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Commentary

Dioxygen reduction, reduced oxygen species, oxygen toxicity and antioxidants — A commentary

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Molecular oxygen, a diradical, needs intervention of redox metal ions or other radicals to receive electrons for its reduction. The oxygen radicals thus produced are responsible for oxygen toxicity and oxidative stress. But, autoxidation, relevant in ischemia-reperfusion injury, is absent in any discussion on oxygen toxicity. Naturally occurring compounds which prevent formation or action of the reactive oxygen species (ROS) are generally referred as antioxidants. The reduced oxygen species, superoxide, peroxide and hydroxyl radicals, are formed in a variety of systems in the cell and are useful in selective oxidations. Currently, the popular method for assaying ROS with fluorescence of dichlorofluorescein actually measures a hemeprotein-Fe-oxo complex. The Fe-oxyl radicals are the likely oxidants in damaging proteins, nucleic acids and lipids. Such major lesions are normally repaired or replaced in the cells.

The antioxidants counter the damaging oxidant actions. Among these, occurring in large concentration, are glutathione and ubiquinol, synthesized in the body and ascorbic acid and α-tocopherol, drawn from the food. A large number of plant-derived phenolic compounds, especially the flavonoid variety, are also absorbed, albeit poorly, from the food. At the natural low concentrations, these compounds show wide ranging biological effects. Increased benefit on increasing them in circulating blood needs individual verification. The polyphenolic compounds demonstrated powerful antioxidant effects in laboratory experiments. But the clinical studies did not support the consequent expectations of countering the oxidative stress, the purported crucial factor in pathology in several diseases. Antioxidant action against ROS causing oxygen toxicity needs to be reassessed.

This commentary is a reappraisal of formation and reactivity of ROS in different cells, the active cellular oxidant forms, products of oxidant action on proteins, nucleic acids and lipids as marker of oxidant injury, bulk antioxidants of endogenous and exogenous origin, limited absorption occurrence and functions of polyphenolic classes.

Keywords: Ascorbic acid, Autoxidation, Glutathione, Hydrogen peroxide, Oxyl radicals, α-Tocopherol, Ubiquinol

Introduction

Breathing large volumes of air non-stop from birth to death sustains life. Gaseous oxygen thus consumed in average human at rest is estimated at about 800 g per day. Consumption of oxygen in the Warburg apparatus with tissue slices, and in later years in the Gilson oxygraph with mitochondria, is a revealing spectacle. The O_2 molecule, the ultimate electron sink, is reduced to harmless water during respiration. The combustion occurs with oxygen dissolved in cellular fluids maintained at about 0.2 mM at body temperature, yielding usable energy forms is a vital process. Sudden exposure to oxygen, as in ischemia-reperfusion, causes tissue injury. Could such a benign molecule be toxic? Do cells need protection from it?

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A majority of oxygen consumed is reduced fully by 4-electron transfer through heme-Fe and Cu in the terminal oxidase. Small amounts of oxygen, are found in partially reduced oxygen species, superoxide, hydrogen peroxide and oxyl radicals. They have useful functions and also some injurious effects in different cells. Introduction of an oxygen atom in substrate hydroxylation is the first step in detoxifications of xenobiotics. Hydrogen peroxide is a valuable cellular oxidant, the substrate for many essential peroxidases, and is now recognized as a 'second messenger'. Oxyl radicals (hydroxyl or Fe-oxyl) can selectively damage protein-amino acids, nucleic acids-nucleotides and phospholipid-fatty acids. Such modifications always happen and mechanisms of repair and replacement are evolved in cells. They are taken to represent cell injury by reactive oxygen species (ROS), particularly hydroxyl radicals, and thus led to phobia and fear of free radicals. These actions form the basis of oxygen toxicity.

Many phenolic compounds of plant origin find their way through food into the body, albeit in traces[1-6]. These are highly potent in neutralizing the oxygen radicals and oxidant-dependent damages in laboratory tests. Some of them elicited limited, characteristic biological effects underscoring their benefits in food items. The name 'antioxidants' became instant hit and they are expected protect the humans subjected to the ravages of pathophysiology. Great disappointments followed in the clinical trials. Expectations far exceeded the true potential.

We need some fresh understanding of antioxidant defence, ROS and oxygen toxicity. The phenomenon of autoxidation in oxygen toxicity, somehow, did not receive due attention. This article attempts a reappraisal of formation and reactivity of reduced oxygen species in different cells and components, the active cellular oxidant forms, products of oxidant action on proteins, nucleic acids and lipids as marker of oxidant injury, bulk antioxidants of endogenous and exogenous origin, limited absorption occurrence and functions of polyphenolic classes. Available literature is vast and only few appropriate references are selected for the theme developed. The focus is on reduced oxygen species with opinions expressed, queries raised and interpretations offered based on my encounters and experiences from the work done in our laboratories.

Oxygen toxicity: How is inert oxygen toxic?

Strict anaerobes are intolerant to molecular oxygen and oxygen is toxic to these cells. Paradoxically, molecular oxygen is chemically inert. It is a poor oxidant. It requires high temperature to combust organic matter to oxidize carbon by O_2 to CO_2. Many organisms live with abundant atmospheric oxygen around. Anaerobes choose low oxygen habitats and aerobes develop defence against oxygen toxicity.

