH dehydrogenase (Moriya et al., 2010). These observations presented that increased intracellular Fe and oxidative stress, in turn, aggravates HCV-induced mitochondrial damage.

In animal models of HCV infection, increased ROS, decreased GSH and NADPH levels in liver mitochondria, and increased intrahepatic lipid peroxidation in response to CCl_4 have been observed (Okuda et al., 2002; Korenaga et al., 2005a, b). Further, altered mitochondrial function has shown that not only results in hepatic fat accumulation but also leads to increased ROS that induces inflammatory response, thereby activating stellate cells and

fibrogenesis (Fromenty et al., 2004; Rolo et al., 2012). HCV core proteins induced ROS generation leads to a decreased hepcidin expression also contribute to Fe accumulation (Nagashima et al., 2006). Fe overload induced hepatic 8-oxo-dG and eventually increased mitochondrial injury and the risk of hepatocellular carcinoma development (Furutani et al., 2006; Moriya et al., 2010). Thus, increased oxidative stress and altered mitochondrial function both in vitro and in vivo is proven to be involved in chronic hepatitis C infection and is thought to contribute to its progression.

6.1. Alterations in enzymatic antioxidants and cofactors

Chronic HCV-infected patients were observed to have an increase or decrease in plasma and erythrocyte SOD and GPx activity, higher, lower, or unchanged catalase levels (Ko et al., 2005b; Kaya et al., 2006; Levent et al., 2006), increased serum and plasma Fe, and decreases in serum, plasma, and erythrocyte Zn and Se concentrations (Czuczejko et al., 2003; Ko et al., 2005b; Himoto et al., 2011; Khan et al., 2012). Associations have been observed between plasma MDA, SOD, and GPx levels with viral loads (Ko et al., 2005b). There were significant negative relationships between MDA and HCV-RNA levels with Zn contents in erythrocytes and whole blood. Se deficiency has been observed to be inversely associated with HCV-RNA loads, the severity of hepatic fibrosis, and IR in HCV-infected patients (Ko et al., 2005b; Himoto et al., 2011; Chen et al., 2012). On the other hand, serum, plasma, and erythrocyte levels of Fe and Cu were significantly higher in hepatitis C patients. Positive correlations were also noted between plasma Cu and hepatic Fe levels with HCV-RNA in these patients (Fargion et al., 1997; Ko et al., 2005b; Guo et al., 2012).

6.2. Inadequate vitamins and glutathione status

The evidence regarding antioxidants, some nutrients along with substances play an important role in mitochondrial resuscitation (Liu and Ames, 2005). GSH and vitamin B complex (B1, B2, B3, B6, pantothenic acid, biotin, and folic acid) protect mitochondria from oxidative damage, improve mitochondrial function, act as cofactors or substrates to protect mitochondrial enzymes, and restore GSH content. Further, these components can enter cells and mitochondria following exogenous treatment (Liu et al., 2009). Deficiency in vitamin B complex and GSH leads to decreased mitochondrial membrane potential, decreased ATP synthesis, and increased oxidative stress and inflammatory responses (Depeint et al., 2006).

Patients with chronic HCV infection have significantly lowered plasma vitamin B1, B2, B6, C, and folic acid levels. Anti-HCV therapy causes further decrease in vitamin B1, B2, B6 and E concentrations and reduces SOD and GPx activity (Lin and Yin, 2009). These patients were also observed to have significantly higher plasma homocysteine (a sulfur-containing amino acid, which is influence by vitamin B2, 6, 12, and folic acid) concentrations and lower concentrations of folic acid and vitamin B12 (Roca et al., 2012). The plasma homocysteine levels were inversely correlated with the concentrations of folic acid in HCV-infected patients (our unpublished observation). SVR patients have been observed to have lower

plasma homocysteine levels than non-SVR patients (Borgia et al., 2009). Pre-treatment with IFN- α and ribavirin in chronic HCV-infected patients, serum vitamin B12 levels are positively correlated to end-of-treatment response (Rosenberg and Hagen, 2011).

Besides the above-noted findings, HCV-infected patients have lower GSH and higher GSSG concentrations in blood, plasma, liver, and the lymphatic system. However, the ratio of GSSG to GSH increases, indicating a high GSH turnover and oxidative stress (Seronello et al., 2007; Lin and Yin, 2009). Thus, GSH depletion might be one reason for the low rate of patient response to treatment (Bernhard et al., 1998). Taken together, these observations suggest that the antioxidant defense is clearly depleted in patients suffering from hepatitis C.

