

Strategies of antioxidant drug discovery

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Abstract—Oxidative stress, a state of imbalance between oxidants and antioxidants in favor of the oxidants, is known to be implicated in a wide range of diseases including cancer and many inflammatory, cardiovascular and neurodegenerative diseases. The high reactivity of the oxidants (known as reactive oxygen species or ROS) allows them to attack biological structures; membrane lipids, nucleic acids, proteins and enzymes, resulting in irreversible cellular damage. To counter this harmful wave, chemical antioxidants have been widely used as food complements despite their side effects. Recently, more attention has been offered to natural antioxidants, found mostly in plants, because of their extraordinary diversity and above all their inoffensive nature. Normally, living organisms are equipped with an arsenal of enzymatic and non-enzymatic endogenous antioxidants. In oxidative stress condition, this natural wall of defense is overwhelmed by oxidants (coming from the outside, or produced by the cellular enzymes) causing the balance to succumb. That's the reason behind the importance of providing the organism with exogenous antioxidants. An antioxidant is defined as "a molecule capable of slowing or preventing the oxidation of other molecules". To do so, it can act as a free radical scavenger, a chelating agent (catching bivalent ions involved in primary ROS to much more toxic species), a reducing agent or an inhibitor of one the most important producers of ROS in living cells (xanthine oxidase and NADPH oxidase). This work offers an enlarged overview of the recent and most effective strategies used in discovering antioxidants in natural sources, from in vitro tests to clinical trials.

Keywords—Oxidative stress, antioxidants, scavenger, animal models, NADPH oxidase

I. INTRODUCTION

The "oxygen paradox" is defined by the fact that aerobic organisms require oxygen for survival but oxygen is also inherently toxic to these organisms due to its association with free radical generation and oxidative stress. Various free radicals are common products of respiration and other biochemical reactions in cells that are normal physiological processes essential for survival. To survive in an unfriendly oxygen environment, living organisms generate water- and lipid-soluble antioxidants that can neutralize these highly reactive free radicals [1]. For healthy living, a delicate balance must be maintained between oxidative stress and antioxidant defense of the body. If the body's antioxidant mechanism does

not operate optimally, excess free radicals can damage various biomolecules, including lipids, proteins, carbohydrates, and nucleic acids [2].

Dietary, natural or synthetic antioxidants are essential to maintain a healthy redox statue. The objective of this paper is to show the different steps followed in evaluating antioxidant properties of a given substance in order to be used in antioxidant therapy.

II. FREE RADICALS AND OXIDATIVE STRESS

A. Free Radicals and Their Sources

Free radicals are defined as molecules or molecular fragments containing one or more unpaired electrons in the outer orbit. This unpaired electron(s) is unstable and usually gives a significant degree of reactivity to the free radical. Reactive Oxygen species (ROS) includes superoxide ($O_2^{\bullet -}$), hydroxyl ($\bullet OH$), peroxy (ROO^{\bullet}), lipid peroxy (LOO^{\bullet}), alkoxyl (RO^{\bullet}) radicals. Reactive Nitrogen Species (RNS) includes nitric oxide (NO^{\bullet}) and nitrogen dioxide (NO_2^{\bullet}). Oxygen and nitrogen free radicals can be readily converted to other non-radical reactive species which are also dangerous for health. Hydrogen peroxide (H_2O_2), ozone (O_3), singlet oxygen (1O_2), hypochlorous acid ($HOCl$), nitrous acid (HNO_2), peroxyntirite ($ONOO^-$), dinitrogen trioxide (N_2O_3), lipid peroxide ($LOOH$) are not free radicals and generally named oxidants and can easily lead to free radical reactions in living organisms. Oxidants are also capable of nitrosylating proteins thereby disrupting biological function. Thus, ROS and RNS include radical and non-radical species. These reactive species are produced in animals and humans under physiologic and pathologic conditions [3-6].

ROS and RNS can be produced from both endogenous and exogenous substances. Production of these reactive species in the body is continuous and a normal part of our physiology. The biological process associated with free radical generations includes the following [6-9 cited 10]:

- *Immune system*: Immune system cells generate ROS in response to pathogens.

- *Metabolic process*: Free radicals can generate during metabolism of arachidonic acid, platelets, macrophages and smooth muscle cells. Lipid peroxidation an important source of free radicals and can be formed from several sources like mitochondrial cytochrome oxidase, xanthine oxidases, NADPH oxidase of neutrophils. Mitochondria generate continuously and abundantly oxy-radicals and ROS as toxic waste through a number of metabolic processes, each of which can produce different free radicals.