Damages mediated by oxygen radicals have been implicated in oxygen toxicity in radiation, ischemia-reperfusion and many pathological conditions. Hydroxyl radicals become notorious "electron-sucking, chain forming indiscriminate destroyers" during their fleeting existence[7]. Molecules which prevent formation of reduced oxygen species or 'neutralize' those formed, protect cells from oxidant damage. Thus, the antioxidant concept emerged.

Autoxidation sidelined in the evaluation of oxygen toxicity

Autoxidation or self-oxidation of some selected compounds such as catechols, quinonls and thiols occurs by direct consumption of oxygen, distinct from that dependent on metals. Browning is a well-known observation on the cut surfaces of an apple or a potato. This is due to exposure of the native phenol compounds undergoing autoxidation and then polymerization on exposure to oxygen. Is it a defence strategy to seal of the opened surface?

Autoxidation rates of quinols and catchols are high at alkaline pH. A lag in the rate of autoxidation usually observed at physiological pH is abolished on adding the quinone product. The quinhydrone intermediate formed instantly between quinol and quinone, facilitates the electron transfer[8]. Substitutions make big difference in rates – pyrogallol > gallic acid, dihydroxyphenylalanine > catechol, ubiquinol > *p*-benzoquinol. Autoxidation requires phenols with two or more hydroxyl groups in *ortho*- or *para*- position. Resorcinol with meta-dihydroxyl groups is poorly autoxidized but forms the base structure of flavonoids whose phenolic substitutions make them good antioxidants.

Oxygen reduction in autoxidation yields H_2O_2. Contrary to the expectation, this H_2O_2 generating reduction is inhibited by superoxide dismutase, also a H_2O_2 generator. Working with quinol autoxidation, the mechanism of superoxide dismutase action was explained as an oxidoreductase of the two products, semiquinone and superoxide[9]. This remained buried in literature unnoticed for nearly three decades. Inhibition of pyrogallol autoxidation by superoxide dismutase is indeed due to reverse dismutation of the two oxygen radical products[10]. Superoxide dismutase can dismutate any two radicals that can exchange electrons with it copper atoms, not just superoxide. Thus, it is truly an antioxidant enzyme as always believed. Some proteins have the intrinsic ability to perform more than one cellular function, as illustrated by superoxide dismutase protein[11].

Calcineurin is a serine/threonine protein phosphatase involved in many regulatory events, involving neurotransmitters, neural excitation, hormones, ion-channels, cell cycle and apoptosis. It occurs to the extent of 1% of the protein in neural tissue, a high oxygen consuming tissue. The enzyme is inactivated on exposure to air, and this is prevented by superoxide dismutase[12]. Calcineurin is protected from oxygen inactivation by superoxide dismutase interpreted as dismutation of the two radical species, $S^{\bullet}$-radical and superoxide, formed on autoxidation[13]. Oxygen is thus toxic to some proteins with facile thiols.

The reduced oxygen species

Molecular oxygen (dioxygen, O=O) is a diradical. It has an unpaired electron on each oxygen atom in separate orbitals with parallel spin in its outer shell. Addition of another electron is spin-forbidden and makes it mercifully inert. Each of its atoms can receive the second electron only from a metal center or another radical for activation of dioxygen. Ironically a reduction is crucial to produce oxidant species.

Hemin-catalyzed oxidation of dithiothreitol provides a chemical model for a direct 4-electron reduction of O_2 to H_2O, specific for dithiol; and for free heme oxygen radicals do not seem to be involved. H_2O_2 is not required for this oxidation of dithiothreitol and does not appear to be an intermediate in the reduction of O_2 to H_2O. Hemin-mediated oxidation of dithiothreitol reduces oxygen all the way to H_2O[14].

Sequential reduction of O_2 produces superoxide anion radical, peroxide anion and oxo-radical (Table 1). Formation of traces of these is ubiquitous in aerobic cells, albeit in, a tiny fraction of consumed oxygen. The protonated forms exist and act in acid pH. Their anionic forms at physiological pH are likely to associate with a cation/metal to gain enhanced activity. Together, these reduced oxygen species are part of the 'reactive oxygen species' popularly known as ROS.

Superoxide

Many membrane systems and oxidase enzymes (eg. xanthine oxidase) do produce superoxide. Superoxide, formed by trapping electrons from coenzyme Q-radical during electron transport in mitochondria, is probably its major source in the cell. Being a one-electron reduction product of dioxygen, it should be called "semiperoxide", analogous to semiquinone. Superoxide is a radical, but is "singularly unreactive"[15]. It undergoes both enzymic and non-enzymic dismutation producing H_2O_2. It donates its extra electron to redox-active metal ion. Reduction of Fe^{3+} in cytochrome *c* and in non-heme iron proteins is its well-known reaction. Such reduction in iron storage proteins releases free Fe^{2+}-iron which in the presence of H_2O_2 can generate the feared hydroxyl radical. In the case of ferritin, the common iron storage protein in tissues, this is limited to the few surface iron atoms and not the bulk core[16]

