

Review Article

# Antioxidant Networks *In vivo*

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## Abstract

Oxygen interacts with cells and can form highly reactive compounds *in vivo* known as reactive oxygen species (ROS). High levels of ROS can lead to cellular damage, oxidative stress, heart diseases and cancer. Known substances that are capable of halting the physiological process of oxidation in tissue are called antioxidants.

This review covers developments in the field of antioxidant chemistry including interactions between antioxidants and ROS, topics about sources and natural occurrences, classification of antioxidants and a discussion of possible mechanisms. We also summarize examples of oxidative stress biomarkers.

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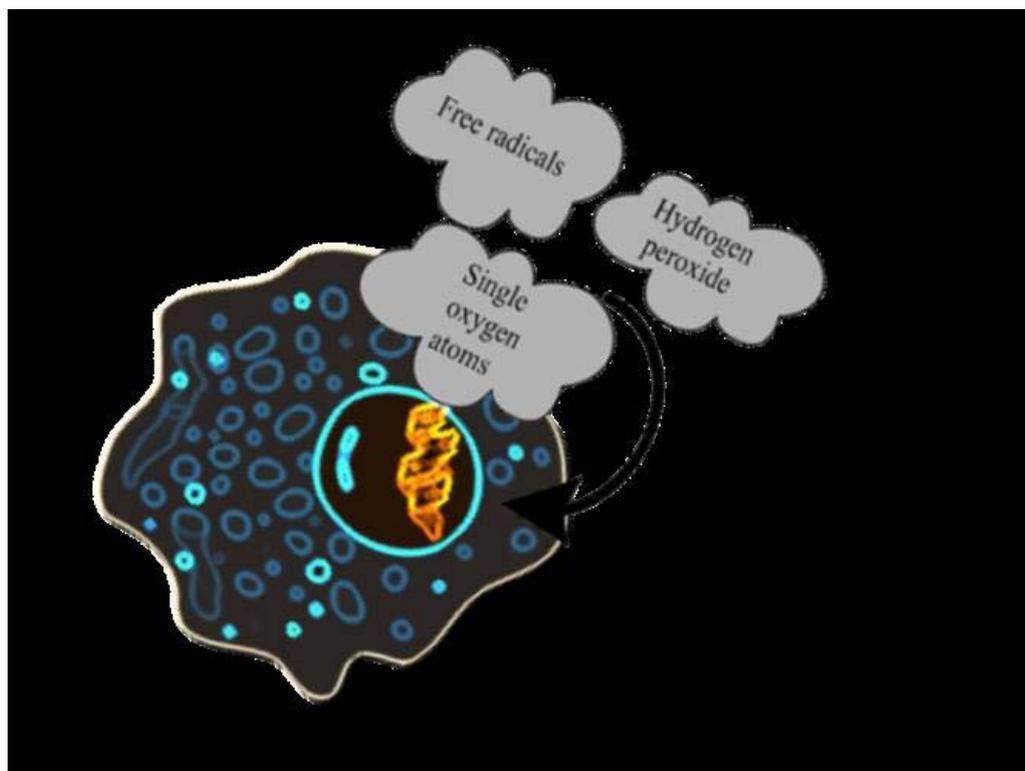
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## Introduction to Interactions between Antioxidants and Reactive Oxygen Species