- *Inflammation*: Inflammation releases cytokines and initiates neutrophils and macrophages to produce free radicals.

- *Stress*: Mental and body's stress can trigger the production of free radicals as a toxic by-product. Additionally, the hormones that mediate the stress reaction in the body like cortisol and catecholamine themselves degenerate into destructive free radicals.

- *Pollution*: The different type of pollutants like air pollutants (asbestos, benzene, carbon monoxide, chlorine, formaldehyde, ozone, and toluene), chemical solvents (cleaning products, glue, paints, paint thinners, perfumes, and pesticides), and water pollutants (chloroform and other trihalomethanes) are all potent generator of free radicals. Burning of organic matter during cooking, forest fires, and volcanic activities also can generate free radicals.

- *Radiation*: UV radiations, medical and dental x-rays, gamma rays, and microwave radiation can lead to free radical generation.

- *Dietary factors*: Additives, alcohol, coffee, foods from animal origin, foods that have been barbecued, broiled fried, grilled, or otherwise cooked at high temperatures, foods that have been browned or burned, herbicides, hydrogenated vegetable oils, pesticides, sugar and processed foods containing high levels of lipid peroxides, and can produce free radicals.

- *Toxins and drugs*: Carbon tetrachloride, paraquat, benzo pyrene, aniline dyes, toluene and drugs like adriamycin, bleomycin, mitomycin C, nitrofurantoin, chlorpromazine etc. increases free radical productions.

- *Other factors*: Automobile exhausts fumes, smoking of tobacco products, cause free radicals generation.

B. Damage of Biomolecules by Free Radicals

Due to high reactivity, free radicals can damage virtually all biomolecules, including lipids, proteins, carbohydrates, and nucleic acids.

Lipids are major targets for oxidative damage induced by free radicals.

Lipid peroxidation can cause cell membrane damage by alteration of membrane fluidity and permeability. Lipid peroxidation mediated by free radicals is a series of chain reactions involving initiation, propagation, and termination.

Free radicals that can initiate lipid peroxidation include the hydroxyl radical (most reactive), alkoxyl radicals, peroxy radicals, and peroxyxynitrite. Metal ions such as cuprous and ferrous ions can also contribute catalytically in chain initiation. All polyunsaturated fatty acids can undergo lipid peroxidation, but the rate of reaction for lipid peroxidation is docosahexaenoic acid, eicosapentaenoic acid, arachidonic acid, linoleic acid [2]. Major end products of lipid peroxidation are aldehydes such as malondialdehyde, acrolein, and 4-hydroxy-2-nonenal. Acrolein and, to some extent, 4-hydroxy-2-nonenal, are highly reactive compounds that can damage proteins, DNA, and phospholipids. Secondary oxidation products are a series of prostaglandin-like products known as isoprostanes as well as monocyclic and serial cyclic peroxides. Isoprostanes, especially F2-isoprostane, are excellent markers of endogenous lipid peroxidation and oxidative stress [11].

Amino acids and proteins are susceptible to oxidative damage by hydroxyl radicals. Oxidation of proteins by reactive oxygen species and reactive nitrogen species involves side chains of all amino acid residues of proteins. In particular, sulfur-containing cysteine and methionine residues of proteins are very susceptible to oxidation by both reactive oxygen and reactive nitrogen species. Oxidation of cysteine residue leads to the formation of disulfides, and methionine is oxidized to methionine sulfoxide residues. However, disulfide reductase enzymes present in the human body can repair such damage, but these are the only oxidized form of protein that can be repaired. Peptide bond cleavage may occur due to reaction of reactive oxygen species with glutamyl, aspartyl, and prolyl side chain. As a result of protein oxidation, protein carbonyl derivatives are generated that can be used as a marker of the extent of free radical-induced protein damage [12].

Hydroxyl radicals can damage DNA and are implicated in mutagenesis, carcinogenesis, and aging. Reactive oxygen and reactive nitrogen species can lead to DNA oxidation including direct modification of nucleotide base, formation of apurinic/apyrimidinic sites, DNA single-strand breaks, and, less frequently, DNA double-strand breaks. Of all the nucleotides, guanine is the most susceptible to oxidative damage where the hydroxyl radical can interact with C4, C5, and C8 positions in the imidazole ring of guanine, and formation of 8-hydroxyguanine (also known as 8-oxo-7,8-dihydroguanine) can be utilized as a marker of DNA damage. Peroxynitrite can also react with guanine with formation of 8-nitroguanine, which is considered a marker of nitrosative DNA damage. Single-strand break is a result of interaction of hydroxyl radicals with deoxyribose and subsequent generation of peroxy radicals responsible for nicking phosphodiester bonds that form the backbone of each helical strand of DNA [13].