Table 1—Reduced oxygen species

Form	Dioxygen	Superoxide	Peroxide	Oxo-radical
Anionic	O=O	$^{-}$O-O$^{\bullet}$	$^{-}$O-O^{-}	$^{-}$O$^{\bullet}$
Protonated		HO-O$^{\bullet}$	HO-OH	HO$^{\bullet}$
Metal-		MO-O$^{\bullet}$	MO-OM	MO$^{\bullet}$

Hydrogen peroxide

Dihydrogen peroxide (H_2O_2) is commonly produced from superoxide by dismutation, chemical or enzymic. By definition, cellular oxidases and autoxidation of substrates do reduce O_2 and to H_2O_2 either directly or via superoxide. Biomembranes do generate most of cellular H_2O_2[17].

Mitochondria supply bulk of H_2O_2 in the animal cell. A small, ~1% or less of oxygen consumed in mitochondria of different tissues is insensitive to cyanide and antimycin. This activity, known as alternative oxidase or shunt pathway, generates H_2O_2. This is made possible due to passing of electrons leaked from NADH flavoprotein (complex I) and from the Q-cycle (complex III) to an alternative coenzyme Q pool. The resultant Q-radical reduces O_2 first to superoxide and then to H_2O_2. Characteristically, only this small consumption of oxygen, but not the bulk by cytochrome oxidase, is sensitive to a variety of antioxidant phenolic acids[18].

Plasma membranes have distinct redox systems that oxidize NADH, commonly known as NOX. One is intracellular NADH oxidase (possibly the previously known DT-diaphorase flavoprotein) linked to coenzyme Q for electron transfer to O_2 by an extra cellular quinol-oxidase (ectoNOX)[19]. The NOX system in plasma membrane is also possibly used in cellular aging/senescence and apoptosis of the cells on suitable activation by Rac proteins[20].

A transmembrane voltage-dependent anion channel (VDAC), porin, is lined with proteins with exposed sulfhydryl groups that carry electrons from NADH to reduce the natural acceptor, ascorbate radical. Ferricyanide can substitute as acceptor in laboratory experiments[21]

Another type of NAD(P)H oxidase occurs in plasma mambranes whose low activity is enhanced during respiratory burst in phagocytosis in killing invading bacteria. Suppressing this activity is necessary for successful parasitism[22]. It is known for a long time that a complex assembled with two membrane-bound proteins, $gp91^{phox}$ and $p22^{phox}$ (cytochrome b_{558}, a flavohemoprotein); and two cytosolic proteins, $p47^{phox}$ and $p67^{phox}$ becomes active on interaction with a prenylated Rac, a guanine nucleotide-binding protein[23]. In these systems,

electrons from NAD(P)H are transferred by protein complexes, with no definitive evidence for the involvement either a heme or flavin.

A model of burst oxygen consumption was found when microsomes oxidize NADH in the presence of decavanadate sensitive to SOD and catalase. The initial slow rate was enhanced by 40-50 fold with 1:1 ratio of NADH:O_2 indicating formation of H_2O_2. A novel phenomenon of burst of oxygen uptake during decavanadate-dependent oxidation of NADH[24]. This represents a specialized version of NADH oxidation activity present in endomembranes, including endoplasmic reticulum and plasma membranes. The "peroxovanadate cycle" was formulated by the work developed in our laboratory to explain the catalytic role of vanadium and H_2O_2 whereby the two electrons from NADH are transferred to otherwise spin-forbidden dioxygen. The membranes provide the enzyme for the initial reduction of the decamer-V^V. The subsequent non-enzymic steps include formation of peroxo- bridged mixed valency dimer that rapidly oxidizes NADH forming V radical species (V^{IV} and V^{III}) which by autoxidation reduces O_2 to peroxide held as diperoxovanadate[25]

Hydroxyl radicals

Hydroxyl radicals ($HO^{\bullet}$) are formed in cells by endogenous sources of H_2O_2 and Fe^{2+} by the hemolytic split H_2O_2, a Fenton-type reaction typically sensitive to catalase and metal chelators. "The pair Fe^{3+} + $^{\bullet}OH$ would not separate at all" and remain as the species $[Fe…OH]^{3+}$, or as ferryl $[Fe^{IV}=O]^{2+}$ or Fe-oxyl radical [Fe-$O^{\bullet}$] in such a reaction[26]. "The active form of the metal oxo is not always M=O as traditionally shown in textbooks, but the newly recognized oxyl radical form of M-O"[27]. Some metal-oxo species qualify as 'pseudo' and 'crypto' hydroxyl radicals as the standard $HO^{\bullet}$ radical scavengers do not intercept their formation.

Currently most popular assay of ROS – limitations and precautions

A fluorogenic dye, 2',7'-dichlorofluorescein (DCF) diacetate (DCFH-DA), is used widely to measure ROS species. It is deacetylated by cellular esterases to a non-fluorescent compound, which on non-enzymic oxidation yields the fluorescent product[28]. Quantitation of this product apparently was never considered worthy of attention. There is no clue on the control values even in terms of the measured arbitrary units and what they represent. The active oxygen species remains vague. Some reports claim H_2O_2, but alone H_2O_2, or its more active diperoxovanadate[20, 28], cannot oxidize DCFH. Customarily, the results on fluorescence are given as fold increase of ROS, and strangely this is acceptable to the workers and the referees/editors. Scores of papers continue to use this method and claim to have measured ROS notwithstanding the cautions highlighted below.