The human body has developed an efficient antioxidant protection system. The body relies on several endogenous defense mechanisms to help protect against free radical induced cell damage. The term antioxidant refers to species that prevent oxidation reactions. The antioxidants are substances that, when present at low concentrations, significantly delay or inhibit oxidation [1]. The possible mechanisms of antioxidant action are often discussed based upon solubility. Antioxidants are hydrophilic (water soluble) or hydrophobic (soluble in lipids). In general, hydrophilic antioxidants react with oxidants in the cell cytosol and the blood plasma, while hydrophobic antioxidants protect cell membranes from lipid peroxidation. Both antioxidant groups, hydrophilic and hydrophobic, are substances that may protect cells from the damage caused by molecules known as reactive oxygen species (ROS). ROS are molecules with an incomplete electron shell and are more chemically reactive than those with complete electron shells. In the first step, the ROS releases an electron, and in the second step a new radical is formed. The reaction continues until ROS termination occurs when either the radical is stabilized or it simply decays into a new product. Incomplete electron shells of ROS make them more chemically reactive than those with complete electron shells. In biological systems, ROS are continuously being produced intracellularly by oxidation-reduction (redox) reactions [2,3]. ROS generated by electron-transfer reactions are superoxide anion radical ( $O_2^{\cdot-}$ ), hydrogen peroxide ( $H_2O_2$ ), hydroxyl radicals ( $HO^{\cdot}$ ), lipid alkoxyl ( $ROO^{\cdot}$ ) and peroxy radicals ( $HOO^{\cdot}$ ). In other ways, ROS are generated by energy-transfer reactions such as singlet oxygen ( $^1O_2$ ) and triplet carbonyl compounds. At lower concentrations ROS play a role in mutagenic activity and in response to pathogen attack. However, if ROS are present in higher concentrations it gives rise to oxidative stress [4]. Briefly, the antioxidants are called a chain breaking compound. Insufficient levels of antioxidants, or inhibition of the antioxidant enzymes cause oxidative stress and may damage or kill cells. The term oxidative stress represents a shift towards the ROS amount in the ROS - antioxidant equilibrium balance that occurs in metabolism. There are multiple sources of ROS generation *in vivo* and thus ROS are classified into two groups (group 1) mitochondrial and (group 2) non-mitochondrial in origin. The mitochondrial ROS are generated in a non-enzymatic process respiratory chain [5]. Hyperglycemia induced generation of free radicals at the mitochondrial level is thought to be the major driver of the vicious cycle of oxidative stress in diabetes [6]. Briefly, intracellular glucose leads to an abundance of electron donors generated during the Krebs cycle driving the inner mitochondrial membrane potential upward into a state that is associated with mitochondrial dysfunction and increased ROS production [7,8].

Oxidative damage to deoxyribonucleic acid (DNA), proteins, and other macromolecules has been implicated in the pathogenesis of a wide variety of diseases, most notably heart disease and cancer [9]. When an oxygen molecule ( $O_2$ ) becomes electrically charged it removes electrons from other molecules, causing damage to the DNA and other molecules. Over time, such damage may become irreversible and lead to disease including cancer [10]. ROS can also mediate an indirect attack to DNA, primarily by reacting with other cellular components (i.e., phospholipids), resulting in the generation of secondary reactive intermediates that irreversibly couple to DNA bases, forming DNA adducts. Formation of DNA adducts is central in the carcinogenic process. Antioxidants have therefore been considered as a means to modify and minimize the toxic effect of free radicals. When a cell with a damaged DNA strand divides, a mutation can arise which turn in carcinogenesis. Antioxidants decrease oxidative damage to DNA and diminish abnormal cell division [11,12].



**Figure 1** Reactive Oxygen Species Cause Damage to DNA which may Result in the Formation of Cancer Cells.

Oxidation products of DNA bases (i.e., 5-hydroxyuracil, hydroxyguanine and thymine glycol) [13] have been used as oxidative stress biomarkers. Also oxidation products of lipids (i.e., oxidized lipids, oxysterols and conjugated dienes) [14] and proteins (i.e., nitroamino acids, albumin dimer and crosslinked protein) [15] are recognized as oxidative stress biomarkers.

## Sources and Classification of Antioxidants and Reactive Oxygen Species

Antioxidants represent types of endogenous and exogenous compounds that neutralize free radicals [16]. The enzymatic antioxidants include superoxide dismutase (SOD), glutathione peroxidase and catalase. Vitamin A, C, E, carotenoids, thiol antioxidant (e.g. thioredoxin, lipoic acid, glutathione) and flavonoids are non-enzymatic antioxidants. Vitamin C, vitamin E and carotenoids are among the most widely studied dietary antioxidants [17]. Vitamin C is considered the most important water-soluble antioxidant in extracellular fluids and helps to maintain the collagen production, and also helps with healing of wounds. It is a water soluble compound found mostly in oranges, red peppers, kiwifruit and grapefruits. Vitamin C can be found in high abundance in many fruits and vegetables and is also found in cereals, beef, poultry, and fish. Vitamin E was the first vitamin where scientists recognized the antioxidant effect which works in the cell membranes. Vitamin E is found in almonds, safflower, soybean oils, corn, and is also found in nuts, mangos and broccoli.  $\beta$ -carotene is found in sweet potatoes, carrots, squash, apricots, pumpkin, mangos and some green, leafy vegetables, including collard greens, spinach, and kale. Another antioxidant, vitamin A is found in three main forms: retinol (vitamin A<sub>1</sub>), 3,4-didehydroretinol (vitamin A<sub>2</sub>), and 3-hydroxy-retinol (vitamin A<sub>3</sub>). Foods rich in vitamin A include liver, sweet potatoes, carrots, milk, egg yolks, and cheese. Lutein is abundant in green, leafy vegetables such as collard greens, spinach, and kale. Lycopene is a potent antioxidant found

in tomatoes, watermelon, guava, papaya, apricots, pink grapefruit and red blood oranges.

