Antioxidants: Basic Principles, Emerging Concepts, and Problems

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The radical scavenging antioxidants play an essential role in the maintenance of health and prevention of diseases, and a thorough understanding of the action and capacity of antioxidants is critically important. Despite the assumption that antioxidants must exert beneficial effects against oxidative stress, many large-scale randomized controlled trials gave inconsistent and disappointing results on the prevention of chronic diseases. It is now generally accepted that there is no evidence to support the use of non-discriminative antioxidant supplements for prevention of diseases. On the other hand, recent data show that antioxidants may be effective in the prevention and/or treatment of diseases when the right antioxidant is given to the right subject at the right time for the right duration. Now it is accepted that reactive oxygen species (ROS) act as physiologically important signaling messengers as well as deleterious agents. The signaling ROS are produced in a subtly regulated manner, while many deleterious ROS are produced and react randomly. Free radical–mediated lipid peroxidation products which, in contrast to enzymatic oxidation products, are produced by non-specific mechanisms cause oxidative damage, but may also induce adaptive response to enhance the expression of antioxidant enzymes and compounds. This has raised a question if removal of too many ROS by supplementation of antioxidants may upset the cell signaling pathways and actually increase the risk of chronic diseases. However, it is unlikely that antioxidants impair physiologically essential signaling pathways. *(Biomed J 2014;37:106-111)*

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We are protected from oxidative stress by subtle defense network in which multiple antioxidants with diverse functions play their respective roles. Some antioxidants are proteins and enzymes, while others are small molecules[1,2] [Table 1]. Some antioxidants are synthesized *in vivo*, while others have to be taken from diet. From the viewpoint of functions, the preventive antioxidants suppress the formation of reactive oxygen and nitrogen species (ROS and RNS) by reducing hydrogen peroxide and hydroperoxides and by sequestering metal ions. Superoxide is removed by superoxide dismutase (SOD), while singlet oxygen is quenched by carotenoids. Antioxidative and anti-inflammatory action of heme oxygenase-1 (HO-1) has received attention.[3] The role of free radical scavenging antioxidants is to remove reactive the free radicals before they attack biologically important lipids, proteins, and DNA. Oxidative damage is repaired, reconstituted, and eliminated by antioxidative enzymes. Furthermore, adaptation mechanisms are induced to produce appropriate antioxidants and/or antioxidant enzymes in response to oxidative stress. Humans are able to live as long as 100 years under air at ambient temperature thanks to these antioxidants, although foods made of the same materials are easily deteriorated in several days when kept in refrigerator.

However, the capacity, action mechanisms and dynamics, and effects of antioxidants have not been fully elucidated and have remained as a matter of arguments and debate.[2,4-8] In this brief review article, the following three issues will be overviewed focusing primarily on the radical scavenging antioxidants: assessment of radical scavenging capacity, antioxidant efficacy against diseases, and impairment of ROS signaling by antioxidants.

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Table 1: Antioxidant defense network against oxidative stress *in vivo*

Preventive antioxidants: Suppress the formation and enhance the removal of ROS/RNS
Reduction of hydrogen peroxide and hydroperoxides: Catalase, glutathione peroxidases, selenoprotein P, glutathione S-transferase, peroxiredoxins, thioredoxin
Sequestration of metal ions: Transferrin, lactoferrin, haptoglobin, hemopexin, ceruloplasmin, albumin
Heme oxygenase 1, Glucose-6-phosphate dehydrogenase
Quenching of superoxide: Superoxide dismutase
Quenching of singlet oxygen: Carotenoids
Radical scavenging antioxidants: Scavenge the free radicals to inhibit chain initiation and break chain propagation
Hydrophilic: Vitamin C, uric acid, bilirubin, albumin, flavonoids
Lipophilic: Vitamin E, ubiquinol, carotenoids
Repair and *de novo* enzymes: Repair damage and reconstitute membrane
Lipase, protease, DNA repair enzyme, transferase
Adaptation: Induces appropriate antioxidants and enzymes at the right time and site in the right amount

Abbreviation: ROS: Reactive oxygen species; RNS: Reactive nitrogen species; SOD: Superoxide dismutase