It is instructive to note that the H_2O_2-Fe^{2+}-derived oxidant, but not superoxide, H_2O_2, or free hydroxyl radical, is mainly responsible for the non-enzymatic oxidation of DCFH[28]. A team of specialists in the field presented a position paper with a critical analysis of the challenges and limitations of the most widely used fluorescent probes for detecting and measuring reactive oxygen and nitrogen species[29].

Worth noting in the present context are the following observations arising out of the experimental work and experience of active workers in the field. DCFH does not directly react with H_2O_2 and the DCF fluorescence cannot be used to measure H_2O_2. Fe^{2+} and other redox-active metals in the presence of H_2O_2 promote DCFH oxidation by an active Fe-oxo form, being insensitive to standard hydroxyl radical of scavengers. In the presence of H_2O_2 and hemoproteins, such as cytochrome *c* and peroxidases, do produce active Fe-oxo forms that produce DCF fluorescence[28,29]. Thus, the high DCF fluorescence yield indicates release of free Fe^{2+} or hemoproteins and specifically H_2O_2 more than ROS.

Control of H_2O_2 generation on one side, and the release of free iron from sequestered forms on the other, protect the cells from oxidant injury. Concept of ROS served its purpose to focus on the active oxidant forms. It is time to identify actions with the individual reduced oxygen species.

The active oxidant forms

Cellular metabolism is regulated by oxidative modifications by hydrogen peroxide, the most relevant cellular oxidant. This is recognized from the published literature as early as 1990. The findings supported rapid activation or inactivation of several enzymes[30]. The list is growing in the last three decades and now it is accepted that H_2O_2 can act as a redox signaling and control molecule. How does H_2O_2, occurring in cells in small, micromolar concentrations, act in presence of abundant cellular 'peroxicidal' enzymes such as catalase, glutathione

peroxidase and peroxiredoxin We found that the diperoxo-complex diperoxo-complex between H_2O_2 and vanadate is stable to catalase, yet efficiently substitutes in peroxide actions[31]. H_2O_2 is no longer considered a toxic metabolic byproduct but is a redox regulator, a common mediator in cell signaling and a promoter of aging and senecesence[32,33].

The hydroxyl radicals, $HO^{\bullet}$, in acid pH and the metal-oxyl radicals, $M\text{-}O^{\bullet}$ (M = Fe, V, Mo, Mn), in physiological pH are the reactive oxo species. Ferryl $[Fe^{IV}=O]^{2+}$ or Fe-oxyl radical $[Fe\text{-}O^{\bullet}]$ are good and selective oxidants of organic compounds that extract electrons from –CH groups yielding carbon radicals. In the presence of abundant oxygen their $COO^{\bullet}$ derivatives are immediately formed. On their reduction by glutathione, ascorbate and other reducing agents some useful hydroperoxy- or hydroxy products, especially of the unsaturated fatty acids, are obtained (see reference 7). Others which are unusable or toxic are modified and destroyed as in the case of amino acids and nucleotides.

The 'footfalls' of the oxidant radicals on proteins, nucleic acids and lipids

It is not easy to verify the formation of short lived, highly reactive radicals. Giving a spin trap of a radical elicits increased urinary excretion of its adduct[34]. It is known they are indeed formed in the body and are detected by indirect means. Products of oxidation of a variety of cell constituents considered due to oxidant injury under these conditions can now be measured. Examples of protein, nucleic acids and lipids are given below. These confirm the footfalls of the oxidant radicals visiting the cell.

Oxidations in protein amino acids

Typically, the oxidant action by oxo radicals (hydroxyl or ferryl) on the amino acid residues (lysine arginine, threonine and proline) yields aldehydes and ketones[35]. The protein-carbonyl products, typical indicators of oxidation, are biomarkers of damaged proteins. Usually, many copies of a protein exist and those oxidized and inactivated can be degraded and replaced by fresh synthesis. The cells that lose some essential proteins by such inactivation will face damage.

The starting point in oxidation of tyrosine by oxo-radicals is to generate a ring carbon radical which then attacks another tyrosine to form C-C linked dityrosine. In special cases this activity on membrane proteins leads to crosslinked proteins used as a strategy used in sealing the membrane after fertilization[20].

The amino acid residues of proteins are oxidatively modified. Oxidation of cysteine (-SH) to cystine (S-S), of methionine and of cysteine to their sulfoxides, usually by H_2O_2, is reversible by reducing compounds. These reversible interconversions are part of the redox regulatory process. *Sulfenic acids,* the single *cysteine-SH* oxidation product, are increasingly observed in regulating activities of proteins[36].