7. Effects of antioxidants and nutrient substances in Hepatitis C

The supplementation of antioxidants or cofactors may show greater benefits in mitochondrial function and antiviral therapy in patients infected with HCV. Recently, the combination of antioxidant with antiviral therapy is recommended for hepatitis C.

7.1. Zn supplementation

Zn as a cofactor of Cu,Zn-SOD and thus is a potential modulator of mitochondrial oxidative phosphorylation. Decreased serum and plasma Zn may serve as a potential inflammatory marker, similar to CRP, but it may also reduce hepatic inflammation in chronic hepatitis C patients through induction of Zn metallothionein, which functions as a free radical scavenger and immune-modulator (Ko et al., 2005a; Guo et al., 2012). Zn has been shown to influence antigen-specific immune response and unspecific immune mechanisms (Grüngreiff and Reinhold, 2010). Disturbances in Zn homeostasis can lead to a shift in the Th1/Th2 balance towards a Th2 response (Rink and Haase, 2007; Prasad, 2009). HCV replication enhances activation of the NF- κ B-signal pathway triggered by TNF- α (Kanda et al., 2006); however, Zn inhibits NF- κ B activation results in decreasing inflammatory cytokine levels (Prasad, 2008). The non-structural protein NS5A is an active component of HCV replicase and is a Zn metalloprotein, suggesting complex interaction between Zn and NS5A activation (Tellinghuisen et al., 2004). In addition, some of the adverse side effects seen during antiviral treatment were similar to the symptoms of Zn deficiency (Saper and Rash, 2009). The effects of Zn administration on these side effects, oxidative stress, and inflammatory responses remain to be determined.

The concentrations of serum Zn were declined further in hepatitis C patients receiving treatment with IFN- α and ribavirin; whereas Zn concentrations were remediable by daily administration of 50 mg elemental Zn from Zn gluconate for six months. Serum Zn level was also found to be significantly higher in complete responders to IFN- α therapy than in non-responders. No apparent difference was seen in virologic response, but adverse side effects including gastrointestinal disturbance, weight loss, and mild anemia were significantly decreased (Ko et al., 2005a).

In clinical observation, the daily dose of polaprezinc includes 34 mg elemental Zn for six months that markedly decreases both ALT and aspartate aminotransferase (AST) levels and enhances the response to IFN- α therapy (Takagi et al., 2001; Nagamine et al., 2000; Matsuoka et al., 2009). However, Zn administration did not affect virologic response (Takagi et al., 2001), SVR, and adverse side effects except for gastrointestinal disturbance (Suzuki et al., 2006). On the other hand, Zn responders were observed to have a clearly lower cumulative incidence of hepatocellular carcinoma in patients suffering from chronic HCV infection and liver cirrhosis. For those Zn non-responders, suggesting a higher daily Zn dose may be needed to increase the response to IFN- α treatment (Matsuoka et al., 2009). Polaprezinc was administered at 51 mg elemental Zn per day for six months to hepatitis C patients, the rate of reduction of ALT levels was observed to positively correlate with that of ferritin (i.e., a clinical marker of iron storage protein, inflammation and oxidative stress), whereas Zn administration did not affect virologic response (Himoto et al., 2007).

These observations suggest that Zn supplementation in HCV-infected patients may improve nutritional status, and thereby decrease inflammation and liver enzyme levels. Administration of Zn supplement has shown to reduce potential oxidative stress and stabilize erythrocyte membrane, but not to inhibit virus.

7.2. Vitamin C and E supplementation

Vitamin C is an essential and water-soluble antioxidant molecule efficiently protects biological materials against damaging free radicals such as OH * and O $_2^*$. Vitamin C serves as a cofactor for enzymes involved in synthesis of collagen or carnitine (essential for the transport of fatty acids into mitochondria), and the mitochondrial reduction of vitamin E, ferricytochrome c, lipoic acid, and GSH (Sagun et al., 2005; Levine et al., 2011). In vivo study has shown that administration of vitamin C supplementation markedly increases plasma, leukocyte, and mitochondrial vitamin C concentrations and mitochondria themselves can produce vitamin C (May et al., 2007). Vitamin C supplementation is observed to significantly enhance NK cells activity, monocytes, T- and B-lymphocytes, and increase the Th1/Th2 ratio, balancing the immune function (Heuser and Vojdani, 1997; Chang et al., 2009). Deficiency in vitamin C can cause oxidative stress and lead to decreased immune response, impaired membrane integrity, and altered membrane fluidity (Maggini et al., 2007). On the other hand, vitamin E (α -tocopherol) is a fat-soluble antioxidant that prevents lipid peroxidation and scavenges lipid peroxy radicals. Vitamin E administered in the diet predominantly localizes in the mitochondrial inner- and outer-membranes (Lauridsen and Jensen, 2012). Effects of vitamin E were also observed which involving in heme biosynthesis, immune system modulation, Se-containing proteins formation, and the integrity of mitochondrial membranes (Mabalirajan et al., 2009).

