

Chapter 2

Free Radicals and Oxidative Stress: Basic Concepts and Misconceptions

Jinze Xu and Christiaan Leeuwenburgh

Abbreviations

8-oxodGuo	8-Oxo-7,8-dihydro-2'-deoxyguanosine
8-oxoGuo	8-Oxo-7,8-dihydro-guanosine
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
EC	Epicatechin
ECG	Epicatechin gallate
EGC	Epigallocatechin
EGCG	Epigallocatechin gallate
ELISA	Enzyme-linked immunosorbent assay
ESR	Electron spin resonance
GC/MS	Gas chromatography-mass spectrometry
GPX	Glutathione peroxidase
HPLC	High-performance liquid chromatography
MDA	Malondialdehyde
NADP	Nicotinamide adenine dinucleotide phosphate
RNSs	Reactive nitrogen species
ROs	Reactive oxygen species
SODs	Superoxide dismutases
TBAR	Thiobarbituric acid reactive substances

J. Xu (✉) • C. Leeuwenburgh
Department of Aging and Geriatric Research, Institute on Aging, University of Florida,
Gainesville, FL, USA
e-mail: jinze.xu@gmail.com; cleeuwen@ufl.edu

2.1 Overview and Introduction

In the past few decades, remarkable progress has been made toward understanding both the deleterious and beneficial roles of free radicals in mammals. While it is widely recognized that excessive amounts free radicals, including reactive oxygen species (ROSs) and reactive nitrogen species (RNSs), can be harmful and damage cell structures, in strong contrast, at low concentrations they can act as cellular and intracellular signaling molecules that facilitate normal biological processes (Buetler et al. 2004; Bienert and Chaumont 2014; Valko et al. 2007). Truly reactive radicals are those chemical species with one or two highly reactive unpaired valence electrons in open electron shell(s). A large body of evidence suggests that free radicals are linked to the pathogenesis of tumors (Manjunath et al. 2010; Valko et al. 2006; Dwivedi et al. 2008), hearing loss (Yamasoba et al. 2013; Rewerska et al. 2013), tonsillitis (Garca et al. 2013b; Cvetkovic et al. 2009; Yilmaz et al. 2004; Li et al. 2011), allergic rhinitis (Aksoy et al. 2009, 2012), chronic otitis media (Garca et al. 2013a; Baysal et al. 2013; Testa et al. 2012), and rhinosinusitis (Citardi et al. 2006; Uslu et al. 2003) and implies that antioxidant therapy eventually may be employed as a strategy to ameliorate these disorders and diseases (Ding et al. 2013; Tian et al. 2013; Someya et al. 2010). As has been shown recently, however, many antioxidants that are effective in animal models are not effective in human clinical trials.

2.2 Free-Radical Biology

Many sources of free-radical production exist, such as nicotinamide adenine dinucleotide phosphate (NADP) oxidases and mitochondria. The primary function of mitochondria is to produce energy by utilizing oxygen to generate adenosine triphosphate (ATP) for cells. However, mitochondria are also one major source of intracellular ROSs and can generate several reactive radicals and molecules, such as superoxide ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and hydroxyl radical (HO^{\cdot}) (Boveris et al. 1976; Chance et al. 1979; Boveris and Chance 1973; Pollack and Leeuwenburgh 1999). Superoxide anions, but mostly H_2O_2 , are diffusible within cells and may act as essential signaling molecules to facilitate normal biological processes and physiology (Buetler et al. 2004; Bienert and Chaumont 2014). Oxidants can react with specific thiol groups on proteins and/or affect a variety of enzymes involved with phosphorylation. In stark contrast, harmful hydroxyl radicals may be formed via Fenton chemistry in the presence of labile transition metals and react immediately and indiscriminately with surrounding biomolecules, including lipids, proteins, and DNA (Koskenkorva-Frank et al. 2013; Halliwell and Gutteridge 1992), and cannot act as specific signaling and/or important redox modification of proteins. Moreover, nitric oxide (NO^{\cdot}), which belongs to the RNS family, is involved in several cellular signaling pathways and also is produced by mitochondria (Giulivi et al. 1998).

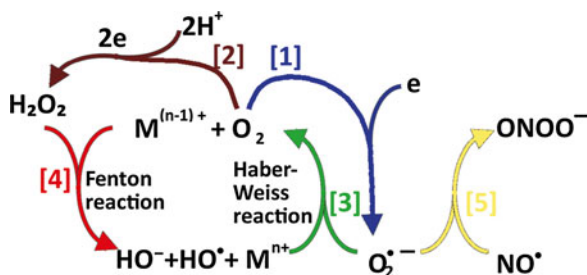


Fig. 2.1 Redox chemistry of free radicals in vivo. Oxygen (O_2) reacts slowly to accept a single electron and produce superoxide ($O_2^{\cdot -}$) in vivo [1], while two electrons can reduce oxygen with the addition of two protons ($2H^+$) to generate hydrogen peroxide (H_2O_2) [2]. Reactive metal ions (M^{n+}), such as ferric iron and cupric ion, play a major role in oxidative stress via Haber–Weiss reaction, which is reduced by $O_2^{\cdot -}$ to reduced form ion ($M^{(n-1)+}$) and oxygen [3]. If H_2O_2 coexists, reduced metal ion will be oxidized to generate a hydroxyl radical (HO^{\cdot}) and a hydroxide anion (HO^-) via Fenton chemistry [4]. In addition, superoxide can react with nitric oxide (NO^{\cdot}) to form peroxynitrite ($ONOO^-$), which is a major RNS pathway in vivo and also considered harmful without fulfilling signaling roles