An inorganic exogenous antioxidant, selenium, is found in plant foods like rice and wheat. The amount of selenium in soil, which varies by region, determines the amount of selenium in the foods grown in that soil. It is capable of neutralizing ROS in the aqueous phase before lipid peroxidation is initiated. Sulfur containing phytochemicals, such as the allyl sulfides are found in the allium family of garlic, onions, and leeks. Isothiocyanates and sulphoraphane are in cabbage, broccoli, and cauliflower and have been shown to inhibit various steps in tumor development [18]. Indole antioxidants are also found in cruciferous vegetables, terpenes and in citrus oils [18]. Endogenous antioxidants are present in a wide range of concentrations in body fluids and tissues, with some such as glutathione or ubiquinone mostly present within cells, while others such as uric acid are more evenly distributed. Antioxidants presented in Table 1 include nutrient-derived antioxidants, antioxidant enzymes, and metal binding proteins. Numerous other antioxidant phytonutrients are present in a wide variety of plant foods.

**Table 1** Classifications of Endogenous and Exogenous Antioxidants and their Representative Compounds.

Class of Antioxidants	Representative Antioxidants
Endogenous antioxidants [19]	bilirubin
	thiols, lipoic acid, N-acetyl cysteine
	NADPH and NADH
	ubiquinone (coenzyme Q10)
	uric acid
	superoxide dismutase (SOD)
	iron-dependent catalase
	glutathione peroxidase
	albumin with copper
	ceruloplasmin with copper
Enzymes [20]	metallothionein with copper
	ferritin with iron
	myoglobin with iron
	transferrin with iron
	vitamin C
	vitamin E
Metal binding proteins with interacted metal [21,22]	carotenoids and oxycarotenoids
	polyphenols
Exogenous dietary antioxidants [20]	

In addition, metal binding proteins, such as ferritin, lactoferrin, albumin, and ceruloplasmin sequester free iron and copper ions that are capable of catalyzing oxidative reactions. Another group is synthetic antioxidants such as butylatedhydroxytoluene (BHT), butylatedhydroxyanisole (BHA) and propylgallate which are added to packaged and prepared foods to prevent oxidation and spoilage [23].

ROS are capable of reacting with membrane lipids, nucleic acids, proteins and enzymes, and other small molecules, resulting in cellular damage. The main pathways to generate ROS are a consequence of aerobic metabolism, oxidative burst from phagocytes, and xenobiotic metabolism during general antioxidants actions. ROS and associated antioxidants [19-22] are presented in Table 2.

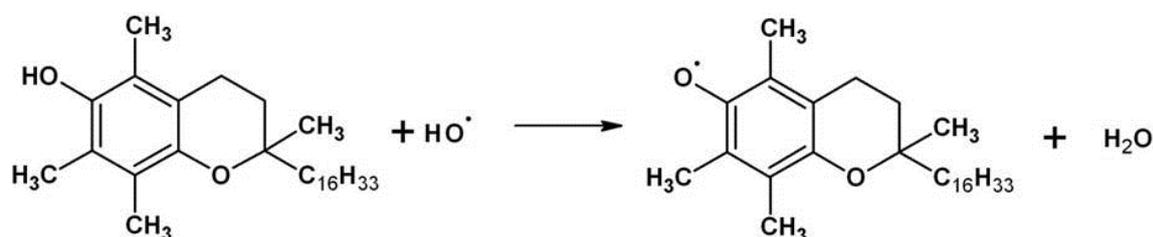
**Table 2** Reactive Oxygen Species (ROS) and Corresponding Neutralizing Antioxidants

Neutralizing Interacting Antioxidants	Reactive Oxygen Species (ROS)
vitamin C	HO <sup>•</sup> and O <sub>2</sub> <sup>•-</sup>
glutathione	HO <sup>•</sup> and O <sub>2</sub> <sup>•-</sup>
flavonoids	HO <sup>•</sup> , ROO <sup>•</sup> and O <sub>2</sub> <sup>•-</sup>
lipoic acid	HO <sup>•</sup>
vitamin E	ROO <sup>•</sup>
beta carotene	ROO <sup>•</sup>
ubiquinone	ROO <sup>•</sup>
glutathione peroxidase	ROO <sup>•</sup>
superoxide dismutase (SOD)	O <sub>2</sub> <sup>•-</sup>

Under certain conditions, an excess of any one type of antioxidant in the absence of balance with the others may be counter-protective [24]. Superoxide dismutase (SOD) converts superoxide to hydrogen peroxide, while carotenoids scavenge singlet oxygen. Furthermore, there is now evidence that some antioxidants or ROS act as a cellular signaling to regulate the concentrations of antioxidants.