C. The organism's antioxidant defense

Continuous exposure to various types of oxidants from numerous sources has led the cell and the entire organism to develop defense mechanisms for protection against reactive metabolites.

Various antioxidants with different functions play their respective roles in the defense network in vivo. Some antioxidants are proteins and enzymes, while others are small molecules. The body's defense antioxidants consist of both endogenous and diet-derived exogenous antioxidants [2] (Table 1). From the viewpoint of mechanistic functions, the antioxidants may be classified into preventing antioxidants, scavenging antioxidants, and repair and de novo antioxidants (Fig. 1) [14].

The preventing antioxidants function as the first line defense by suppressing the formation of reactive oxygen and nitrogen species (ROS/RNS) by, for example, reducing hydrogen peroxide and lipid hydroperoxides to water and lipid hydroxides, respectively, or by sequestering metal ions such as iron and copper [15]. Antioxidants, which directly interact with ROS of various kinds, belong to two major groups: antioxidant enzymes and low-molecular-weight antioxidants. The enzyme-containing group is composed of direct-acting proteins, such as SOD (superoxide dismutase). The enzyme activity itself is capable of enhancing the spontaneous dismutation of superoxide radicals to H_2O_2 [16]. The end product of the dismutation reaction (H_2O_2) can be removed

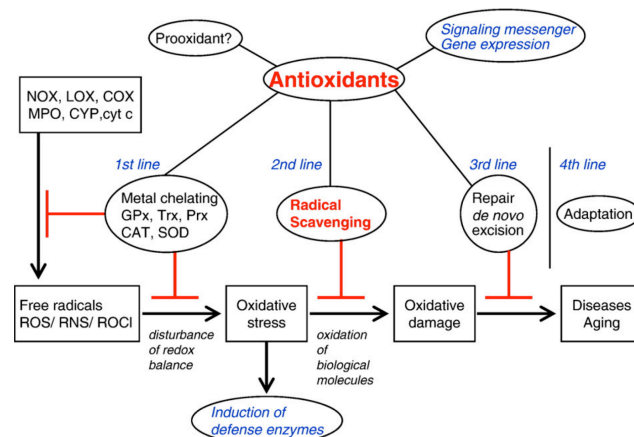


Fig. 1. Defense network in vivo against oxidative stress. Various antioxidants with different functions play their roles in the defense network, the free radical scavenging antioxidants being one of the players [15].

by the activity of the enzyme catalase and members of the peroxidase family including glutathione peroxidase [17].

Scavenging antioxidants act as the second line defense in vivo [15]. These molecules share a similar chemical trait that allows them to donate electrons to the oxygen radical so that they can scavenge the radical and prevent it from attacking the biological target. Scavengers possess many advantages over the group of enzymatic antioxidants. Because scavengers are small molecules, they can penetrate cellular membranes and be localized in close proximity to the biological target. The cell can regulate their concentrations, and they can be regenerated within the cell. They possess a wide spectrum of activities toward a large variety of ROS. The scavenging mechanism can proceed only if the concentration of the scavenger is sufficiently high to compete with the biological target on the deleterious species [18].

Scavengers originate from endogenous sources, such as biosynthetic processes and waste-product generation by the cell, and exogenously from diet. That the number of LMWA synthesized by the living cell or generated as waste products is so limited is surprising (eg, histidine dipeptides [19], glutathione [20], uric acid [21], lipoic acid [22], and bilirubin [23]); most LMWA are derived from dietary sources [24].

Various enzymes function in the third line defense by repairing damages, clearing the wastes, and reconstituting the lost function [15].

The DNA repair system, for example, can identify a DNA-oxidized adduct, remove it, and incorporate an undamaged base [25-26]. Molecules that can donate hydrogen atoms to damaged molecules are also considered repair compounds; one such example is the donation of a hydrogen atom by ascorbate or tocopherol to a fatty acid radical that was previously attacked by a radical and lost its hydrogen [24].

TABLE 1. Classification of antioxidants [10].