Transition metals, including iron and copper, play an important role in free-radicals-linked damage. Thus, cellular iron, mostly in the ferric form (Fe^{3+}) at physiological pH, can be reduced by $O_2^{\cdot -}$ to ferrous iron (Fe^{2+}) via the Haber–Weiss reaction (Fig. 2.1) (Leeuwenburgh and Heinecke 2001; Xu et al. 2008). While two electrons and two protons coexist, oxygen can be reduced to H_2O_2 reactions, typically accelerated in the presence of enzymes such as glutathione peroxidase (GPX). In the presence of free metals, more highly ROSs can be formed if enzymes like GPX are not readily available. The Fe^{2+} can react with H_2O_2 to generate the extremely reactive HO^{\cdot} by the Fenton reaction. Numerous studies on detecting cellular and mitochondrial labile iron by a wide variety of iron chelators have shown that cells normally maintain a labile iron pool, which exists in a state of dynamic equilibrium between free and ligand iron as well as ferrous and ferric iron (Kozlov et al. 1992; Sohal et al. 1999; Rothman et al. 1992). Certainly in pathophysiological conditions, the quantity of free iron is increased. Iron taken up by eukaryotic cells must reach mitochondria for heme and iron–sulfur cluster biosynthesis (Fig. 2.2) to fulfill one of its biological roles. Upon exposure to oxidants, a minor amount of iron, either loosely bound or poorly liganded to proteins, escapes from storage sites and undergoes the Fenton and Haber–Weiss reactions, particularly within the same compartment (Kakhlon and Cabantchik 2002; Cabantchik et al. 2002; Cantu et al. 2009).

Given the fact that reactive intermediates are transient with extremely short half-lives (10^{-6} – 10^{-12} s), it is very difficult to detect them directly in vivo (Leeuwenburgh and Heinecke 2001). A substantial research effort has been directed at exploring end products of oxidative damage to biomolecules, including lipids, proteins, and DNA. The detection of radicals can be performed by direct and indirect measures. Electron spin resonance (ESR) spectroscopy provides a valid tool to investigate free radicals directly by measuring the absorption microwave radiation of unpaired electrons when free radicals are exposed to a strong magnetic field

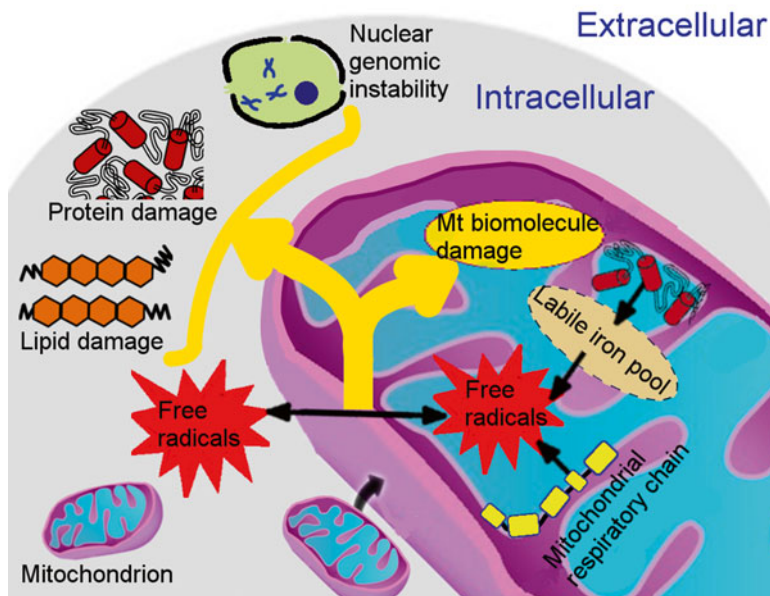


Fig. 2.2 Mitochondrial oxidative stress. The mitochondrial respiratory chain is one major source of free radicals. Mitochondria also express nitric oxide synthase, which produces nitric oxide (NO^{\bullet}). Moreover, mitochondria are the major site of heme and iron–sulfur cluster biosynthesis. Iron taken up by eukaryotic cells is transported into the mitochondrial matrix by iron import proteins (e.g., mitoferrin) for mitochondrial iron storage, iron–sulfur cluster biosynthesis, heme metabolism, or other currently unknown pathways. Defects in these processes (e.g., faulty biosynthesis of heme and iron–sulfur cluster) or impaired mitochondrial iron homeostasis contributes to elevated labile iron levels in mitochondria, which has a strong potential to catalyze the reaction of free radicals. Free radicals within mitochondria are directly associated with extensive damage to mitochondrial DNA, which may further lead to mitochondrial dysfunction. More stable ROSs can reach other intracellular areas, resulting in cytosolic damage of other organelles