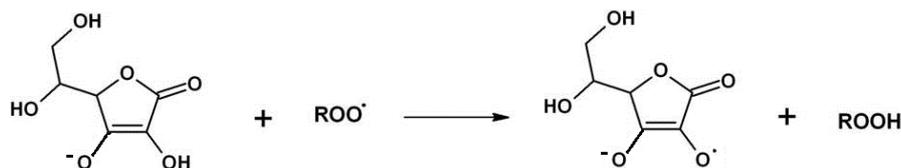
### *In vivo* Network of Antioxidants

Antioxidants neutralize radicals preventing them from taking electrons from other molecules. When highly reactive oxidants breach specific intracellular defenses, the cells use antioxidant protection systems which are constituted by small antioxidant molecules, such as vitamin E and C, and carotenoids. Vitamin E is a lipid soluble vitamin, which concentrates mainly in the interior of membranes and blood proteins. It is the major lipid soluble antioxidant in human blood plasma. Vitamin E reacts at considerable rates with a variety of free radical species, with emphasis on lipid peroxyl radicals formed during lipid peroxidation. During the course of this reaction, as in any other antioxidant mechanisms, a free radical form of vitamin E is formed generated (Figure 2).

**Figure 2** Direct Reactions of Vitamin E with Hydroxyl Radical.

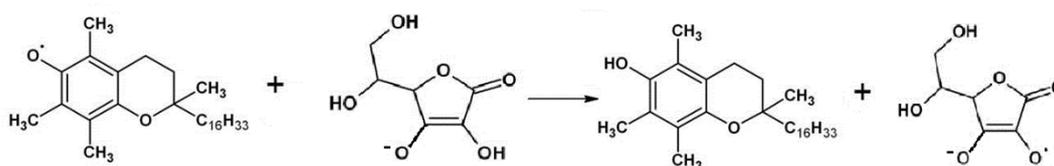
The new radical species, the vitamin E radical, has a chemical reactivity lower than the original free radicals. Hence, the transfer of the radical character proceeds toward creating a less oxidizing species. Vitamin C, on the other hand, is a water-soluble vitamin that reacts with several radical species producing semidehydroascorbic acid or

ascorbyl radical (Figure 3).



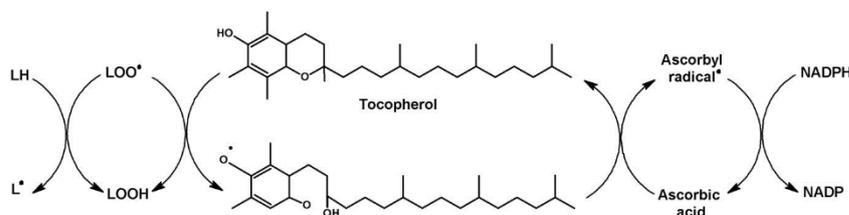
**Figure 3** Direct Reactions of Vitamin C with Peroxyl Radical.

Vitamin E and vitamin C act independently to destroy free radicals and also work together by reaction between the radical of vitamin E with vitamin C to produce vitamin E and ascorbyl radical (Figure 4). The interaction between vitamin E and C is the synergistic antioxidant action [25,26].