A. CLASSIFICATION BASED UPON THEIR NATURE	
1. Enzymatic antioxidant	Superoxide dismutase (SOD), Catalase (CAT), Glutathione peroxidase (GPx), and Glutathione reductase (GR).
2. Non-enzymatic antioxidant	
a) Metabolic antioxidants	Reduced glutathione (GSH), lipid acid, L-arginine, coenzyme Q ₁₀ , melatonin, uric acid, bilirubin, metal-chelating proteins, transferrin, etc
b) Nutrient antioxidants	Vitamin E, vitamin C, carotenoids, trace metals (selenium, manganese, zinc), flavonoids, omega-3 and omega-6 fatty acids, etc
B. CLASSIFICATION BASED UPON SOURCE	
1. Endogenous Antioxidants	Bilirubin, glutathione, lipoic acid, N-acetyl cysteine, NADPH and NADH, ubiquinone (coenzyme Q ₁₀), uric acid, enzymes (SOD, CAT, GPx, GR).
2. Dietary Antioxidants	Vitamin C, Vitamin E, Beta carotene and other carotenoids and oxycarotenoids (lycopene and lutein), polyphenols (flavonoids, flavones, flavonols, and proanthocyanidins
3. Metal Binding Proteins	Albumin (copper), ceruloplasmin (copper), metallothionein (copper), ferritin (iron), myoglobin (iron), transferrin (iron).
C. CLASSIFICATION BASED MECHANISM OF ACTION	
1. Catalytic systems to neutralise or divert ROS	SOD, CAT, GPx
2. Binding/inactivation of metal ions prevents production of ROS by Haber-Weiss reaction	Ferritin, caeruloplasmin, catechins
3. Self suicidal and chain breaking antioxidants scavenge, destroy ROS	Vitamin C, vitamin E, uric acid, glutathione, flavonoids
4. Quenching ROS, chemical traps/sinks to 'absorb' energy	Carotenoids, anthocyanidins

In addition, the adaptation mechanism functions as the fourth line defense, in which appropriate antioxidants are generated at the right time and transferred to the right position in right concentration. Furthermore, there is now increasing evidence showing that some antioxidants act as a cellular signaling messenger to regulate the level of antioxidant compounds and enzymes [15].

D. Oxidative Stress

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are the by-products resulting from the cellular redox process. These reactive species play a dual role in human as both toxic and beneficial compounds. The delicate balance between their two opposite effects is undoubtedly a key aspect of life [10]. At low or moderate levels, reactive species exert beneficial effects on cellular redox signalling and immune function, but at high concentrations, they produce oxidative stress, a harmful process that can damage cell function and structures [6,9].

The term oxidative stress refers to a condition where the levels of ROS significantly overwhelm the capacity of antioxidant defenses in a biological system. Oxidative stress condition can be caused by either increased ROS formation or decreased activity of antioxidants or both in a biological system. Oxidative stress condition is associated with oxidative damage to biomolecules, including proteins, lipids, and nucleic acids. Moderate oxidative stress may cause cell dysfunction, whereas overt oxidative stress usually causes cell death [27].

The disease conditions in which oxidative stress plays a role usually involve sustained formation of relatively large amounts of ROS via various mechanisms, including activation of inflammatory cells.

Oxidative stress and inflammation are intimately related processes. On the one hand, inflammation leads to the production of ROS/RNS from cellular sources, including NAD(P)H oxidase and inducible nitric oxide synthase. Such formed ROS/RNS mediate the detrimental effects associated with dysregulated inflammation. On the other hand, ROS/RNS cause increased expression of proinflammatory cytokines and adhesion molecules, thus augmenting or propagating the dysregulated inflammatory process. Such a dysregulated inflammatory state is sometimes referred to as inflammatory stress. As oxidative stress and inflammation are closely intertwined, the compound term oxidative/inflammatory stress has been used in the literature to describe settings where both oxidative stress and dysregulated inflammation contribute to disease pathophysiology [27].

E. Oxidative stress and human disease

It has been increasingly realized that because of the ubiquitous biological toxicity of ROS, the similarity of ROS-induced pathology to that of many spontaneous diseases, and the relative ease with which ROS are produced, much disease,

from malignancy to cardiovascular disease and dementia, is associated with ROS [28].

Increased formation of ROS/RNS and subsequent oxidative damage to biomolecules have been consistently observed under various disease conditions. However, these observations do not necessitate a causal relationship between oxidative stress and disease development. Possible relationships between oxidative stress and diseases, and the effects of controlling ROS/RNS on disease development are summarized below [27].

- ROS/RNS act as the only initial cause of the disease. Control of the ROS/RNS will prevent or stop the disease development.
- ROS/RNS act as one of the initial causes of the disease. Control of the ROS/RNS will mitigate the disease development.
- ROS/RNS act as a contributing factor in the disease progression. Control of the ROS/RNS will slow the disease progression.
- ROS/RNS are formed as a consequence of the disease and contribute to the progression of the disease pathophysiology. Control of the ROS/RNS will slow the disease progression.
- ROS/RNS are formed as a consequence of the disease and play no role in disease initiation or progression. Control of the ROS/RNS will have no effects on disease process. Table 2 lists some of the disease processes and related conditions in which oxidative stress plays a role.