(Davies et al. 1982). Lai et al. successfully used ESR to demonstrate that the superoxide dismutases (SODs) predominantly localized in epithelial cells of rat nasal mucosa scavenge microenvironmental $\text{O}_2^{\bullet-}$ radicals (Lai et al. 1997). Moreover, spin trap compounds generally are necessary to detect short-lived radicals. A spin trap reagent can react with the radical to form a spin adduct that is relatively stable and can be detected with ESR spectroscopy. However, in biological systems where multiple radicals or oxidants are present, many may form the same radical adduct with a spin trap reagent. Thus, use of spin trap compounds with ESR spectroscopy may raise an issue of their selectivity and specificity.

Except for biological fluids, radical detection protocols are based mostly on cell-disruptive methods, which inevitably alter the equilibrium between radical species as well as between radicals and various cellular components. Current non-disruptive techniques for detecting radicals and labile ions *in vivo* dramatically rely on the application of fluorescent probes, which comprise a fluorescent group

coupled with a high-affinity chelator (Cabantchik et al. 2002; Kakhlon and Cabantchik 2002). Moreover, probes must be lipophilic, membrane-permeable, and able to compete with endogenous ligands and polypeptides (Petrat et al. 2002; Rauen et al. 2007). For instance, labile iron in cells and tissues can be visualized by fluorescent probes, such as Phen Green SK and calcein. A recent study by Cantu et al. employing an iron indicator rhodamine B 4-[(1,10-phenanthroline-5-yl)amino-carbonyl]benzyl ester has shown that mitochondrial aconitase releases labile iron under oxidative stress in primary ventral mesencephalic cells (Cantu et al. 2009). This event was followed by mitochondrial dysfunction and cell death. Furthermore, this study's observation that removal of H_2O_2 and labile iron, using catalase and an iron chelator (*N,N'*-bis(2-hydroxybenzyl)ethylenediamine-*N,N'*-diacetic acid), mitigated cell death strongly suggests that H_2O_2 and labile iron mediate oxidative damage and cell death.

More indirectly, radical activity—damage—can be detected on a variety of biomolecular fingerprints. The polyunsaturated acids of the fatty acid membrane contain more than one double bond in their alkyl chain, which are extremely vulnerable to ROSs. The end products of oxidant reactions with these vulnerable structures (lipid peroxidation reactions) include alkanes, isoprostanes, malondialdehyde (MDA), and isoketals. The thiobarbituric acid reactive substance (TBARS) assay is a typical nonspecific test that measures lipid peroxidation by detecting MDA levels. Thiobarbituric acid reacts with MDA in acidic condition to form a pink pigment, which has an absorption maximum of 532 nm. Absorbance changes at 532 nm are linearly correlated with chromogen concentration, and the amount of chromogen can be quantified by spectrophotometer (Buege and Aust 1978). In addition, gas chromatography/mass spectrometry (GC/MS) is a powerful technique for measuring MDA or other more robust lipid peroxidation markers, e.g., F_2 -isoprostane, which present in all normal biological fluids and tissues (Morrow and Roberts 1997).

Reactive radicals' attacks can modify amino acid residues in the proteins. Reactive hydroxyl radical (HO^\bullet) converts the amino acid phenylalanine to ortho-, meta-, and para-tyrosine. Peroxynitrite ($ONOO^-$), which is formed by the reaction of O_2^\bullet with NO^\bullet , can mediate the nitration of the ortho position of tyrosine residue to form 3-nitrotyrosine. Trace amounts of tyrosine isomers and 3-nitrotyrosine can be detected by GC/MS and high-performance liquid chromatography (HPLC).

Oxidation of specific amino acid residues (lysine, arginine, proline, and threonine) of proteins forms carbonyl species (aldehydes and ketones). Lipid oxidation with the covalent adduction of lipid aldehydes and sugar reduction also contribute to the increase in carbonyl levels (Dalle-Donne et al. 2003). Hence, the carbonyl assay indicates not only protein oxidation but also lipid peroxidation products. Carbonyl levels, however, can be quantified by various techniques, including spectrophotometry, Western blot immunoassay, enzyme-linked immunosorbent assay (ELISA), and GC/MS, which are accessible and can be performed in any biomedical research laboratories. Thus, carbonyl assays are widely accepted for evaluating protein oxidation, with the caveat that additional analysis is required to exclude the carbonyl groups from lipids and sugar.

Free-radical-induced damage to DNA and RNA yields numerous products. The most popular biomarkers of DNA and RNA are guanine base oxidation products, 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodGuo) and 8-oxo-7,8-dihydroguanosine (8-oxoGuo), respectively, which can be quantified simultaneously using HPLC with an electrochemical detector (Hofer et al. 2006).