Radical scavenging capacity

Scavenging of free radicals is one of the important functions of antioxidants. The assessment of "radical scavenging capacity" has been the subject of extensive studies and arguments.[9,10] Especially, the capacity of natural antioxidants contained in foods, fruits, beverages, spices, and supplements have attracted much attention of the public as well as scientists in anticipation of their role in the maintenance of health, prevention of diseases, and retardation of aging. Various methods have been developed and applied to assess the capacity to scavenge radicals, from simple *in vitro* model to *in vivo* systems.[2,9,11,12] However, very often, there is a lack of correlation between the capacities determined on the same material by different assays and between the capacities determined by the same assay in different laboratories. There is no simple universal method by which the radical scavenging capacity can be assessed accurately and quantitatively. Needless to say, the results of *in vitro* tests cannot be extrapolated directly to *in vivo* system. Further, it should be noted that the free radical scavenging capacity does not always correlate with the capacity to inhibit oxidative stress and even lipid peroxidation. The scavenging capacity depends on the type of free radicals and on the reaction environment. It is also important to appreciate that considering the physiological concentrations of antioxidants and the reactivity of free radicals and active species, the peroxyl radicals are the major target for antioxidants to scavenge efficiently *in vivo*.[13] Vitamin E, the major lipid-soluble radical scavenging antioxidant *in vivo*, inhibits lipid peroxidation efficiently in combination with vitamin C, but it is not effective against many other radicals such as hydroxyl, alkoxyl, and thiyl radicals.[13] Scavenging of peroxyl radicals is important since they act as the chain-carrying species in lipid peroxidation, which may play a key role in the oxidative modification of biological molecules including proteins and DNA bases and in the development of various diseases.

It is often required to measure the free radical scavenging capacity of antioxidant compounds or products containing mixtures of antioxidants. The capacity of antioxidants for scavenging free radicals has been assessed frequently by either the reaction with stable reference radical such as 2,2-diphenyl-1-picrylhydrazyl (DPPH) and galvinoxyl or by competition methods using reference compound as a probe.[2,9,11,12] Among others, Oxygen Radical Absorbance Capacity (ORAC) has been used most widely, in which the radical scavenging capacity is assessed from the effect of antioxidant on the decay of probe induced by constant flux of free radicals.[9] It should be pointed out that ORAC method suffers from the inherent drawback that the rate and amount of scavenging radicals cannot be distinguished and that the capacity depends on the probe used.[2,10,14] A high ORAC value may be due to high reactivity toward radical or high concentration of the antioxidants contained or both. A probe with low reactivity toward free radical, such as fluorescein, may overestimate the radical scavenging capacity. When assessing the scavenging capacity, it is important to measure the reactivity and amount of antioxidants separately by using probes with low and high reactivity toward free radicals. A method for assessment of antioxidant radical scavenging capacity (ARAC) has been developed using fluorescein and pyrogallol red as probes.[14]

The antioxidant capacity is determined by multiple factors in addition to the reactivity toward free radicals, as listed below:

1. The chemical reactivity toward free radicals
2. Fate of antioxidant-derived radicals
3. Interaction with other antioxidants
4. Concentration, distribution, mobility, and metabolism at the micro-environment

One such factor is the effect of heterogeneity of the medium. There are both hydrophilic and lipophilic antioxidants localized in intra- and extracellular fluids and lipophilic domains. Some lipophilic antioxidants are localized predominantly in the membrane raft, while others are in the non-raft domain, and some are on the membrane surface while others are in the interior.[15] The dynamics of radical scavenging by vitamin E in the membranes have been studied extensively.[16,17] The efficacy of scavenging radicals depends on the localization of antioxidants and also on free radicals.[16] To understand the capacity of radical scavenging and dynamics of antioxidant action, the rate of antioxidant consumption is often measured. The "pecking order," order of consumption of antioxidant during oxidation

in the presence of more than two antioxidants,[18] depends on these factors.[2] It should be emphasized that bioavailability is one of the key factors that determine the antioxidant capacity *in vivo*.

It is reported that many radical scavenging antioxidants including polyphenolic compounds and curcumin may also enhance the antioxidant capacity by inducing phase 2 antioxidant genes through Kelch-like ECH-associated protein 1 (Keap1)-nuclear factor erythroid 2-related factor 2 (Nrf2)-antioxidant (electrophile) responsive element.[8]

Are antioxidants effective for prevention/ treatment of diseases?

Oxidative stress has been implicated in the pathogenesis of various diseases. Oxidative stress was originally defined as an imbalance between oxidants and antioxidants in favor of the oxidants, potentially leading to damage. In fact, there is ample evidence that biomarkers of oxidative stress are increased in subjects with certain diseases or associated risk factors. This implies that these diseases are, at least in part, caused by oxidative damage to critical biomolecules and that intake of, or supplementation with, antioxidants may lower disease risk or be useful in disease prevention and treatment. Large-scale epidemiological studies support such effects.[19] However, many randomized clinical trials gave inconsistent and disappointing results on the effects of antioxidants such as vitamin E and carotenoids. Such "antioxidant paradox" has raised extensive arguments.[6] Possible reasons for failure of antioxidant treatment are listed below:

1. Oxidative event is not a cause, but rather a consequence of disease.
2. Multiple oxidants with different reactivity and selectivity contribute to etiology. Therefore, multiple antioxidants with different functions are required.
3. Healthy subjects who already have enough antioxidants may have limited potential beneficial effect of supplemental antioxidant.
4. Choice of antioxidants, dose, and duration of antioxidant supplementation
5. Choice of clinical trials and end points to be included in meta-analysis
6. Oxidative stress may be pivotal for the initiation of diseases, but may become progressively less important during the later stages of the disease. In most human studies, antioxidant is given to the subjects who are older than 50, which may result in limited effects.