III. ASSESSMENT OF ANTIOXIDANT CAPACITY

An antioxidant may be defined as a substance which, when present at low concentrations compared with those of an oxidizable substrate, such as fats, proteins, carbohydrates or DNA, significantly delays or prevents the oxidation of the substrate [29].

A compound might exert antioxidant actions *in vivo* or in food by inhibiting generation of ROS, or by directly scavenging free radicals. Additionally, *in vivo* an antioxidant might act by raising the levels of endogenous antioxidant defenses (e.g. by upregulating expression of the genes encoding SOD, catalase or glutathione peroxidase) [30]. In the stressful unhealthy 21st century's way of life, antioxidants have gained a considerable attention, and demand for intake of antioxidant food or dietary antioxidant is increasing with the hope of keeping the body healthy and free from diseases [31,32].

It is reasonable to expect the beneficial effects of antioxidants in maintaining our health and lowering disease risk. Thus the assessment of antioxidant capacity has been the subjects of extensive studies. There is no general, reliable method for assessment of antioxidant capacity even *in vitro*. The effects of antioxidants *in vivo* are far less understood. It is difficult to show unequivocally whether or not antioxidants are in fact effective for prevention and/or treatment of diseases and, if so, whether it is by the action of scavenging free

radicals. Solid data showing the correlation between antioxidant capacity and successful pharmacological properties has not been reported [15]. Fig.2 summarizes the overall strategy in evaluation antioxidant action, capacity and efficacy of antioxidants of different natures (natural or synthetic).

TABLE 2. Disease processes and related conditions in which reactive oxygen and nitrogen species play a causal or contributing role [27].

Disease	Description
Cardiovascular Diseases	Hypertension, Atherosclerosis Myocardial ischemia-reperfusion injury Heart failure, Cardiotoxicity
Diabetes and Metabolic Syndrome	Diabetes, Diabetic complications Obesity, Insulin resistance
Neurological Diseases	Alzheimer's disease, Parkinson's disease Stroke, Amyotrophic lateral sclerosis
Pulmonary Diseases	Chronic obstructive pulmonary disease Asthma, Hyperoxia-induced lung injury Pulmonary toxicity
Hepatic and Gastrointestinal Diseases	Alcoholic fatty liver disease Nonalcoholic fatty liver disease Hepatotoxicity, Inflammatory bowel disease Ischemia-reperfusion injury
Renal Diseases	Diabetic nephropathy Ischemia-reperfusion injury, Nephrotoxicity
Eye Diseases	Cataract Age-related macular degeneration
Skin Diseases	Ultraviolet light-induced skin injury Scleroderma, Contact dermatitis, Psoriasis
Cancer	Chemical carcinogenesis Spontaneous cancer development Angiogenesis, Cancer metastasis
Aging	Lifespan Aging-related organ degeneration
Arthritic Diseases	Rheumatoid arthritis Other types of arthritis
Sepsis	Septic shock Multiple organ dysfunction
Infections	Viral infections Bacterial infections

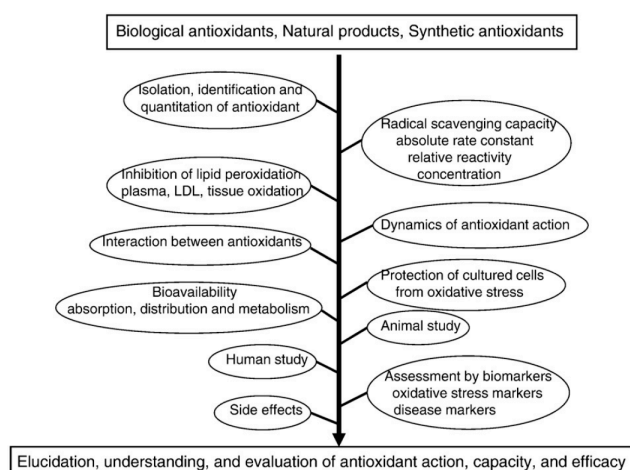


Fig. 2. Unit processes and factors for assessment of antioxidant capacity in vitro and in vivo [15].