2.3 Select Misconceptions in Free-Radical Biology

As briefly discussed earlier, under normal (non-pathophysiological) conditions, a minor amount of free radicals within cells is essential for cellular and intracellular signaling systems. In addition, cells are well protected against oxidative damage by delicate and sophisticated defense systems. The biological activity of free radicals is maintained in balance by endogenous antioxidants and antioxidant enzymes. A sudden acceleration in the production of free radicals (i.e., ischemia reperfusion) or a chronic deficiency in the antioxidant defense system may alter this balance, which leads to free-radical-induced damage to biomolecules, and may be further involved in the pathogenesis of various disorders and diseases (Valko et al. 2007). Levels of beta-carotene, retinol, vitamin E, and vitamin C, for example, were observed to be significantly decreased in children with acute otitis media and tonsillitis (Cemek et al. 2005). Manjunath et al. reported that serum MDA levels were significantly elevated and that ferric reducing antioxidant power was dramatically decreased in patients with laryngeal and hypopharyngeal cancer (Manjunath et al. 2010). Moreover, Aksoy et al. revealed an association of patients with allergic rhinitis to increased serum protein oxidation levels (Aksoy et al. 2009, 2012). Other findings add to the evidence that elevated serum oxidant levels and declined antioxidant status levels were observed in patients with chronic long-term otitis media (Garca et al. 2013a; Baysal et al. 2013; Testa et al. 2012).

Current understanding of the involvement of free radicals in pathology suggests that antioxidant therapy through attenuating free radicals may be important to optimize treatment (Ding et al. 2013; Tian et al. 2013; Someya et al. 2010; Cemek et al. 2005). Most studies using animal models indicate the benefits of antioxidants in the prevention of acute oxidative conditions and diseases. Bonabi et al., for instance, reported that resveratrol had a significant protective effect against gentamicin-induced toxicity in cultured organ of Corti explants from newborn Sprague–Dawley rats (Bonabi et al. 2008). A recent study on noise-induced hearing loss in Fischer 344 rats showed that resveratrol dramatically reduced noise-induced cyclooxygenase-2 expression and ROS formation (Seidman et al. 2013). Moreover, the antioxidant N-acetylcysteine combined with disodium 2,4-disulfophenyl-N-tert-butyl nitron as a therapeutic intervention for noise-induced hearing loss in rats was found to be effective in reducing both temporary and permanent threshold shifts across all frequencies (2–16 kHz) in auditory brainstem response tests (Lu et al. 2014). A clinical study of radix astragali, a natural antioxidant herb, indicated its beneficial effect in recovering hearing in patients with sudden deafness (Xiong et al. 2012).

For decades, a substantial research effort has focused on coenzyme Q10 as a therapeutic approach to ENT pathology. A study in a guinea pig model of noise-induced hearing loss by Fetoni et al. demonstrated that coenzyme Q10 decreased active caspase-3 expression and the number of apoptotic cells and improved outer hair cell survival (Fetoni et al. 2009). The same research group also found that coenzyme Q10 significantly mitigated noise-induced auditory function and hearing loss in rats (Fetoni et al. 2012). (Other studies demonstrating the efficacy of antioxidant intervention in the prevention of noise- and drug-induced and age-related hearing loss can be found in Chaps. 10, 11, 12, 13, 14, 15 and 16 of this book.)

Though scientists have made considerable progress in support of antioxidant supplements in pathology, human research findings remain controversial and inconsistent, particularly in epidemiological and clinical studies. A prospective study of vitamin intake and the risk of hearing loss in men aged 40–74 years by Shargorodsky et al. found a lack of correlation between higher intake of vitamins C, E, beta-carotene, or B12 and the risk of hearing loss (Shargorodsky et al. 2010). Consistently, 5-year longitudinal analyses on the link between diet quality and hearing loss in the elderly did not provide robust efficacy data (Gopinath et al. 2014). In a previous study on the glutathione supplement in 30 patients with hearing loss, the treatment failed to modify significantly the progression of hearing loss (Sataloff et al. 2010). On the other hand, studies by Choi et al. and Spankovich et al. both demonstrate relations between use of antioxidant supplements and reduced risk of age-related hearing loss in elderly humans. Despite the differences in the protocols, the conflicting study observations may stem from several factors or misconceptions involved in free-radical biology.

2.4 Antioxidants May Blunt Benefits that Depend on ROS Signaling

Previous research has demonstrated that ROSs actively participate in a variety of biological signaling processes. Appropriate increases in oxidative stress as effector molecules can trigger stress-inducible genes, which participate in cellular repair. Thus, modest stimulation of ROSs is beneficial. Indeed, caloric restriction, with an associate increase in ROS formation, has been shown to extend life span in *C. elegans* (Schulz et al. 2007). Exposure to H₂O₂ can also activate transcription of the DNA-damage-response gene and induce DNA repair in HeLa cells (Furukawa-Hibi et al. 2002). Another example of hormetic increases in ROSs is moderate aerobic exercise, which exerts beneficial health effects mediated through direct ROS increases (Mendelsohn and Larrick 2013). In general, acute exercise increases oxidant levels and oxidative stress in untrained animals, but long-term exercise may counter this effect by increasing the activity of antioxidant enzymes and reducing oxidant production. These defenses may be critical for preventing chronic oxidative muscle damage during exercise and even at rest. We have assayed the activities of antioxidant enzymes in rat skeletal muscle samples following exercise training.

