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Factors affecting *in vitro* and *in vivo* antioxidant effects. Experimental conditions and nature of oxidants determine antioxidant efficacy

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Abstract. Reactive oxygen and nitrogen species have been implicated in the onset and progression of various diseases and the role of antioxidants in the maintenance of health and prevention of diseases has received much attention. The action and effect of antioxidants have been studied extensively under different reaction conditions in multiple media. The antioxidant effects are determined by many factors. This review aims to discuss several important issues that should be considered for determination of experimental conditions and interpretation of experimental results in order to understand the beneficial effects and limit of antioxidants against detrimental oxidation of biological molecules. Emphasis was laid on cell culture experiments and effects of diversity of multiple oxidants on antioxidant efficacy.

Keywords: Antioxidant, cell culture medium, oxygen concentration, reactive oxygen species

1. Introduction

It is now accepted that oxidative stress is deeply involved in the human health and diseases and that oxidative stress has dual faces, *eustress* and *distress* [1, 2]. Oxygen is indispensable for our life. It is essential for energy production, that is, molecular oxygen functions as the terminal acceptor of electrons during mitochondrial electron transport. Oxygen is also required for synthesis of structural components and trace constituents such as cholesterol and signaling mediators, respectively. Furthermore, it is used for disposition of foreign compounds in detoxifying system and for host defense, that is, for the destruction of invading microorganisms. For such purpose, stable dioxygen molecule is converted to various forms termed reactive oxygen species (ROS) by redox reactions in a regulated manner. ROS also oxidizes essential lipids to produce oxygenated lipid mediators selectively to maintain homeostasis.

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At the same time, ROS are produced *in vivo* randomly as well and attack biological molecules by non-regulated manner giving rise to diverse mixtures of oxidatively modified products. Such random oxidation of lipids, proteins, and nucleic acids has been implicated in many diseases including atherosclerosis, liver diseases, neurological disorders, and cancer.

We are protected from detrimental oxidative stress by an excellent antioxidant network which we acquired in a process of long evolution. Many kinds of antioxidants having different functions play their respective roles in the physiological defense network. Multiple enzymes, proteins, and small molecules act as essential antioxidants. The role of antioxidants present in natural products including berries has received much attention of the public as well as scientists [3–6]. The action and effects of antioxidants against *in vitro* oxidation have been the subjects of extensive studies and are now fairly well understood [7, 8], but there still remain many issues that have to be addressed and elucidated to fully reveal the beneficial effects and limit of antioxidants in the maintenance of health and prevention of diseases [9]. The aim of this review article is to discuss some of these issues, especially the factors that determine experimental results.

2. Antioxidant effects in cultured cells

Cell culture is widely used to study redox events in cells aiming at elucidating the action and effects of antioxidants against detrimental oxidation mediated by ROS. Cell culture studies have provided a huge amount of valuable information. Cultured cells may become more important with increasing restriction of animal experiments. However, the results of cultured cell experiments cannot be directly extrapolated to the *in vivo* system. Further, the antioxidant effects depend on experimental conditions, that is, methods used to determine the antioxidant activity. It has been pointed out that a greater awareness of the potential artifacts in cell culture studies is needed to obtain scientifically sound information [10]. Several factors are discussed below.

2.1. "Physiological concentration"

Lipophilic antioxidants such as vitamin E are localized in lipid domain of cellular membranes and within lipoproteins. The behavior of vitamin E in membranes including uptake, localization, retention, diffusion, and excretion has been studied extensively [reviewed recently in 11]. Cultured cells have often been used for assessment of antioxidant capacity against oxidative stress. Antioxidant may be pre-treated or added to cell culture medium simultaneously with the oxidative insult. The choice of antioxidant concentration is critically important.

The plasma concentration of antioxidant is expressed in different units such as micromolar (μM) and milligram per deciliter (mg/dL). In cell culture, antioxidant concentration has been reported in mass or moles per number of cells or per mg protein. In the context of lipid peroxidation, the more useful unit may be the mole amount of antioxidant per mole of lipid such as cholesterol, phospholipids or polyunsaturated fatty acids (PUFA). The proportions of antioxidant to lipids in the membranes are different not only with respect to total lipids but also to individual PUFAs [12–14]. In general, the physiological molar ratio of vitamin E to total lipids in the cellular membranes is approximately one to several hundreds.