A. Assessment of Free Radical Scavenging Capacity in vitro

Various assays have been described in which the putative antioxidant is added to a reaction mixture in which free radicals are generated. The mechanism involved and the type of assessment, antioxidant capacity assays can be divided in two main categories. The first category is an “assessment of antioxidant efficacy in relation to free radical species”. This category includes different reaction mechanisms models such as: (1) Hydrogen atoms transfer reactions model (HAT) based on the transfer of hydrogen atoms; (2) Single electron transfer reactions model (SET) based on the transfer of a single electron; (3) Hydrogen-electron transfer reactions model combining the two mechanisms HAT and SET [33].

The second category is an “assessment of antioxidant efficacy using biological significant markers and significant substrates” [34-36]. This category involves the determination of antioxidant efficacy via evaluation of the damaging effects on a biological substrate produced by reactive species of oxygen (ROS) or related nitrogen oxide species (RNOS) when reacting lipids, lipoproteins, DNA etc. [37].

According to the Halliwell's definition of an antioxidant [29], not all reductants involved in a chemical reaction are antioxidants; only those compounds which are capable of protecting the biological target meet these criteria. This protection may be based on several mechanisms of action, namely: (i) inhibition of generation and scavenging capacity against ROS/RNS; (ii) reducing capacity; (iii) metal chelating capacity; (iv) activity as antioxidative enzyme; (v) inhibition of oxidative enzymes [38]. Table 3 summarizes the most important in vitro tests for the assessment of antioxidant capacity:

TABLE 3. List of In-Vitro antioxidant methods [39]

Name of the method
Hydrogen Atom Transfer methods (HAT)
Oxygen radical absorbance capacity (ORAC) method
Lipid peroxidation inhibition capacity (LPIC) assay
Total radical trapping antioxidant parameter (TRAP)
Inhibited oxygen uptake (IOC)
Crocin bleaching Nitric oxide radical inhibition activity
Hydroxyl radical scavenging activity by p-NDA (p-butiridunethyl aniline)
Scavenging of H ₂ O ₂ radicals
ABTS radical scavenging method
Scavenging of super oxide radical formation by alkaline (SASA)
Electron Transfer methods (ET)
Trolox equivalent antioxidant capacity (TEAC) decolourization
Ferric reducing antioxidant power (FRAP)
DPPH free radical scavenging assay
Copper (II) reduction capacity
Total phenols by Folin-Ciocalteu
N,N-dimethyl-p-Phenylenediamine (DMPD) assay
Other Assays
Total oxidant scavenging capacity (TOSC)
Inhibition of Briggs – Rauscher oscillation reaction
Chemiluminescence
Electrochemiluminescence
Fluorometric Analysis
Enhanced chemiluminescence (ECL)
TLC bioautography
Cellular antioxidant activity (CAA) assay
Dye-substrate oxidation method

Measurements of lipid peroxidation should be the first line of tests to establish the potential antioxidant action of dietary antioxidant compounds. An antioxidant index based on the ability to scavenge peroxy radicals may then provide support for antioxidant efficacy in *in vitro* systems [40,41].

The deoxyribose assay allows the determination of rate constants of reactions with OH \cdot radicals, the assessment of abilities to exert prooxidant action, and the assessment of abilities to chelate metal iron. Assays involving DNA damage have also been developed for assessing pro-oxidant actions. These assays have unique features. The positive pro-oxidant actions in the deoxyribose system rely on the ability of the compounds to interact with metal ions (i.e. to promote reduction of Fe³⁺ to Fe²⁺ chelates) and hence, to promote OH \cdot formation in the presence of H₂O₂. The assays involving DNA rely on the ability to reduce the metal ions in the iron-bleomycin-DNA or the copper-1,10-phenanthroline-DNA complex [40].

During *in vitro* testing, it is essential to examine the action of a compound over a concentration range that is relevant to its intended use. For example, if the compound is present *in vivo* at low concentrations (less than 1 μ mol), its ability to inhibit lipid peroxidation only at high millimolar concentrations is irrelevant unless there is good reason to suspect that it concentrates at a particular site *in vivo*. The same is true if the compound exerts a pro-oxidant effect at high concentrations *in vitro* and is only present at low concentrations and exerts antioxidant action to different species [40].

There is considerable debate about which method is best and it is critical to understand that these tests are done in test tubes, not in people, since, there are different ways to measure antioxidant power, leaving research people seriously confused [39].

The commonly accepted methods for evaluating antioxidant capacity rely on the inhibition of radical chain reactions caused by a presumed antioxidant.

Most of the methods are based on the decrease of specific absorbance of a long-lived free radical in the presence of the antioxidant. The decrease of the 2,2'-diphenyl-1-picrylhydrazyl radical (DPPH) and of the radical cation derived from 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) are the stable radicals mainly used in 'in vitro' assays as they provide easily comparable results.