In most of our studies, the activities of two major antioxidant enzymes, mitochondrial SOD and cytosolic glutathione peroxidase, were significantly higher in exercising animals than in sedentary animals. There was little difference in catalase and cytosolic SOD activities (Leeuwenburgh and Heinecke 2001). This suggests that mitochondria are a source of constant free-radical production, which may cause beneficial adaptations. We also consistently detect lower levels of oxidative stress markers following the exercise training. These results indicate that increased activities of GPX and mitochondrial SOD might account in part for the decline in oxidative damage in exercise-trained animals. What may be surprising is the fact that increased mitochondrial radical production with moderate bouts of exercise can provide long-term beneficial adaptations. During the short burst of exercise, metabolism is characterized by a high rate of adenosine diphosphate (ADP) formation because ATP breakdown increases with workload. High ADP levels activate oxidative phosphorylation, thereby dramatically increasing the levels of electron flow in the respiratory complexes. This flow of electrons toward oxygen reduces the amount of superoxide production but appears sufficient to increase the induction of major antioxidant enzymes GPX and SOD. While some studies now show that antioxidant supplementation can blunt the normally required free-radical production of SOD and GPX induction, more human research is needed. Together these results suggest that antioxidant supplements can blunt the beneficial effects in humans by counteracting with transient hormetic responses in ROSs.

2.5 Many Factors Can Impact the Bioavailability of Antioxidants

In laboratory animal studies, diet compositions are precisely controlled and animals are genetically homogeneous. With heterogeneous backgrounds and different dietary habits in human research participants, individual differences in dietary nutrients, plasma cholesterol profile, gender, and age could have major influences on the bioavailability of antioxidant supplementation.

Antioxidant therapies generally are either hydrophilic or hydrophobic compounds. The use of hydrophobic antioxidants in humans usually proves unsatisfactory, most likely because of their relatively low oral bioavailability and complex absorption process. Coenzyme Q10, for example, is relatively insoluble in aqueous solutions, has a relatively high molecular weight, and has a limited solubility in lipids, which directly results in about 2–3 % absorption when orally administered to rats. Other factors also impact plasma coenzyme Q10 levels after oral administration. There is evidence showing that dietary fat enhances coenzyme Q10 absorption. On the contrary, uptake of vitamin E together with coenzyme Q10 intervened and decreased coenzyme Q10 absorption. These brief examples demonstrate the many effects of administering a single antioxidant in research studies, thus making interpretation complicated.

2.6 Antioxidants May Act as Prooxidants

Antioxidants are highly reactive and prone to be oxidized. Many factors can influence their stability and trigger oxidation reaction, including light, temperature, pH, and metallic ions.

Previous research *in vitro* has demonstrated the beneficial outcomes of tea polyphenols, which are potent hydrophilic antioxidants and can scavenge ROSs, RNSs, and redox-active transition metal ions. Epigallocatechin gallate (EGCG) is the primary bioactive compound in green tea, followed by epigallocatechin (EGC), epicatechin gallate (ECG), and epicatechin (EC). Green tea polyphenols are stable in acidic solutions with pH values ranging from 1.0 to 6.0. EGCG with a galloyl moiety is the most vulnerable to pH among them, which results in its instability at physiological pH 7.4. Much attention has therefore focused on the potential prooxidative role of EGCG, particularly in a number of *in vitro* assays. A decade ago, several lines of evidence suggested EGCG was involved in inducing H₂O₂ generation and DNA damage in HL-60 cells (Furukawa et al. 2003; Oikawa et al. 2003). Suh et al. reported similar results, in which EGCG was positively associated with apoptotic cell death and H₂O₂ production in a dose-dependent manner in HIT-T15 pancreatic beta cells (Suh et al. 2010). In the same study, the observation that removing iron using iron chelators inhibited ROS formation strongly suggests that transition metals are essential catalysts during the process. In this case, some oxidants can be used to kill harmful (cancer) cells but may also damage noncancerous cells in the body. Because there is substantial evidence that selected antioxidants also exhibit prooxidant activity, it will be essential to study antioxidant compounds in various cell types and under physiological conditions.