Oxidations in nucleotides in DNA and RNA

More than 20 damaged DNA base lesions were identified on oxidation[37]. Guanine is the most common target for oxidant damage in DNA indicated by increase in its only direct oxidation product, 8-hydroxyguanine of the seven most common damages found[38]. Oxidation of DNA by oxo-radicals is random including coding and non-coding forms. Electron holes in nucleotide bases are created and these radical cations migrate along the DNA strand and become localized on the guanine base, specifically at a GG-doublet[39]. This product increases several fold in a variety of diseases and conditions including ionizing radiation. This aberration is corrected in the normal course within minutes by inherent repair of excision and replacement of the oxidized bases[40]. Damages are always found in the native DNA and a steady-state balance of oxidized bases is maintained after repair. It is estimated that about 2400 per cell of 8-hydroxyguanine residues are retained, perhaps in harmless positions[41].

In the absence of a suitable guanine, radical cation occurs at adjacent thymine bases and forms a TT-dimer between them. This is somewhat like tyrosine crosslinking in proteins. In DNA repair of TT-dimer is achieved by excision-replacement or by reduction with electrons supplied by a flavin enzyme[42]. By implication electron transfer occurs over a range through the π–stack of DNA by a mechanism now known as "chemistry at a distance"[43].

Oxidation of RNA occurs on similar lines. In fact, RNA is more susceptible to attack. Bases in RNA are more accessible being single stranded, distributed in the cytosol and not compartmentalized, unlike DNA[44,]

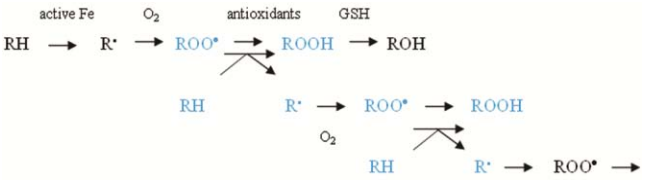
The major lesion on oxidation of nucleic acids is the 8-hydroxy derivative of guanine base. This is not part of hydrogen bond pairing with adenine base. Yet it is necessary to remove these modified bases.

Lipid peroxidation –is it a destructive chain reaction?

A common refrain in the action of metal-oxyl radical is the extraction of an electron to form the carbon-radical, its attack by O_2 to form $COO^{\bullet}$ followed by reductions, usually by glutathione, yields the hydroxy or oxo product. This is typically illustrated in lipid oxidation.

Unsaturated fatty acids with at least two double bonds, but not saturated fatty acids, undergo lipid peroxidation. The rates increase with increased unsaturation. Typically the process of lipid peroxidation oxidizes a polyunsaturated fatty acid (PUFA) molecule to its first stable product, hydroperoxy-PUFA, still bound in phospholipid form, on the way to other oxidation products and degradation. Lipid-peroxide (ROOH) being the product of oxygen consumption seems to have prompted the name lipid peroxidation[45].

The initiation reaction is the removal of H-atom (electron + proton) from a fatty acid (RH) carbon atom forming lipidC$^{\bullet}$-radical ($R^{\bullet}$) by an "active Fe" [Fe-O$^{\bullet}$, Fe=O, perferryl) and Fe-O-O-Fe, with Fe being the preferred metal in lipid peroxidation. This proposal also validates of metal-oxyl, rather than hydroxyl radical, and peroxo-bridged Fe-dimer, instead of hydrogen peroxide to carry out such functions at physiological pH. The electron-withdrawing agent is often wrongly shown as hydroxyl radical. Standard hydroxyl radical generating system (Fe^{2+} + H_2O_2) does not support lipid peroxidation. Compounds known to scavenge hydroxyl radical do not block lipid peroxidation[46]. Oxygen instantly reacts with the R$^{\bullet}$-radical yielding the lipidC-peroxo radical (ROO$^{\bullet}$). Loss of unsaturated fatty acid occurs on breakdown of these peroxidized products to conjugated dienes, hydroxynonenal, malondialdehyde and a set of isoprostanes, all considered markers of peroxidative loss of lipid.



Propagation of the chain is proposed with the fatty acid (RH) becoming the reducing source. Then R$^{\bullet}$-radical regenerates through interaction of ROO$^{\bullet}$, independent of "active Fe" until the fatty acid is exhausted. Once triggered an uncontrolled, extensive loss of PUFAoccurs. Consequent damage to cellular membranes formed the basis of proposals of pathophysiology of several diseases. But the Fe^{2+}-triggered reaction stops on exhaustion of the reducing agent NADPH in the microsomal enzymic system sparing the residual PUFA. Consumption of excess of O2 over that of NADPH occurs in lipid peroxidation in brain microsomes with the observed stoichiometry for NADPH: O2 of 1:4 exceeding the expected value of 1:2[47]. This implies lipid is the source of extra electrons and thereby supports occurrence of the chain reaction.

Hydroperoxy fatty acids are physiologically useful as lipid-oxidants and a substrate for peroxiredoxin enzyme[48]. Lipid peroxidation is not always wasteful or destructive. It forms the basis of thermogensis with its high rate of oxidation of NADPH accompanied by consumption of oxygen that releases energy as heat[49].

Deciphering antioxidants

"The word 'antioxidant' has become a household term – a popular media flash. Everyone seems to understand instantly" - a simplistic comment I made in the keynote talk at an international forum[50]. Look at the winding definition of an antioxidant: "Any substance that, when present at low concentrations compared to those of an oxidizable substrate, significantly delays or inhibits oxidation of that substrate"[51]. "Simply stated antioxidants protect cells from oxidant damage. But they are not simple reductants"[50].