Despite the possibility of determining antioxidant capacity using important biological markers and substrates, the most frequently used methods are still based on the assessment of antioxidant efficacy as radical scavengers. This choice is due to the fact that methods using biological significant markers are affected by several drawbacks such as:

1. The lack of reproducibility when using the oxygen consumption method that is connected with the general lack of reproducibility of oxygen electrodes.

2. The lack of reproducibility when using lipoproteins which are obtained by extraction from tissues.

3. The complexity of the procedures used to follow the reaction(s).

4. The difficulties of automating these kinds of methods.

Finally, the ORAC, TEAC or DPPH assays are not strictly related to a compound's efficacy against ROS and consequently not strictly related to the antioxidant activity. These methods are more frequently used to assess the radical scavenging behaviour of food and raw materials [37].

In vitro assays can only rank antioxidant activity for their particular reaction system and their relevance *in vivo* health protective activities is uncertain. Therefore, it is prudent to use more than one type of antioxidant assay to measure antioxidant activities, and to include at least one assay that has biological relevance [39].

As the total antioxidant capacity is dependent of a multitude of factors, a "battery" of assays measuring different aspects of the behaviour of antioxidants is strongly recommended to generate a complete antioxidant profile. In this context, a primary factor to consider when selecting a method is the mechanism of reaction and its relationship to what might occur in the envisioned application. It is also advantageous to select methods that are commonly accepted, validated and standardized, with a large body of comparable data available in the literature [38].

B. Antioxidant Capacity in Cultured Cells

The advantage of using cultured cells is that various different stressors and cell types including model systems for some specific disease can be used for evaluation of the antioxidant effects. The effects of antioxidants have been measured against oxidative stress in cultured cells for the suppression of ROS formation, oxidation of lipids, proteins and DNA, and cell death. The antioxidants are added to the cell culture medium simultaneously with the stressor or preincubated beforehand to incorporate into the cells. It is very important to measure the intake of antioxidant into the cells for sound evaluation of the antioxidant effect, because the rate of intake into the cells varies markedly with the antioxidants [15].

The amount of antioxidants, especially lipophilic antioxidant, added into the culture medium should be chosen carefully so they would not differ much from the physiological conditions.

C. Antioxidant capacity in vivo

Studies in order to evaluate the pharmacology of antioxidants (drug derived or plant-derived antioxidants) should balance the use of *in vivo* biomarkers with the choice of population, formulation and dose of antioxidants being used, the expected outcome variables, and the pathologic variables. Fig. 3 suggests one such rationale. The global direction would be for food and drug antioxidants to be

evaluated for their inherent properties using *in vitro* models. Assessment of their protective effects in human health and disease should then consider how the steady state levels of markers of oxidative damage are affected by the antioxidants [30].

The capacity of antioxidants *in vivo* is determined by many factors which should be taken into consideration in its assessment (Fig. 2). One of such factors is the bioavailability. The antioxidants should be absorbed, transported, distributed, and retained properly in the biological fluids, cells and tissues. The bioavailability of various antioxidants and effect of dosage and duration have been studied by analysis of biological fluids and tissues of humans and experimental animals after intake of antioxidants. The time-course change in the concentrations of metabolites as well as the parent antioxidant has been analyzed. Metabolism also affects the antioxidant capacity *in vivo* [15].

The capacity and efficacy of antioxidants *in vivo* may be assessed most accurately by the effect of antioxidant compounds and materials on the level of oxidation in biological fluids and tissues, such as plasma, erythrocytes, urine, and cerebrospinal fluids, from humans and experimental animals. Saliva and tear may also be used. Reliable biomarkers are essential for this purpose and many biomarkers for oxidative stress have been applied to measure the level of oxidation *in vivo* [42-45]. Oxidation products of lipids [46-51], oxidative modification and expression of proteins and sugars [52,53], strand breaks of DNA and oxidation products of DNA bases [54] have been used as oxidative stress biomarkers. Measurement of oxidative damage in humans is summarized in Table 4.