As described subsequently, the effects of ROS/RNS and also of antioxidants are quite complex. It may be said that although indiscriminative supplementation of antioxidants may not be recommended, antioxidant should be beneficial when given to the right subject at the right time and for the right duration. In support of this, recent data show that vitamin E is effective to the subgroups under oxidative stress. Three examples are given below.

Non-alcoholic fatty liver disease (NAFLD) is now the most common liver disease affecting a high proportion of the population worldwide. Oxidative stress has been implicated in the pathogenesis of NAFLD and a strong correlation has been observed between free radical mediated lipid peroxidation and progress of NAFLD and non-alcoholic steatohepatitis (NASH).[20,21] There has been no established pharmacologic treatment for NAFLD/ NASH. Several encouraging pilot studies of various agents indicate potential beneficial effects which may be related to their antioxidant effects. A recent phase 3, multicenter, randomized, placebo-controlled, double-blind clinical trial showed that vitamin E was superior to placebo in regard to the resolution of NASH.[22]

Haptoglobin (Hp) is a hemoglobin (Hb) binding protein whose major function is to bind the free hemoglobin released daily from intravascular hemolysis in an essentially irreversible, non-covalent association.[23,24] Free hemoglobin is highly toxic and has the ability to induce considerable oxidative tissue damage through the release of its heme iron. Hp, by binding to free hemoglobin, inhibits heme iron release and, thus, prevents heme iron mediated oxidation. In the human population, there exist two classes of alleles, Hp1 and Hp2, giving rise to three Hp genotypes, Hp 1-1, Hp 1-2, and Hp 2-2. Hp1 sequesters hemoglobin more strongly than Hp2. Interestingly, it was observed that vitamin E supplementation reduced cardiovascular events in individuals with Type 2 diabetes mellitus and Hp 2-2 genotype.[24]

A recent study also suggests potential ameliorating effect of vitamin E against Smith–Lemli–Opitz syndrome (SLOS).[25] SLOS is caused by mutations in the gene encoding the last enzyme in cholesterol biosynthesis, 7-dehydrocholesterol reductase.[26] SLOS is characterized by multiple congenital malformations and defects, impaired cognitive function, and behaviors of autistic spectrum disorders. The concentration of 7-dehydrocholesterol in plasma with SLOS is markedly elevated compared with that in healthy human plasma and it is quite readily oxidized to give various biologically active oxysterols. It was shown that vitamin E suppressed the formation of oxysterols from 7-dehydrocholesterol, which must be critical for countering the detrimental effects of 7-dehydrocholesterol reductase mutations.[25]

Do antioxidants impair physiological signaling of ROS/RNS?

ROS and RNS induce oxidative modification of biologically essential molecules, leading to functional impairment and loss of biological membranes and proteins and resulting in various disorders and diseases. In addition to such

deleterious effects, it is now clear that some ROS and RNS play an important physiological role as well under certain conditions.[27,28] It has been known that superoxide produced by phagocytosing cells kills the invading bacteria and that up-regulation of endogenous antioxidant enzymes is necessary for the metabolism and elimination of xenobiotics and pro-carcinogens. It has also been shown that ROS is required for normal force production in skeletal muscle and for the development of training-induced adaptation in endurance performance.[29]

ROS and RNS act also as signaling messengers in physiological settings. Considering the specificity of production, reaction, and elimination and also their kinetics, hydrogen peroxide has been suggested to be the most important signaling messenger *in vivo*.[27] Further, superoxide and nitric oxide may also function as physiological signaling messengers. These ROS and RNS produced in an exquisitely regulated manner regulate the gene expression, activate receptors and nuclear transcription factors, and induce adaptive response.

Lipids are oxidized *in vivo* by multiple oxidants and mechanisms.[30] Enzymatic lipid oxidation proceeds in a specific and regulated manner, while non-enzymatic oxidation proceeds randomly. The enzymatic lipid oxidation products act as physiological signaling messengers. On the other hand, free radical mediated lipid peroxidation produces numerous products and has been implicated in the pathogenesis of several diseases. In fact, elevated levels of lipid peroxidation products have been observed in the plasma and urine of patients with the progression of certain diseases.