**Figure 4** Vitamin E Radical Reaction with Vitamin C.

Vitamin E and coenzyme Q have a long side chain, which are indispensable for incorporation in membranes and lipoproteins. At the same time, the side chain reduces the mobility within and between the membranes and lipoproteins, reducing the apparent antioxidant capacity [27]. The efficacy of scavenging radicals by antioxidants in the membranes and lipoprotein particles depends on the physical factors such as mobility of antioxidant and its chemical reactivity. Thus, the rate of scavenging peroxy radicals by  $\alpha$ -tocopherol in the membrane is smaller than that in homogeneous solution [28]. Cells contain two enzymes that can reduce the ascorbyl radical or semidehydroascorbate radical back to ascorbate: dehydroascorbate reductase and NADH-semidehydroascorbate reductase. Vitamin E is reacting mainly with peroxy radicals formed during lipid peroxidation. The reactivity of vitamin E with lipid peroxy radicals at the membrane yields the corresponding antioxidant-derived radical, vitamin E radical or tocopheroxyl radical. Vitamin C is a water-soluble vitamin and antioxidant that reacts with a variety of free radical species. The antioxidant-derived radical, ascorbyl radical, is recovered via dehydroascorbate reductase. Vitamin C is also present in the cytosol. The different compartmentalization of vitamins E and C provides a synergistic antioxidant mechanism by which the free radical character is transferred from the lipid phase (membrane) to the cytosol (Figure 5).



**Figure 5** *In vivo* Reaction of Vitamin E (tocopherol) and C (ascorbic acid) [25,26].

Enzyme systems protect the cell against oxygen radical attack. Specific preventive antioxidants remove superoxide anion or hydrogen peroxide, the two required precursors of hydroxyl radical (HO<sup>•</sup>). The product of the superoxide dismutase-catalyzed reaction above is hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>). Although the latter is less reactive than superoxide anion radical, it is still a strong oxidant and a precursor of hydroxyl radical (HO<sup>•</sup>) via a Fenton reaction. The cell possesses mechanisms by which hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) is readily reduced to water. The enzymes catalyzing this reaction are catalase and glutathione peroxidase. Catalase is located in peroxisomes and glutathione peroxidase occurs in cytosol and in the mitochondrial matrix. It requires glutathione, a tripeptide present in high concentrations in most mammalian cells.

## Clinical Use of Antioxidants

Clinical applications use only a few antioxidants including N-acetylcysteine (for acetaminophen toxicity), alpha-lipoic acid (for diabetic neuropathy) and some flavonoids (polyphenolic compounds present in dietary plants), such as micronized purified flavonoid fraction (diosmin and hesperidin) and oxerutins (for chronic venous insufficiency) as well as baicalein and catechins (for osteoarthritis) [29,30]. Increasing evidence suggests that gamma-tocopherol (the form of vitamin E) might be effective for preventing colorectal cancer [31]. Epidemiological evidence consistently relates low antioxidant intake or low blood levels of antioxidants with increased cancer risk [32].

Plasma concentrations of carotenoids (alpha- and beta-carotene, canthaxanthin, beta-cryptoxanthin, lutein, lycopene, zeaxanthin) and vitamins A (retinol), C and E and dietary consumption of beta-carotene and vitamins A, C, and E were determined in colon cancer. An association was observed between higher prediagnostic plasma retinol concentration and a lower risk of colon cancer. Additionally, inverse associations for dietary beta-carotene and dietary vitamins C and E with colon cancer were observed [33]. The plasma concentration of antioxidants are found for vitamin E (10 – 40 μM) [34], vitamin C (50 – 60 μM) [35], vitamin A (1 – 3 μM) [34], glutathione 4 μM [36], lipoic acid (0.1 – 0.7 μM) [37], uric acid (200 – 400 μM) [34] and coenzyme Q (5 μM) [38].

Skin cells are constantly exposed to ROS and oxidative stress from exogenous and endogenous sources. The reduction of oxidative stress can be achieved by increasing levels of antioxidant defense in order to scavenge ROS [39]. Antioxidant therapy can be in the use of antioxidant enzyme and proenzymes, biogenic elements, drugs, synthetic antioxidants, and drugs with antioxidant activity [12]. The enzymatic antioxidant systems, such as copper, zinc, manganese superoxide dismutase, glutathione peroxidase, glutathione reductase, and catalase may remove the ROS directly or sequentially, preventing their excessive accumulation and consequent adverse effects. Non-enzymatic antioxidant systems consist of scavenging molecules that are endogenously produced such as glutathione, ubiquinol, and uric acid or dietary such as vitamins C and E, carotenoids, lipoic acid and selenium [40]. To date, antioxidants are proven to be beneficial to protect healthy cells from anticancer therapy. In particular, amifostine is a radioprotectant drug that when dephosphorylated, protects normal but not malignant cells against oxygen-based radicals, alkylator or organoplatinum anticancer drugs. Physical exercise results in an up-regulation of antioxidant defense mechanisms in various tissues, presumably due to increased levels of oxidative stress that occurs during exercise [41,42]. Although easily available, antioxidants have a limitation. Possible explanation for the lack of benefit in clinical trials is that the trials have not lasted long enough. It may be impossible to show the benefits of antioxidant therapy over several years if the therapy is trying to reverse the results of several decades of oxidative stress. It is critical to remember that the lack of benefits seen in clinical trials to date does not disprove the central role of oxidative stress in atherosclerosis. Rather, these results challenge us to evaluate optimal antioxidant therapies [43]. Well-established antioxidants derived