Studies in an animal model demonstrates that combined administration of vitamin E and C, markedly decreases the carbonyl group content in mitochondrial proteins and enhances SOD and citrate synthase activity (Rosa et al., 2009). On the other hand, clinical observation has been shown to have significantly lower plasma concentrations of α -tocopherol, ascorbic

acid, and GSH in HCV- infected patients (Lin and Yin, 2009). Plasma α -tocopherol or ascorbic acid levels were negatively correlated with F₂-isoprostane and ALT levels (our unpublished results). Thus, further studies will be needed to clarify the effect of a combination of vitamin C and vitamin E on chronic hepatitis C patients treated with IFN- α and ribavirin.

Administration of vitamin E (804 mg α -tocopherol/day) for eight weeks has been shown to decrease protein carbonyl group levels; whereas did not significantly affect ALT levels, virologic response, and fibrosis process in HCV-infected patients (Houglum et al., 1997). Further studies indicate that plasma and erythrocyte α -tocopherol and plasma ascorbic acid levels increased, and serum levels of ALT decreased significantly after two weeks of treatment with Vitamin E (500 mg α -tocopherol/day) and C (750 mg ascorbic acid/day) supplementation (Murakami et al., 2006). The combined administration of vitamin E (1342 mg α -tocopherol/day) with vitamin C (100 mg ascorbic acid/day) for 48 weeks has been shown to decrease in ribavirin-induced anemia but not SVR in patients with HCV infection (Kawaguchi et al., 2007). Patients undergoing IFN- α and ribavirin treatment have markedly higher AA and decreased EPA levels in PBMC. The combined administration of vitamin E with vitamin C for four weeks prevents the decrease in PBMC EPA and the increase in the ratio of AA to EPA in these patients (Murakami et al., 2006). Eight weeks of such treatment led to increases in hemoglobin levels and significantly elevated erythrocyte EPA concentrations in these patients (Hino et al., 2006).

Studies have demonstrated that ribavirin's toxicity decrease intracellular energy metabolism, increase oxidative membrane damage, and accelerate hemolytic anemia in the combined therapy of IFN- α and ribavirin (Assem and Yousri, 2011). Ribavirin-induced ROS would increase EPA peroxidation and result in alteration in fatty acid compositions of erythrocyte membranes. A combination antioxidant treatment improves the antioxidant capacity than vitamin E alone in HCV-infected patients, thereby protecting erythrocyte EPA depletion. Based on our previous experience with clinical trials, the dosages of vitamin C can range from 1000 mg to 6000 mg. These patients who take greater amount of vitamin C, which can offers greater benefit in raising GSH concentrations. Additionally, a combination of vitamin C and other antioxidants may further increase the efficiency of antiviral therapy.

7.3. Vitamin C, E, and Zn supplementation

There is a need for effective antiviral treatments that decrease the inflammation and increase antiviral response. The availability of such treatments would maintain erythrocyte integrity and resistance to hemolysis. It seems reasonable that co-administration of Zn and antioxidants may be more effective in antiviral therapy.

HCV-infected patients receiving antioxidant supplementation (combination of 800 mg α -tocopherol/day, 500 mg ascorbic acid/day and 40 mg Zn/day for six months) showed significant improvement in antioxidant enzyme activity and ALT reduction (Farias et al., 2012). Polaprezinc supplementation (equivalent to 34 mg elemental Zn) daily for 12 months

has been observed to significantly decrease plasma MDA, HCV-RNA load, and prevent the decrease in polyunsaturated fatty acids of erythrocyte membrane phospholipids in patients during IFN- α plus ribavirin therapy with vitamin E (300 mg α -tocopherol acetate/day) and vitamin C (600 mg ascorbic acid/day) supplementation (Murakami et al., 2007). Results from clinical studies suggest that Zn supplementation is more effective against HCV when given along with antioxidants (combinations of vitamin C and vitamin E).