In cell culture experiments, lipophilic antioxidants dissolved in amphiphilic solvents such as alcohol and DMSO are added to culture medium. The concentration of antioxidant is determined often by referring to the physiological concentration. The plasma concentrations of α-tocopherol and γ-tocopherol in healthy subjects are around 20–30 and 1–3 μM, respectively. Often, 20 to 30 μM vitamin E is added to culture medium containing 10^4 to 10^6 cells/mL. However, it should be noted that the concentration of total lipids in the cell culture medium is much smaller than that in plasma which is roughly 10 mM [15], making the molar ratio of vitamin E/lipids in cultured cells much higher than physiological ratio. This implies that the concentrations of vitamin E used in cell culture experiments are often extremely high causing a "culture shock" and the results obtained under such conditions may lead to physiologically irrelevant argument. In fact, it has been observed in many studies

that tocopherol less than 0.1 μM is sufficient to prevent cell death induced by various oxidative insult including selenium deficiency [16] and glutamate [17]. It is important to select appropriate concentration of antioxidant in cell culture medium considering the concentration of lipids and also, as discussed below, to measure actual concentration of antioxidants in the cells.

2.2. Potential pro-oxidant effects of antioxidant

Cell culture media are frequently deficient in the antioxidants such as vitamins E and C, a situation which can lead to overinterpretation of the actions of added antioxidants. Further, ascorbate, as well as flavonoids and many other polyphenolic compounds are unstable in the commonly used cell culture media, undergoing autoxidation to generate superoxide and hydrogen peroxide, which may cause artifacts.

Many reactive antioxidants often act as reductant. Ascorbic acid reduces ferric ion and cupric ion to ferrous and cuprous ions, which decompose hydrogen peroxide and hydroperoxides much faster than Fe^{3+} and Cu^{2+} to produce hydroxyl and alkoxyl radicals, respectively. Under such cases, ascorbic acid may act as a prooxidant. In fact, the combination of ascorbic acid and iron has often been used to initiate oxidation *in vitro*. Similar effects have been observed for tocopherols as well [18–20]. It was found that α-tocopherol and α-tocotrienol reduced cupric ion but that β-, γ-, and δ-tocopherol and tocotrienol did not [20]. It may be noted that the transition metal ions sequestered by proteins *in vivo* may not be reduced readily by biological antioxidants.

Biological specimens such as plasma are sometimes treated with reducing agent to convert hydroperoxides to more stable hydroxides for quantitative analysis by HPLC or GC coupled with mass spectrometer. Sodium borohydride used to reduce hydroperoxides to hydroxides may also reduce sequestered metal ions to active low-valency forms, leading to induction of artificial oxidation. In fact, it was found in the analysis of lipid oxidation products in human plasma that the treatment of plasma with sodium borohydride induced artifactual lipid oxidation giving rise to higher levels of lipid hydroxides than those treated with triphenylphosphine, which reduces hydroperoxides to hydroxides but does not react with metal ions [21].

2.3. Uptake and distribution of antioxidants in cells

Hydrophilic and lipophilic antioxidants dissolved in water and amphiphile solvent respectively are added to the cell culture medium. Hydrophilic antioxidants remain in the extracellular fluid or taken into the cellular cytosol. On the other hand, lipophilic antioxidants are taken up and distributed into cellular membranes. The distribution of antioxidants in aqueous and lipophilic phases depends on the hydrophilicity/lipophilicity of antioxidants, that is, partition coefficient.

The rate of uptake into cellular membranes depends on the nature of antioxidants and determine the apparent antioxidant effects. A well-known example is the difference between tocopherol (T) and tocotrienol (T3). It was found that initial rate of α-T3 uptake into Jurkat cells was about 70 times higher than that of α-T [22]. Similar higher uptake rate for α-T3 than α-T has also been observed for immature primary cortical neurons [17], and endothelial cells [23, 24]. α-T and α-T3 showed similar cellular distribution, which was directly proportional to the lipid distribution [22]. More recently, it was observed that intracellular distribution of α-T, α-T3, γ-T and γ-T3 in cultured liver cells are similar [25].