from the diet are vitamins C, E, A, and carotenoids, which have been studied intensively. In general, exogenous antioxidants can compensate for the lower plasma antioxidant levels often observed in pre-diabetic individuals, whether their diabetes is primarily genetic in origin or due to obesity and a sedentary lifestyle [44]. Vitamin E is the major lipid soluble antioxidant protecting lipids against peroxidative damage. Studies indicate that oxidative cleavage of the phytyl side chain is a major metabolic pathway in humans, operative at saturated vitamin E plasma concentrations [45]. Vitamin C and vitamin E have well-described antioxidant properties. Vitamin C has been shown to scavenge superoxide, hydrogen, peroxide, hydroxyl radical, peroxy radicals efficiently. Ascorbic acid can also protect membranes against peroxidation by enhancing the activity of alpha-tocopherol, the chief lipid-soluble vitamin [46]. Although the value of vitamin C as a potential cancer treatment has been debated for decades, only one randomized clinical trial was found that evaluated vitamin C treatment concurrently with chemotherapy and reported on outcomes [46]. In 1994, over 100 studies have reported that reduction in cancer risk is associated with a diet high in vitamin C [47].

Coenzyme Q or ubiquinone may decrease oxidative stress not only by quenching reactive oxidant species but also by 'recoupling' mitochondrial oxidative phosphorylation, thereby reducing superoxide production. Alpha-lipoic acid, a critical co-factor for mitochondrial dehydrogenase reactions, is another compound with free radical-scavenging activity [48]. Lipoic acid was found to increase glucose transport in cultured muscle cells by stimulating translocation of GLUT4 from internal pools to the plasma membrane. In cultured adipocytes, treatment with lipoic acid protected the insulin receptor from oxidative damage, maintaining its functional integrity. A placebo-controlled explorative study of patients with T2DM indicated that oral administration of lipoic acid significantly increased insulin-mediated glucose uptake, presumably by modulating insulin sensitivity [49].

Carotenoids are natural compounds with lipophilic properties and  $\beta$ -carotene is the most prominent. Most carotenoids contain an extended system of conjugated double bonds, which is responsible for their antioxidant activity. The first large randomized trial on antioxidants and cancer risk was published in 1993. This trial investigated the effect of a combination of  $\beta$ -carotene, vitamin E, and selenium on cancer in healthy men and women at high risk for gastric cancer. The study showed a combination of  $\beta$ -carotene, vitamin E, and selenium significantly reduced incidence of both gastric cancer and cancer overall [50]. Another 1994 study, the Beta-Carotene and Retinol (vitamin A) Efficacy Trial (CARET), also demonstrated a possible increase in lung cancer associated with antioxidants [51]. The 1999 Women's Health Study (WHS) tested effects of vitamin E and beta-carotene in the prevention of cancer and cardiovascular disease among women age 45 years or older. Among apparently healthy women, there was no benefit or harm from beta-carotene supplementation. Investigation of the effect of vitamin E is ongoing [52-54]. A primary mechanism of many chemotherapy drugs against cancer cells is the formation of ROS, or free radicals. Drugs that form ROS include [55-57] melphalan, cyclophosphamide, anthracyclines (doxorubicin, epirubicin), podophyllin derivatives (etoposide), platinum coordination complexes (cisplatin, carboplatin), and camptothecins (topotecan, irinotecan).

## Conclusion

Oxygen interacts with cells *in vivo* and generates species such as hydroxyl radical, superoxide radical, hydrogen peroxide and singlet oxygen. All ROS are cytotoxic and have been implicated in the etiology of cancer. It is well documented that various stresses lead to the overproduction of ROS during oxidative stress process. Antioxidants act *in vivo* as a network that employs a series of redox reactions. A balanced combination of antioxidants may provide maximal efficacy *in vivo* due to their cooperative action, multiple interactions, location and reactivity. The

results presented in this review show antioxidants have potential for the lessening of toxic side effects caused by ROS and offer new strategies for further cancer research.

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