Interestingly, lipid peroxidation products were found to induce adaptive response and stimulate the expression of antioxidant enzymes and production of antioxidant compounds, enhancing the antioxidant capacity against forthcoming oxidative stress. Especially, α, β-unsaturated carbonyl compounds such as 4-hydroxynonenal (HNE) and 15-deoxydelta prostaglandin J_2 (15-dPGJ$_2$) have received much attention as biologically active signaling mediators which induce adaptive response.[31-33] HNE is highly reactive with nucleophiles such as the thiol of cysteine, imidazole group of histidine, and primary amine of lysine in protein via Michael addition and/or Schiff base reaction.[34,35] It has been shown that HNE can stimulate cellular proliferation, differentiation, and cytoprotective response by affecting multiple signaling pathways. HNE is produced in free radical mediated lipid peroxidation.

Furthermore, chemically unreactive lipid peroxidation products such as hydroxy fatty acids and hydroxycholesterol are also capable of inducing adaptive response to enhance tolerance capacity.[36] For example, pretreatment with lipid peroxidation products, such as phosphatidylcholine hydroperoxide, lysophosphatidylcholine, hydroxyoctadecadienoic acid, 7-hydroxycholesterol, and cholesterol 5,6-epoxide, at sublethal concentrations significantly protected cells against subsequent oxidative stress induced by 6-hydroxydopamine.[36] Such an adaptive response has been observed for many kinds of stimuli [Table 2].

Compelling evidence shows that cells have the capacity to adapt to oxidative stress through cell signaling mechanisms. Radiation hormesis, a putative stimulatory effect of low-level ionizing radiation, has been ascribed to protective feedback systems that, upon exposure to low concentrations of toxins, proceed to stimulate metabolic detoxification and repair networks.[37] It was found some time ago that cells being exposed to low levels of hydrogen peroxide are able to survive the subsequent normally lethal oxidative stress by increasing the transcription of stress-related genes, antioxidant defense genes, and/or repair enzymes.[38] Similarly, as described earlier, lipid peroxidation products induce the synthesis of glutathione and the expression of antioxidant enzymes such as HO-1, glutathione *S*-transferase (GST), thioredoxin reductase (TR), and NAD (P) H quinone oxidoreductase 1 (NQO1) by Nrf2-Keap1 system.[39] Lipid peroxidation products enhance the release of Nrf2 from Keap1 and the translocation of Nrf2 into the nucleus, where it binds to electrophile response element and up-regulates the transcription of target genes.

These findings prompted to assume that removal of too many ROS and ROS-derived products by antioxidant supplementation may upset the cell signaling pathways and actually increase the risk of chronic disease.[4] It should be pointed out, however, that physiologically necessary signaling mediator is produced under controlled and regulated manner and reacts with sensing molecules in a fine-tuned, specific, and regulated manner. On the contrary, free radical lipid peroxidation proceeds randomly without specificity. Lipid peroxidation can neither be programmed nor regulated.

Table 2: Adaptive response induced by lipid peroxidation products*

First stimuli	Second stimuli
PC hydroperoxide	6-Hydroxydopamine
HODE	H (P) ODE
7-α, β-Hydroxycholesterol	7- α, β-Hydroxycholesterol
5,6-Epoxycholesterol	Cumene hydroperoxide
15-dPGJ$_2$	Hydrogen peroxide
HNE	Glutamate
Lyso PC	SIN-1 (peroxynitrite)
Nitro-fatty acid	MPTP
γ-Tocopheryl quinone	γ-Ray irradiation
Hydrogen peroxide	γ-Ray irradiation

*: Pretreatment of cultured cells with lipid peroxidation LPO products and other stimuli shown in the first column induce adaptive response to increase cellular tolerance against the subsequent second stimuli shown in the second column. Abbreviations: PC: Phosphatidylcholine; H (P) ODE: Hydro (pero) xyoctadecadienoic acid; HNE: Hydroxy-2-nonenal; SIN-1: 3-Morpholinosydnonimine; MPTP: 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine; 15-dPGJ$_2$: 15-Deoxydelta prostaglandin J_2 (15-dPGJ$_2$)

Lipid peroxidation–derived products such as HNE may induce the adaptive response *in vivo*, but this is considered as a response to xenobiotics, rather than as an essential signaling molecule produced on purpose. Therefore, inhibition of lipid peroxidation by radical scavenging antioxidants should be primarily beneficial for the maintenance of health and prevention of diseases. It is also reported that the radical scavenging antioxidants such as vitamins E and C are not efficient scavengers of physiologically important signaling ROS/RNS such as superoxide, nitric oxide, and hydrogen peroxide.[13]

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