Many studies observed superior antioxidant activity of T3 to the corresponding T against oxidative stress in cultured cells. For example, α-, β-, γ-, and δ-T3 suppressed cell death induced by selenium deficiency more effectively than the corresponding T [16]. However, importantly, it has been observed that the corresponding T and T3 exerted similar resistance against the oxidative damage caused by several different stimuli if the cellular concentrations were adjusted. These results suggest that the apparent higher cytoprotective effect of T3 than T observed is primarily ascribed to higher cellular uptake of T3 than T and that the measurement of intracellular concentration of antioxidants is essential for scientifically sound interpretation of experimental results. In many studies, the actual concentrations of antioxidant in the cells are

not measured. Care should be taken when the effects of added antioxidants to cultured cells are compared.

Interestingly, the cellular distribution of α-T directly corresponded to the lipid distribution, whereas the distribution of coenzyme Q-10 did not show any relationship with the lipid distribution. Instead, ubiquinone-10 and ubiquinol-10, oxidized and reduced form of coenzyme Q-10, were localized mainly in the mitochondrial fraction, which is similar to the localization of endogenous coenzyme Q-10 but different from that of α-T [26].

2.4. Oxygen concentration

Another factor is oxygen concentration in cell culture system, which may affect cell growth, differentiation, signaling, and production of ROS [27]. Cell culture is commonly performed under 95% air/5% CO_2, an oxygen tension of approximately 150 mm Hg. Most cells *in vivo* are exposed to lower oxygen concentrations, in the range of 1–10 mm Hg, although there are obvious exceptions including skin epidermis, cornea of the eye, and the cells lining the respiratory tract.

The oxygen levels in cell culture can fluctuate widely, being high when the cells are first placed in air-saturated culture medium and dropping as the cells grow, proliferate, and consume oxygen. Often an oxygen gradient is established between the surface of the medium and the cells beneath. When the medium is changed, cells may therefore switch from relative hypoxia to hyperoxia and suffer the equivalent of a hypoxia-reperfusion injury [27].

Importantly, the relative importance of competing reactions in free radical mediated lipid and protein oxidation and also antioxidant action depends on oxygen concentration. For example, the production of isoprostanes, which are accepted as golden biomarker of oxidative stress *in vivo* [28], increases with decreasing oxygen concentration [29] and the antioxidant efficacy of carotenoids are enhanced with decreasing oxygen concentration [30]. Semiquinone radicals formed when polyphenols scavenge free radicals may react with oxygen to produce quinone and hydroperoxyl radical/superoxide, which affect antioxidant activity of polyphenols.

Carbon radicals such as pentadienyl radicals derived from PUFA may have longer life with deceasing oxygen concentration. The nitroxide compounds scavenge carbon radicals rapidly by near diffusion-controlled rate. Such scavenging of carbon radicals may become an important antioxidant reaction at low oxygen concentration.

2.5. Antioxidant metabolism

Phenolic antioxidants are metabolized *in vivo* to various conjugated forms. The intact free phenols and their conjugated forms exert different biological functions. For example, α-tocopherol is phosphorylated *in vivo* and the resulting α-tocopherol phosphate does not act as radical scavenging antioxidant by itself but may act as cellular regulator [31, 32].

Terao reported that dietary quercetin was exclusively present in their conjugated form in the blood stream [33]. α-Glucosylation and prenylation of quercetin affect bioavailability and functions. Quercetin acts as both aglycone and metabolites, their relative importance being dependent on conditions.

3. Contribution of multiple oxidants in biological oxidation and effects of antioxidants

Oxidation of biological molecules *in vivo* is mediated by multiple oxidants having different reactivity and selectivity. In general, enzymatic oxidation proceeds by regulated manner at specific site and time, and produces specific products selectively, whereas non-enzymatic oxidation produces diverse products non-selectively.

Major biological oxidants include oxygen radicals ($HO^{\bullet}$, $RO^{\bullet}$, $RO_2^{\bullet}$), singlet oxygen ($^1O_2$), peroxynitrite/peroxynitrous acid ($ONOO^-$/ONOOH), carbonate anion radical ($CO_3^{\bullet-}$), nitrogen dioxide radical ($NO_2^{\bullet}$),

Table 1
Rate constants for the reactions of selected oxidants with linoleate and antioxidants (in M^{-1} s^{-1}) and the ratio of k_{AH}/k_L [a]