2.7 Conclusions

Free radicals have been shown to be involved in various pathology conditions. An increase in research on antioxidants demonstrates that their ability to scavenge free-radical species and labile transition metals could provide therapeutic approaches to treating free-radical-induced conditions and diseases. Some studies in animal models and human subjects, however, have been less conclusive. This raises the concern that the complexities of free-radical biology and antioxidant treatments as well as practical variations of human participants may make it impossible to study and obtain interpretable results on the efficacy of single antioxidant compounds. Physical exercise and a healthy diet that includes a high intake of natural complex antioxidant mixtures, for example, may influence the effects of antioxidant supplementation by (1) interfering with metabolism and bioavailability, (2) failing to meet physiological levels within the cell at critical sites and exacting oxidant production sources, or (3) blunting benefits at other locations that depend on ROS signaling. Moreover, antioxidants are vulnerable to rapid changes in oxygenation (ischemia/reperfusion), light, temperature, and, most importantly, redox-active transition

metals, which could act as prooxidants under certain conditions, both in vitro and in vivo. These issues do not mitigate against the use of supplemental antioxidants to prevent or treat pathologies for which there is a rich basic science database indicating an important role of free radicals in the pathology, but it surely makes it difficult to argue for such use on the basis of empirical human findings.

References

- Aksoy F, Demirhan H, Veyseller B, Yildirim YS, Ozturan O, Basinoglu F (2009) Advanced oxidation protein products as an oxidative stress marker in allergic rhinitis. *Kulak Burun Bogaz Ihtis Derg* 19(6):279–284
- Aksoy F, Yildirim YS, Veyseller B, Demirhan H, Ozturan O (2012) Serum levels of advanced oxidation protein products in response to allergen exposure in allergic rhinitis. *Ear Nose Throat J* 91(8):E32–E35
- Baysal E, Aksoy N, Kara F, Taysi S, Taskin A, Bilinc H, Cevik C, Celenk F, Kanlikama M (2013) Oxidative stress in chronic otitis media. *Eur Arch Otorhinolaryngol* 270(4):1203–1208. doi:10.1007/s00405-012-2070-z
- Bienert GP, Chaumont F (2014) Aquaporin-facilitated transmembrane diffusion of hydrogen peroxide. *Biochim Biophys Acta* 1840(5):1596–1604. doi:10.1016/j.bbagen.2013.09.017
- Bonabi S, Caelers A, Monge A, Huber A, Bodmer D (2008) Resveratrol protects auditory hair cells from gentamicin toxicity. *Ear Nose Throat J* 87(10):570–573
- Boveris A, Chance B (1973) The mitochondrial generation of hydrogen peroxide. General properties and effect of hyperbaric oxygen. *Biochem J* 134(3):707–716
- Boveris A, Cadenas E, Stoppani AO (1976) Role of ubiquinone in the mitochondrial generation of hydrogen peroxide. *Biochem J* 156(2):435–444
- Buege JA, Aust SD (1978) Microsomal lipid peroxidation. *Methods Enzymol* 52:302–310
- Buetler TM, Krauskopf A, Ruegg UT (2004) Role of superoxide as a signaling molecule. *News Physiol Sci* 19:120–123
- Cabantchik ZI, Kakhlon O, Epsztejn S, Zanninelli G, Breuer W (2002) Intracellular and extracellular labile iron pools. *Adv Exp Med Biol* 509:55–75
- Cantu D, Schaack J, Patel M (2009) Oxidative inactivation of mitochondrial aconitase results in iron and H₂O₂-mediated neurotoxicity in rat primary mesencephalic cultures. *PLoS One* 4(9):e7095
- Cemek M, Dede S, Bayiroglu F, Caksen H, Cemek F, Yuca K (2005) Oxidant and antioxidant levels in children with acute otitis media and tonsillitis: a comparative study. *Int J Pediatr Otorhinolaryngol* 69(6):823–827
- Chance B, Sies H, Boveris A (1979) Hydroperoxide metabolism in mammalian organs. *Physiol Rev* 59(3):527–605
- Citardi MJ, Song W, Batra PS, Lanza DC, Hazen SL (2006) Characterization of oxidative pathways in chronic rhinosinusitis and sinonasal polyposis. *Am J Rhinol* 20(3):353–359
- Cvetkovic T, Vlahovic P, Todorovic M, Stankovic M (2009) Investigation of oxidative stress in patients with chronic tonsillitis. *Auris Nasus Larynx* 36(3):340–344
- Dalle-Donne I, Rossi R, Giustarini D, Milzani A, Colombo R (2003) Protein carbonyl groups as biomarkers of oxidative stress. *Clin Chim Acta* 329(1–2):23–38
- Davies KJ, Quintanilha AT, Brooks GA, Packer L (1982) Free radicals and tissue damage produced by exercise. *Biochem Biophys Res Commun* 107(4):1198–1205
- Ding D, Qi W, Yu D, Jiang H, Han C, Kim MJ, Katsuno K, Hsieh YH, Miyakawa T, Salvi R, Tanokura M, Someya S (2013) Addition of exogenous NAD⁺ prevents mefloquine-induced neuroaxonal and hair cell degeneration through reduction of caspase-3-mediated apoptosis in cochlear organotypic cultures. *PLoS One* 8(11):e79817
- Dwivedi R, Raturi D, Kandpal N, Singh R, Puri V (2008) Oxidative stress in patients with laryngeal carcinoma. *Indian J Cancer* 45(3):97–99