"What is an antioxidant?" was the profound question raised in a minireview entitled "Free radical biology – terminology and critical thinking"[52]. It is 'not-so-easy' to comprehend: "A generic definition of an antioxidant is not experimentally constructive unless it is associated with the notion of the oxidant that has to be neutralized." Out of its complexity, one can pull out the essence— antioxidant neutralizes oxidant, as the name implies. The remarks that followed are more complex and incomprehensible: "strong antioxidant activity *in vitro* may have 'non-antioxidant' effects in cells" and "scientists will start to talk about the real molecular function of such compounds" rather than "simple paradigm of bad oxidants and good antioxidants". But the workers in the field, hopefully, will pause and reflect on their sobering suggestion: "Speculation that many (if not all) diseases are related to radical damage needs to be supported by more secure data. The hope that antioxidants can prevent or cure a number of pathological situations also requires reconsideration".

Endogenous antioxidants

Antioxidants control oxidants and radicals by preventing their formation or actions. This perception is used for defining antioxidants in this report.

Glutathione and coenzyme Q, synthesized in animal tissues, play a major role in cellular redox activities. They have significant antioxidant action. Ascorbate and α-tocopherol are obtained from the food like other vitamins. They are not endogenous.

Glutathione

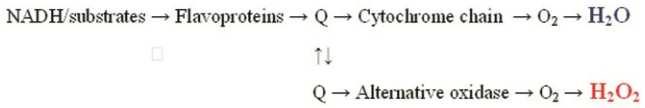
Glutathione (GSH) is the most visible with a high cellular concentration of about 5 mM. It is versatile reducing agent, along with ascorbate. It controls H_2O_2, lipid peroxides, redox metals and oxygen radicals, and thus the cellular oxidant actions. Its oxidation by peroxides yields glutathione disulfide. Increased oxidant activity is indicated by increase in glutathione disulfide was observed in extended hypoxia and sudden introduction of oxygen during the episode of ischemia-reperfusion injury indicating injury to myocardial damage. This tissue is protected on perfusion with superoxide dismutase and catalase that prevent formation of oxygen radicals[53]. A balance between the reduced and oxidized forms is maintained by its oxidation by H_2O_2-dependent glutathione peroxidase and reduction by NADPH-dependent glutathione reductase. This constitutes the glutathione 'redox buffer' that ensures protection from oxidant losses. Loss of glutathione in the form of disulfide from cardiac tissue also occurs after severe ischema during reperfusion oxygen insult[54]. Recovery needs *de novo* biosynthesis of glutathione.

Small thiol proteins with vicinal cysteine residues, such as thioredoxin, also contribute to dithiol-disulfide redox action similar to glutathione.

Coenzyme Q

Coenzyme Q, also known as ubiquinone, is ubiquitous in membranes in animal tissues. It occurs in a wide range of concentration, normally about 10-200 µg/g wet tissue. It is biosynthesized in all tissues. It is the only native redox active lipid antioxidant.

An account of the initial efforts leading to its discovery in beef heart mitochondria and an overview of the functions of coenzyme Q is available[55]. It fits the role of a lipid component interlinking flavoproteins and cytochromes and features in transition of two electron systems up to flavoproteins (Fp) into one-electron cytochrome chain. The semiquinone (one electron depleted quinone, HQ• radical) mediates in electron transfer through the Q-cycle[56]. The Q-radicals generated are the source of electrons to reduce O_2 first to superoxide and then to H_2O_2. Its redox action is central to formation of reduced oxygen species.



The groundwork for broader functions of coenzyme Q in membranes other than mitochondria was laid in our laboratory[57] when coenzyme Q was found in other cell membranes. Its antioxidant function in microsomal membranes was found soon after[58]. Coenzyme Q is enriched in the Golgi apparatus where protein-disulfide formation occurs in export proteins with semiquinone radical acting as the oxidant[59].

Uric acid

Nearly half of the antioxidant capacity of the blood is due to uric acid. It suppresses reactive oxygen species probably by consuming them altruistically in its own degradation. It may be a coincidence that this catabolic product of purine nucleotides happens to be passing through the blood and still be useful as antioxidant. In excess, uric acid has undesirable consequences of gout and kidney stones. Ironic to note that polyphenol-rich foods give increased antioxidant capacity not by the presence of flavonoids but by increased uric acid levels derived from metabolism of purine nucleotides[60].

Exogenous antioxidants

Many compounds with antioxidant activity enter the tissues on absorption from the diets. Two of these compounds, water-soluble ascorbic acid (vitamin C) and fat-soluble α-tocopherol (vitamin E) occur ubiquitously in large concentrations in cells. Both of them are known for multiple actions besides being good antioxidants.