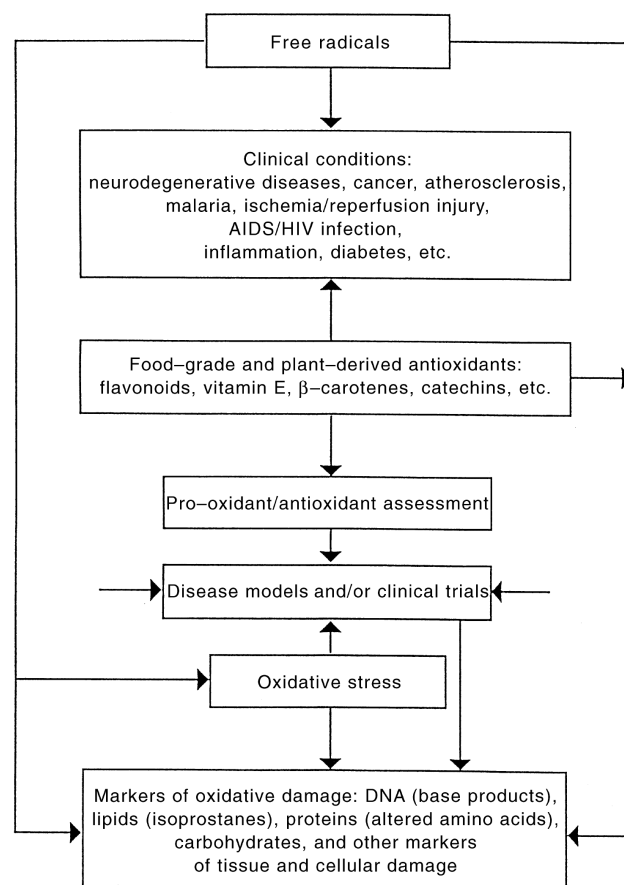


Fig. 3. Human antioxidant strategy [30].

TABLE 4. Measurement of oxidative damage in humans [30].

There are several indicators of the extent of oxidative damage in humans. Some of the most common include measuring:	
Oxidative DNA damage	
GC/MS/SIM detection of oxidized base products	
HPLC-based assays for oxidized base products	
Single gel electrophoresis assay (Comet assay)	
Oxidative damage to lipids	
Measurement of conjugated dienes	
Measurement of isoprostanes	
Measurement of hydroperoxides	
Measurement of thiobarbituric acid reactive materials by HPLC	
Assessment of the levels of antioxidant enzymes	
Catalase, superoxide dismutase and glutathione peroxidase	
Assessment of protein damage	
Steady state protein damage can be quantified in terms of the numbers of protein carbonyls and modified tyrosine residues.	
Total ongoing (repaired) protein damage can be indicated by the concentration of modified tyrosines and fluorescent bityrosines in the urine.	
Assessment of levels of low molecular weight antioxidants and vitamins	
Uric acid/allantoin, glutathione, flavonoids, vitamin E and C, β-carotene	

The effects of various antioxidants on the levels of oxidative stress biomarkers are evaluated in experimental animals under normal conditions and oxidative stress. The effects of antioxidants are further assessed in some clinical intervention cases and model animals for specific diseases. Some studies confirmed the positive effects of antioxidants and antioxidant rich diets to reduce the level of oxidative stress status *in vivo* as assessed by the biomarkers listed in Table 2, while others did not show any significant effects, in spite of the increase in the antioxidants. In contrast to the cultured cells which are often antioxidant-deficient and respond well to the added antioxidants, the beneficial effect of antioxidants is difficult to observe in normal healthy subjects with sufficient amounts of antioxidants [55,56]. Current evidence suggests that the antioxidant intake does not reduce oxidative stress biomarkers appreciably in healthy individuals with sufficient amounts of antioxidants under normal conditions, but that antioxidants lower such biomarkers in subjects in malnutrition, with increased oxidative stress or in patients of diseases related to oxidative stress [57].

Even in well-designed, randomized, cross-over, intervention studies, the data are contradictory and confusing, which may be ascribed to the complex effects of oxidative stress on pathogenesis, inadequate study design such as choice

of antioxidant, its dosage, starting time, duration, and methods of analysis [15]. The choice of subjects may also be critically important. It appears that the higher beneficial effects of antioxidants have been observed in animal studies than in human studies, which may be ascribed, at least in part, to the fact that animal studies are started often before the onset of disease, whereas those for humans after the onset of disease [15].

IV. CONCLUSION

Various biomarkers to determine the antioxidant capacity in a biological system have been developed and advanced. However it seems that there is not yet one system that predicts health outcomes, due to the various factors affecting the antioxidant capacity in a biological system such as interactions of antioxidants, genetic variance, and the origin of reactive oxygen species. Therefore, an important future direction of research would be to elucidate how best to improve our body defense systems against oxidative damage, which in turn might reduce the risk of chronic diseases, by means of dietary modification rather than by taking large amounts of antioxidant supplements [58].

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