7.4. Vitamin C, E, and Se supplementation

Se also plays a vital role in the redox regulation and antioxidant function and immunomodulatory effects. These effects are potentiated by the presence of vitamin E. Se deficiency was observed to have reduced T-lymphocytes, impaired lymphocyte proliferation and function, and altered innate immunity (NK cells, dendritic cells, and neutrophils) (Maggini et al., 2007; Hoffmann and Berry, 2008). On the other hand, decreased Se levels and Se-dependent GPx activity either in plasma or in erythrocytes suggests that the anti-oxidative capability is limited in patients with chronic HCV infection (Ko et al., 2005b; Guo et al., 2012). Significantly higher viral loads correlate with decreased blood Se and GPx activity in HCV-infected patients (Ko et al., 2005b; Himoto et al., 2011; Khan et al., 2012). Associations have been observed between plasma MDA, protein carbonyl group, and ALT levels with plasma Se concentrations (our unpublished results). Serum and plasma Se levels significantly decrease in proportion to the severity of hepatic fibrosis, IR, and HCV-RNA levels, and correlate positively with plasma, erythrocyte GPx activity and Zn concentrations. Also, increased IR is associated with higher HCV-RNA levels (Ko et al., 2005b; Himoto et al., 2011).

Se-dependent GPx modules encoded in RNA viruses have been found (Zhang et al., 1999). HCV-infected patients with early virological response (EVR), which is defined as undetectable HCV-RNA or a less than two log drop in HCV-RNA at week 12, have significantly higher plasma Se concentrations and GPx activity compared to those with non-EVR patients. A similar difference between SVR and non-SVR patients has been observed (C-H Guo, W-S Ko, and P-C Chen; unpublished results). Thus, Se status might be a sensitive indicator for the sustained response to therapy in chronic hepatitis C patients.

HCV-infected patients who received antioxidant supplementation (633 mg α -tocopherol/day, 500 mg ascorbic acid/day, and 200 mg Se/day for six months) had significantly higher plasma levels of ascorbic acid and α -tocopherol and higher erythrocyte GPx activity. However, the supplementation had no effects on ALT, viral load or oxidative markers (Groenbaek et al., 2006).

This finding is difficult to interpret because the potential synergy between vitamin E and Se is well documented. On the basis of the finding, these results might be attributed to viral genotypes or a much high viral load. Based on our previous experience with clinical trials, this dosage may not be enough to be therapeutic for Se therapy, even though the recommended dietary allowances of Se in the USA are 55-70 mg/day for adults.

Additionally, there is variability in the absorption and therapeutic mechanism of Se that is related to the forms of Se. Further large-scale studies are needed to elucidate the effects of Se alone or in combination in chronic hepatitis C patients treated with IFN- α and ribavirin.

7.5. Vitamin C, E, and eicosapentaeic acid supplementation

Two components of fish oil, eicosapentaeic acid (EPA) and docosahexaenoic acid (DHA), are referred to as omega-3 or n-3 fatty acids. Both EPA and DHA have been shown to exert antioxidant, anti-inflammatory activities (efficiently suppressed NF- κ B activation), and reduction of pro-inflammatory lipid mediators (Merzouk et al., 2003; Calder, 2006) and subsequently incorporate into the mitochondrial membranes and maintain the membrane fluidity (Chapkin et al., 2002, 2009). Thus, both EPA and DHA are essential for mitochondrial function, inhibition of HCV-RNA replication (Liu et al., 2010), increases in insulin sensitivity (Ye et al., 2001) and hepatic lipid metabolism (Araya et al., 2004; Al-Gayyar et al., 2011).

Mitochondrial phospholipid composition, particularly in releases of AA and cardiolipin contents are major contributors to trigger MPTP opening. Cardiolipin is composed of four linoleic acid side chains, which is essential for normal mitochondrial respiration; however, substitute such as long chain saturated and monounsaturated fatty acids weaken mitochondrial function (O'Shea et al., 2009). Recent study has shown that increased saturated fatty acids and cholesterol associated with alteration in mitochondrial membrane and cardiolipin oxidation, which are required for HCV replication (Roe et al., 2011). HCV-infected patients have markedly higher AA and decreased EPA levels in PBMC compared to the healthy controls. In addition, IFN- α and ribavirin treatment can further lead to EPA depletion (Murakami et al., 2006). Supplementation with DHA alone or both DHA and EPA significantly delays Ca²⁺-induced MPTP opening in normal and hypertrophied myocardium (O'Shea et al., 2009; Khairallah et al., 2010). These changes were accompanied by an increase in DHA and EPA level in mitochondrial phospholipids and decreased AA level (Khairallah et al., 2010).