- Fetoni AR, Piacentini R, Fiorita A, Paludetti G, Troiani D (2009) Water-soluble Coenzyme Q10 formulation (Q-ter) promotes outer hair cell survival in a guinea pig model of noise induced hearing loss (NIHL). *Brain Res* 1257:108–116
- Fetoni AR, Troiani D, Eramo SL, Rolesi R, Paludetti Troiani G (2012) Efficacy of different routes of administration for Coenzyme Q10 formulation in noise-induced hearing loss: systemic versus transtympanic modality. *Acta Otolaryngol* 132(4):391–399
- Furukawa A, Oikawa S, Murata M, Hiraku Y, Kawanishi S (2003) (-)-Epigallocatechin gallate causes oxidative damage to isolated and cellular DNA. *Biochem Pharmacol* 66(9):1769–1778
- Furukawa-Hibi Y, Yoshida-Araki K, Ohta T, Ikeda K, Motoyama N (2002) FOXO forkhead transcription factors induce G(2)-M checkpoint in response to oxidative stress. *J Biol Chem* 277(30):26729–26732
- Garca MF, Aslan M, Tuna B, Kozan A, Cankaya H (2013a) Serum myeloperoxidase activity, total antioxidant capacity and nitric oxide levels in patients with chronic otitis media. *J Membr Biol* 246(7):519–524
- Garca MF, Demir H, Turan M, Bozan N, Kozan A, Belli SB, Arslan A, Cankaya H (2013b) Assessment of adenosine deaminase (ADA) activity and oxidative stress in patients with chronic tonsillitis. *Eur Arch Otorhinolaryngol*. doi:10.1007/s00405-013-2843-z
- Giulivi C, Poderoso JJ, Boveris A (1998) Production of nitric oxide by mitochondria. *J Biol Chem* 273(18):11038–11043
- Gopinath B, Schneider J, Flood VM, McMahon CM, Burlutsky G, Leeder SR, Mitchell P (2014) Association between diet quality with concurrent vision and hearing impairment in older adults. *J Nutr Health Aging* 18(3):251–256
- Halliwell B, Gutteridge JM (1992) Biologically relevant metal ion-dependent hydroxyl radical generation. An update. *FEBS Lett* 307(1):108–112
- Hofer T, Seo AY, Prudencio M, Leeuwenburgh C (2006) A method to determine RNA and DNA oxidation simultaneously by HPLC-ECD: greater RNA than DNA oxidation in rat liver after doxorubicin administration. *Biol Chem* 387(1):103–111
- Kakhlon O, Cabantchik ZI (2002) The labile iron pool: characterization, measurement, and participation in cellular processes(1). *Free Radic Biol Med* 33(8):1037–1046
- Koskenkorva-Frank TS, Weiss G, Koppenol WH, Burckhardt S (2013) The complex interplay of iron metabolism, reactive oxygen species, and reactive nitrogen species: Insights into the potential of various iron therapies to induce oxidative and nitrosative stress. *Free Radic Biol Med* 65:1174–1194
- Kozlov AV, Yegorov DY, Vladimirov YA, Azizova OA (1992) Intracellular free iron in liver tissue and liver homogenate: studies with electron paramagnetic resonance on the formation of paramagnetic complexes with desferal and nitric oxide. *Free Radic Biol Med* 13(1):9–16
- Lai MT, Ohmichi T, Ogawa T, Nishizaki K, Masuda Y (1997) Electron spin resonance spin trapping assay and immunohistochemical localization of superoxide dismutases in the rat nasal mucosa. *Acta Otolaryngol* 117(3):437–446
- Leeuwenburgh C, Heinecke JW (2001) Oxidative stress and antioxidants in exercise. *Curr Med Chem* 8(7):829–838
- Li XL, Zhou AG, Zhang L, Chen WJ (2011) Antioxidant status and immune activity of glycyrrhizin in allergic rhinitis mice. *Int J Mol Sci* 12(2):905–916
- Lu J, Li W, Du X, Ewert DL, West MB, Stewart C, Floyd RA, Kopke RD (2014) Antioxidants reduce cellular and functional changes induced by intense noise in the inner Ear and cochlear nucleus. *J Assoc Res Otolaryngol*. doi:10.1007/s10162-014-0441-4
- Manjunath MK, Annam V, Suresh DR (2010) Significance of free radical injury in laryngeal and hypopharyngeal cancers. *J Laryngol Otol* 124(3):315–317
- Mendelsohn AR, Larrick JW (2013) Trade-offs between anti-aging dietary supplementation and exercise. *Rejuvenation Res* 16(5):419–426
- Morrow JD, Roberts LJ (1997) The isoprostanes: unique bioactive products of lipid peroxidation. *Prog Lipid Res* 36(1):1–21
- Oikawa S, Furukawaa A, Asada H, Hirakawa K, Kawanishi S (2003) Catechins induce oxidative damage to cellular and isolated DNA through the generation of reactive oxygen species. *Free Radic Res* 37(8):881–890