Oxidant	Peroxyl radical	NO_2 radical	$CO_3^{\bullet-}$	HOCl	$^1O_2$
Antioxidant					
Linoleate	62 [49]	2×10^5 [50]	2×10^5 [50]	18 [51]	1.3×10^5 [52]
α-Tocopherol	3.2×10^6 [39]	1×10^5 [53]	10^7 [54]	1.3×10^3 [51]	3×10^8 [55]
	(**5×10^4**)	(0.5)	(50)	(70)	(**2×10^3**)
Ascorbic acid	2.2×10^6 [56]	3.5×10 [50]	1.1×10^9 [38]	6×10^6 [57]	1.9×10^6 [58]
	(**3×10^4**)	(2×10^2)	(**5×10^3**)	(**3×10^5**)	(15)
β-Carotene	10^5 [59]	11×10^8 [50]		2.3×10^4 [60]	10^{10} [55, 58]
	(**2×10^3**)	(5×10^2)		(**10^3**)	(**8×10^4**)
Uric acid	9×10^5 [61]	2×10^7 [62]	2.9×10^8 [63]	2×10^5 [57]	3.6×10^8 [64]
	(**10^4**)	(10^2)	(**10^3**)	(**10^4**)	(**3×10^3**)
Glutathione (GSH)		2×10^7 [50]	5.3×10^6 [38]	1.2×10^8 [65]	7.4×10^6 [55]
		(10^2)	(26)	(**7×10^6**)	(57)

[a] k_{AH} and k_L are the rate constants for the reactions of oxidant with antioxidant and linoleate, respectively. Numbers in parentheses are the ratio of k_{AH}/k_L. The ratios larger than 10^3 are shown in bold (see text). Numbers in brackets indicate reference number.

hypochlorite (HOCl), and thiyl radical ($RS^{\bullet}$) [34]. Enzymes such as lipoxygenase (LOX), cyclooxygenase (COX), and cytochrome P450 (CYP) also play an important role in the oxidation of unsaturated lipids [35].

Diverse oxidation products are observed in human fluids and tissues. Some products may enable to specify responsible oxidants [9]. Interestingly, oxidation products produced specifically by oxygen radicals, nitrogen dioxide radical, hypochlorite, and singlet oxygen have been detected in human atherosclerotic lesions and their levels were elevated compared with normal tissues, suggesting that multiple oxidants mentioned above are involved in the pathogenesis of atherosclerosis [9]. It may be added that these oxidants induce oxidation of both low density and high density lipoproteins to pro-atherosclerotic forms. Likewise, it is assumed that the oxidation of lipids, proteins and DNA bases mediated by multiple oxidants are involved in the pathogenesis of various diseases.

As mentioned above, multiple antioxidants play their respective roles in the defense network for maintenance of human health and prevention of diseases. Scavenging or removal of reactive oxidants is one of the important functions of antioxidants. Diverse natural products act as radical scavenging antioxidant. Antioxidant capacity for scavenging oxidants has been assessed by many groups by different methods [36, 37 and references cited therein]. The "antioxidant capacity" means many things including capacity to scavenge oxidants, suppress the oxidation, and to prevent oxidation-induced damage or disease. The capacity is expressed by absolute rate constant, rate constant relative to standard antioxidant such as TEAC (Trolox equivalent capacity), ORAC (oxygen radical absorbance capacity), or half maximal effective concentration (EC50). The details of each method are not discussed here, but it is important to note that the capacity of antioxidants to scavenge oxidants depends on the nature of oxidants [7, 9, 38].

The rate constants reported for the reaction of representative substrates with multiple oxidants are summarized in Table 1 [9]. Free and ester forms of linoleic acid are the most abundant PUFA *in vivo* and major target of oxidants. α-Tocopherol, ascorbic acid, β-carotene, and uric acid are major antioxidants *in vivo*, while glutathione and protein thiyl groups may act as both antioxidant and target of multiple oxidants. Peroxyl radical, peroxynitrite/peroxynitrous acid, nitrogen dioxide radical, carbonate anion radical, hypochlorous acid, and singlet oxygen are included in Table 1 as the major oxidants. The rate constants in Table 1 show that the reactivities of substrates and antioxidants depend significantly on the oxidants and that the efficacy of antioxidants is variable.