Both EPA and DHA may induce β -oxidation of fatty acid and upregulation of mitochondrial biogenesis (Ruzickova et al., 2004; Flachs et al., 2005). In rat model, EPA treatment lowered plasma triglyceride and increased β -oxidation of fatty acid in hepatic mitochondria and carnitine palmitoyltransferase-1 activity (Madsen et al., 1999). Treatment with EPA and DHA was observed to reduce in plasma and urinary F₂-isoprostanes, which was due to immuno-modulatory effects via EPA and DHA (Mori et al., 2003). Above observations suggest that EPA and DHA supplementation have potential beneficial effects in HCV-infected patients with and without NAFLD.

It is proposed that administration of either EPA alone or both DHA and EPA may compensate the loss of EPA by ribavirin induction in erythrocyte membrane. After oral treatment with EPA (1.8 g/day) for 12 weeks, patients were observed to have significantly decreased ALT levels and higher Th1/Th2 ratio. These patients had clearly lower plasma

and serum 8-oxo-dG levels after six-months of treatment with IFN- α , ribavirin, and antioxidants (300 mg α -tocopherol/day and 600 mg ascorbic acid/day). EPA supplementation also decreased the ratio of AA to EPA and increased leukocyte levels (Tomioka et al., 2005; Kawashima et al., 2008); suggesting treatment with EPA prevents AA accumulation. Thus, these observations suggest that the combination of EPA and antioxidants (vitamin C and vitamin E) may ameliorate inflammation and oxidative stress and thereby increase the response of antiviral therapy in HCV-infected patients.

The bioavailability and efficacy of fish oils are frequently controversial, although ethyl ester (EE)- or triglyceride (TG)-form, has recently been introduced into clinical practices. EE-form fish oil has shown some unpredictable side effects in clinical application (Data sources from Dr. P-J. Liu). Both ethanol and methanol, the metabolites of EE-form (catalyzed by carboxy ester hydrolase) that may contribute to the adverse events include gastrointestinal disorder, vomiting, and hypertriglyceridemia. Thus, the choice of fish oils for clinical application will have to be considered, particularly in chronic HCV-infected patients with NAFLD.

7.6. Combination of antioxidants and nutrient substances

Beside the use of those antioxidants, some nutrient substances treatments in mitochondrial damage have been reported to produce a positive effect, as reviewed in Tarnopolsky (2008) and Orsucci et al (2009). Further, the combined treatment with antioxidants and other nutrients has been shown to efficiently decrease mitochondrial oxidative injury, increase mitochondrial ATP production, and to arrest the progression of clinical symptoms.

Beneficial therapeutic responses to CoQ (Gane et al., 2010), carnitine (Romano et al., 2008; Malaguarnera et al., 2011), choline (Niederau et al., 1998), or N-acetyl-cysteine (Cimino et al., 1998; Neri et al., 2000) have been observed in patients with hepatitis C. Furthermore, standard treatment with multiple nutrient supplements (including 2000 mg/day of ascorbic acid, 150 mg/day of GSH, 150 mg/day of LA, 800 IU/day of d- α -tocopherol as well as silymarin, glycyrrhiza, and schizandrae) for six months leads to significant declines in ALT levels, improvements in liver histological status, and decreased HCV-RNA loads. Such supplements also produce mild beneficial effect in the inflammatory response of patients who are non-responders to IFN- α (Melhem et al., 2001; Gabbay et al., 2007). Patients with chronic hepatitis C who received a combination of natural supplements (CoQ, EPA/DHA, Se, and vitamin B complex) for six months demonstrated significant improvements in immune function, reduced adverse side effects, and decreases in HCV-RNA loads. Reductions in the rate of non-responders were also observed (manuscript from Dr. Simon Hsia). These observations suggest that the synergistic effects of antioxidants and mitochondria-related nutrient substances may be effective in antiviral therapy.

8. Summary

In conclusion, significant increases in oxidative stress and alterations in mitochondrial function have been observed in patients infected with HCV, as well as in animal and cell

models of HCV. HCV-induced mitochondrial oxidative damage and increased ROS production facilitate HCV replication and contribute to the progression of hepatitis C. Additionally, mitochondrial dysfunction induced by HCV reduces the β -oxidation of fatty acid and accelerates ROS formation, causing fat accumulation and hepatic lipid peroxidation. Reduced mitochondrial biogenesis also contributes to development of IR. Clinical observations indicate that therapeutic approaches targeting mitochondrial biogenesis that decrease oxidative damage and increase the response to antiviral therapy are clinically beneficial for chronic HCV-infected patients undergoing IFN- α and ribavirin treatment.

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