- Petrat F, de Groot H, Sustmann R, Rauen U (2002) The chelatable iron pool in living cells: a methodically defined quantity. *Biol Chem* 383(3–4):489–502
- Pollack M, Leeuwenburgh C (1999) Molecular mechanisms of oxidative stress in aging: free radicals, aging, antioxidants and disease. In: Sen CK, Packer L, Hannien O (eds) *Handbook of Oxidants and Antioxidants in Exercise*. Elsevier Science, Amsterdam
- Rauen U, Springer A, Weisheit D, Petrat F, Korth HG, de Groot H, Sustmann R (2007) Assessment of chelatable mitochondrial iron by using mitochondrion-selective fluorescent iron indicators with different iron-binding affinities. *Chembiochem* 8(3):341–352
- Rewerska A, Pawelczyk M, Rajkowska E, Politanski P, Sliwinska-Kowalska M (2013) Evaluating D-methionine dose to attenuate oxidative stress-mediated hearing loss following overexposure to noise. *Eur Arch Otorhinolaryngol* 270(4):1513–1520
- Rothman RJ, Serroni A, Farber JL (1992) Cellular pool of transient ferric iron, chelatable by deferoxamine and distinct from ferritin, that is involved in oxidative cell injury. *Mol Pharmacol* 42(4):703–710
- Sataloff RT, Bittermann T, Marks L, Lurie D, Hawkshaw M (2010) The effects of glutathione enhancement on sensorineural hearing loss. *Ear Nose Throat J* 89(9):422–433
- Schulz TJ, Zarse K, Voigt A, Urban N, Birringer M, Ristow M (2007) Glucose restriction extends *Caenorhabditis elegans* life span by inducing mitochondrial respiration and increasing oxidative stress. *Cell Metab* 6(4):280–293
- Seidman MD, Tang W, Bai VU, Ahmad N, Jiang H, Media J, Patel N, Rubin CJ, Standing RT (2013) Resveratrol decreases noise-induced cyclooxygenase-2 expression in the rat cochlea. *Otolaryngol Head Neck Surg* 148(5):827–833
- Shargorodsky J, Curhan SG, Eavey R, Curhan GC (2010) A prospective study of vitamin intake and the risk of hearing loss in men. *Otolaryngol Head Neck Surg* 142(2):231–236
- Sohal RS, Wennberg-Kirch E, Jaiswal K, Kwong LK, Forster MJ (1999) Effect of age and caloric restriction on bleomycin-chelatable and nonheme iron in different tissues of C57BL/6 mice. *Free Radic Biol Med* 27(3–4):287–293
- Someya S, Tanokura M, Weindruch R, Prolla TA, Yamasoba T (2010) Effects of caloric restriction on age-related hearing loss in rodents and rhesus monkeys. *Curr Aging Sci* 3(1):20–25
- Suh KS, Chon S, Oh S, Kim SW, Kim JW, Kim YS, Woo JT (2010) Prooxidative effects of green tea polyphenol (-)-epigallocatechin-3-gallate on the HIT-T15 pancreatic beta cell line. *Cell Biol Toxicol* 26(3):189–199
- Testa D, Guerra G, Marcuccio G, Landolfo PG, Motta G (2012) Oxidative stress in chronic otitis media with effusion. *Acta Otolaryngol* 132(8):834–837
- Tian G, Sawashita J, Kubo H, Nishio SY, Hashimoto S, Suzuki N, Yoshimura H, Tsuruoka M, Wang Y, Liu Y, Luo H, Xu Z, Mori M, Kitano M, Hosoe K, Takeda T, Usami SI, Higuchi K (2013) Ubiquinol-10 supplementation activates mitochondria functions to decelerate senescence in senescence-accelerated mice. *Antioxid Redox Signal*. doi:10.1089/ars.2013.5406
- Uslu C, Taysi S, Bakan N (2003) Lipid peroxidation and antioxidant enzyme activities in experimental maxillary sinusitis. *Ann Clin Lab Sci* 33(1):18–22
- Valko M, Rhodes CJ, Moncol J, Izakovic M, Mazur M (2006) Free radicals, metals and antioxidants in oxidative stress-induced cancer. *Chem Biol Interact* 160(1):1–40
- Valko M, Leibfritz D, Moncol J, Cronin MT, Mazur M, Telser J (2007) Free radicals and antioxidants in normal physiological functions and human disease. *Int J Biochem Cell Biol* 39(1):44–84
- Xiong M, He Q, Lai H, Huang W, Wang L, Yang C (2012) Radix astragali injection enhances recovery from sudden deafness. *Am J Otolaryngol* 33(5):523–527
- Xu J, Knutson MD, Carter CS, Leeuwenburgh C (2008) Iron accumulation with age, oxidative stress and functional decline. *PLoS One* 3(8):e2865
- Yamasoba T, Lin FR, Someya S, Kashio A, Sakamoto T, Kondo K (2013) Current concepts in age-related hearing loss: epidemiology and mechanistic pathways. *Hear Res* 303:30–38
- Yilmaz T, Kocan EG, Besler HT (2004) The role of oxidants and antioxidants in chronic tonsillitis and adenoid hypertrophy in children. *Int J Pediatr Otorhinolaryngol* 68(8):1053–1058