Antioxidant scavenges oxidants in competition with the target substrates such as lipids and proteins (SH). In order for the antioxidant (AH) to act as an efficient antioxidant, the rate of scavenging oxidant ($X^{\bullet}$) by

antioxidant (reaction 1) should be larger than that of oxidant with the substrate (SH) (reaction 2), that is, $k_{AH}[X][AH]/k_{SH}[X][SH]»1$, where k_{AH} and k_{SH} are the rate constants for the reactions 1 and 2, respectively. In general, the molar ratio of substrate/antioxidant *in vivo* is larger than 100. Therefore, it is estimated that the ratio k_{AH}/k_{SH} should be larger than 10^3 for the antioxidant to scavenge 90% of the oxidant before the oxidant reacts with the substrate.

$$X^{\cdot} + AH \overset{k_{AH}}{\rightarrow} XH + A^{\cdot} \quad (1)$$

$$X^{\cdot} + SH \overset{k_{AH}}{\rightarrow} XH + S^{\cdot} \quad (2)$$

In other words, the efficacy of antioxidants is determined by the reactivities of both oxidant and substrate. The ratios k_{AH}/k_{SH} which are larger than 10^3 are shown in bold in Table 1. It can be seen from Table 1 that α-tocopherol acts as a potent inhibitor of free radical mediated lipid peroxidation, but not against nitrogen dioxide radical, carbonate anion radical, and hypochlorite.

Statement such as "antioxidant A is more reactive toward ROS than B" is not scientifically sound unless the ROS is specified. It should be noted that the rate constant depends on the environment. For example, the rate constant for the scavenging of peroxyl radical by α-tocopherol is 3.2×10^6 M^{-1} s^{-1} in chlorobenzene solution [39], but it is reduced to 5×10^5 M^{-1} s^{-1} in alcohol solvent due to hydrogen bonding [40, 41], further to $3.7 \sim 6.0 \times 10^4$ M^{-1} s^{-1} in SDS micelles [42, 43] and 5.8×10^3 M^{-1} s^{-1} in PC liposomal membranes [44].

The antioxidant activity depends also on other factors such as localization and interaction with other antioxidants. In the oxidation of plasma induced by 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH), the endogenous antioxidants were consumed in the order of ascorbate, sulfhydryl group, bilirubin, uric acid, and α-tocopherol [45], while in the oxidation of whole blood induced by AAPH, the order was ascorbate, bilirubin, uric acid, plasma α-tocopherol, erythrocyte α-tocopherol, and erythrocyte sulfhydryl group [46]. In the oxidation of low density lipoprotein (LDL) induced by AAPH, both ascorbate and uric acid spared α-tocopherol, but when free radicals were generated within LDL particles by lipophilic azo initiator, α-tocopherol was consumed after depletion of ascorbic acid even though uric acid was remaining [47]. This is because ascorbic acid readily reduces α-tocopheroxyl radical to regenerate and spare α-tocopherol, but uric acid cannot scavenge radicals within LDL particles faster than α-tocopherol, nor can it reduce α-tocopheroxyl radical to produce α-tocopherol.

The data summarized in Table 1 suggest that neither vitamin E nor any other antioxidant alone can cope with deleterious oxidative damage of biological molecules mediated by multiple oxidants. This may explain why many human intervention studies using a single antioxidant failed to show beneficial effect of antioxidant consistently and suggests that multiple antioxidants having different selectivity are required [9]. The extracts from natural products containing mixtures of antioxidants must be more useful than single antioxidant.

It has been pointed out that most attempts to validate beneficial effects of exogenous small molecule antioxidants on the maintenance of human health and prevention of diseases have failed or even increased health risks, in particular for supplements of lipophilic antioxidants such as vitamin E [48]. It is sometimes claimed that small molecule antioxidants are useless. Possible explanations for this paradox have been argued extensively. It has been pointed out that ROS regulate several physiologically essential processes and scavenging of such ROS may cause paradoxical reductive stress and thereby induce or promote disease. It is also said that physiological ROS that are relevant for signaling need to remain untouched, while disease-triggering ROS should be effectively reduced. However, it is unlikely that small molecule antioxidants such as vitamin E, scavenge physiologically important signaling ROS. This again suggests that it is important to specify ROS when antioxidant effects are discussed.

In summary, it may be stated with confidence that antioxdants with multiple functions are indispensable to human health and that it is important to take multiple antioxidants that are effective against all biological oxidants.

Conflict of interest

The author has no conflict of interest to report.

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