Ascorbic acid (vitamin C)

Ascorbic acid, called vitamin C, is essential in many cellular activities. Diet is the source since it is not synthesized in humans. Ascorbate enters the cells along with sodium by the co-transporter. Its oxidized form, dehydroascorbate, on the other hand, uses the glucose transporter (GLUT1) and is converted back to ascorbate at the expense of reduced glutathione or thioredoxin, and ultimately NADPH. Glutathione buffering may thus be compromised by a high dose of dehydroascorbate[61, 62].

Vitamin C acts as an antioxidant because it can easily donate its electrons. It prevents other compounds from being oxidized. Ascorbate becomes the relatively stable, free radical semidehydroascorbic acid (ascorbyl radical) on losing an electron to a variety of cellular oxidants. Only, partial reduction back to ascorbic acid accounts for turnover necessitating the dietary supply. Several lines of evidence suggest that vitamin C is a powerful antioxidant in biological systems *in vitro.* However, its beneficial role in human diseases has not been supported by currently available clinical studies[61].

Orally administered ascorbic acid (400 mg/day) passes to the tissues through plasma with concentration reaching a plateau level of 80 μM. It rarely goes up to 200 μM after daily dose of 100-200 mg of ascorbate circulating blood cells build up relatively high concentrations of 1-4 mM[61]. A level of 10 mM ascorbate in plasma, reached after intravenous administration, induces death of metastatic tumor cells while passing through blood. They cannot survive in the highly reducing environment thus created[62]

α-Tocopherol (vitamin E)

The lipid soluble α-tocopherol, the preferred form among its isomers present in foods, is absorbed, distributed in all tissues and located in the endomembranes. Its major function is to scavenge peroxy radicals in membrane lipids and to maintain the integrity of long-chain polyunsaturated fatty acids and "the membrane qualities, fluidity, phase separation and lipid domains"[63]. It is regenerated on reduction by ascorbate of the tocopheryl-oxy radical. Yet α-tocopherol and its turnover occur. For antioxidant function naturally occurring ubiquinol is perhaps better than α-tocopherol[64]. But α-tocopherol has the advantage that it can function without local redox activity that is required for ubiquinone. Both α-tocopherol and ubiquinone, given orally, are poorly absorbed. In contrast to α-tocopherol's general distribution, ubiquinone goes only to the liver[65].

Polyphenolics

A large number and a broad spectrum of phytochemicals compounds having nutraceutical activities are present in plants[1-6]. The variants and quantities are characteristic of each plant. Of these, polyphenolics are the major group. Plants have evolved these secondary metabolites to withstand physical, chemical and biological stresses they are exposed in the environment.

Basically, they constitute a variety of phenolic compounds (PC). They possess oxygen radical absorbance capacity (ORAC), antioxidant activity (AOA) that includes DPPH radical scavenging activity (DPPH: 2,20-diphenyl-1-picryl hydrazyl) and ferric reducing antioxidant power (FRAP). They also bind metals and inhibit lipid peroxidation, the two activities that can be readily demonstrated experimentally with any plant extract. Food items are evaluated for antioxidant activity[66]. Many such investigations found good correlation between phenol content and antioxidant activity. This laid the foundation for the belief that plant-derived polyphenolics, with the wide-ranging biochemical effects, can be manipulated to protect humans from oxidant damages.

Phenols

A large number of phenolic compounds occur in different plants[67-70]. They have multiple hydroxyl groups with substitutions. Some have more than one ring with different number of hydroxyl groups (eg. resveretrol (Fig. 1).

Flavonoids

Flavonoids are a group of compounds with two rings, one resorcinol and the other heterocyclic with an oxygen atom. Together they form flavonoid structure consisting of chroman (saturated) and chromene (unsaturated) with many substitutions and occur as glycosides. The two benzene rings are at right angles in the saturated flavonoids (flavanones, flavans). The molecules are planar with unsaturation (anthocyanins, flavones and flavonols)[71]. The activity of interaction with oxidants and radicals depends on the substitutions of phenol, catechol or pyrogallol groups the heterocyclic ring (Fig. 1).

All these generally referred as phenolics have the basic properties of metal binding, iron releasing from iron stores (ferritin core iron), radical quenching, and anti-inflammation. They are potent inhibitors of lipid peroxidation often used as a marker of cellular oxidant activity.

Biological activities of the source plants containing these compounds and health benefits from many diseases are recorded from experience over long periods. Typical examples are turmeric, neem, aloe vera, ginkgo biloba, ginger and pepper. Some examples where active phenolic component is identified: eugenol in cloves relieves tooth pain, quercetin in fruits and vegetables is a weak

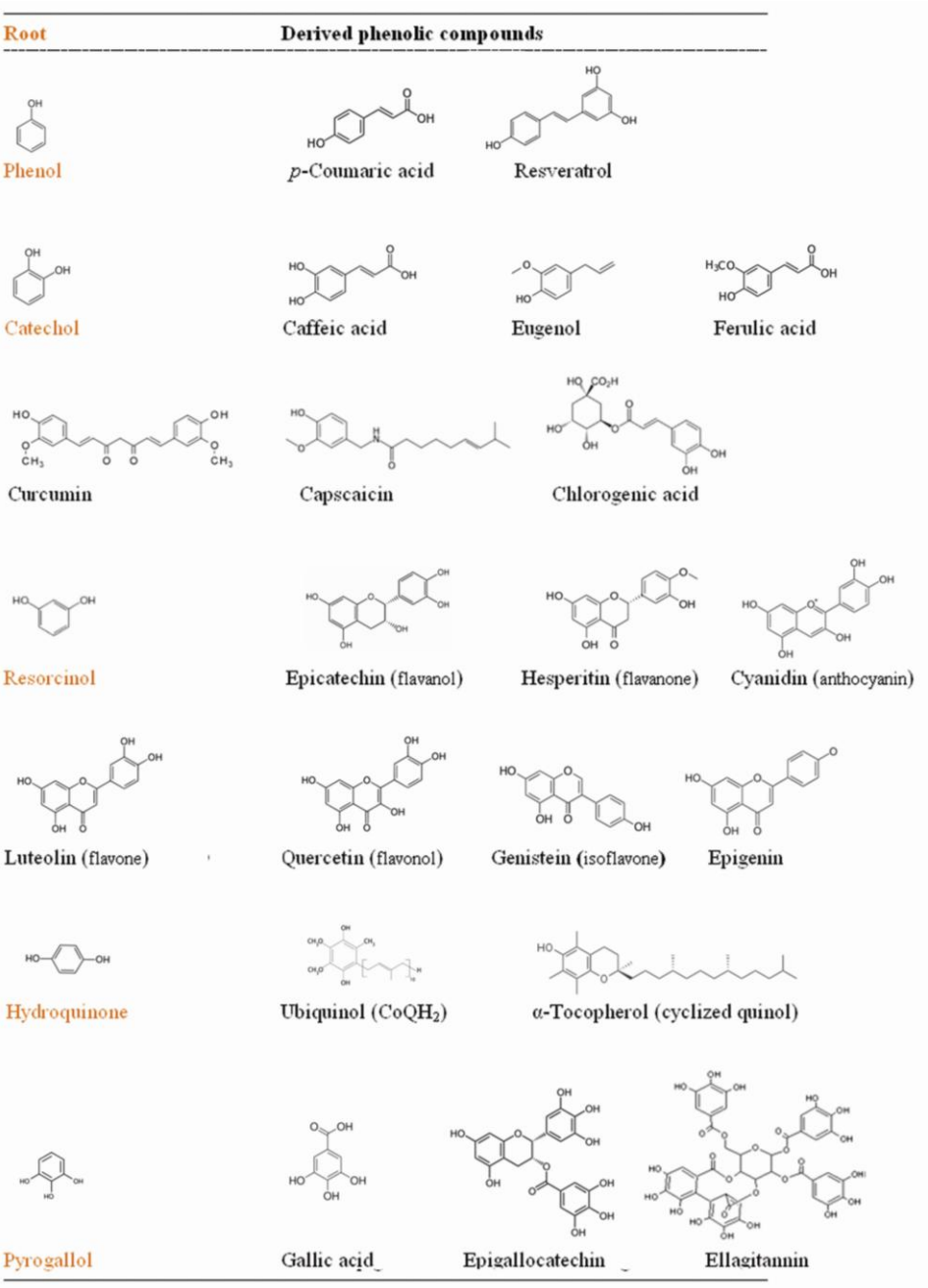


Fig. 1— Structures of some naturally occurring phenolic compounds. [These are derived from the root with one, two and three hydroxyl groups: mono (phenol), di (catechol and resorcinol), quinol (hydroquinone) and tri (pyrogallol). A large number of these are present in plants with varying substitutions. Resorcinol-derived flavonoids is a large group with a variety of accredited biomedical benefits]

phytoestrogen and an agonist of the G protein-coupled estrogen receptor and genistein in soyabean is a selective, specific inhibitor of protein tyrosine kinase.

Experiments to validate the benefits of their consumption in humans encountered some problems. Tea extracts containing flavanoid catechins, for example, are powerful inhibitors of lipid peroxidation, measured as malondialdehyde or the isoprostane products, in the laboratory experiments. On regular ingestion of stimulant tea in humans isoprostanes continue to be formed and excreted in urine with no sign of decrease[72].

The active compounds not reaching the tissues and the cells may be one of the causes. The animals have evolved mechanisms to absorb and retain what they need. If more of an exogenous compound is needed

the cells would have evolved mechanism to take in more. Only the vitamins, ascorbic acid and tocopherol, occur in large quantities occur in human tissues. In general the phenolic compounds are found in tissues in traces and for short periods. Lest we forget these phytochemicals are intended for the use in plants that make them.

Absorption of phenolics

It is axiomatic that plant-specific chemicals are not absorbed in animals. Thus phytosterols and plastoquinone are not found in animal tissues. Limited amounts of a few carotenes selectively enter and even the natural homologue of coenzyme Q (Q_{10}) is poorly absorbed exclusively into the liver (<5%) and not the lower homologues. Thus, it is not surprising to see the secondary metabolites, synthesized for local use, in plants are not favored for absorption. Low, variable amounts of some are found in animal tissues and their excretion products are rapidly eliminated in urine within hours. This is by way of proof that these are absorbed and goes through the body. The polyphenols are absorbed in the following order: gallic acid, isoflavones catechins, flavanones, quercetin glucosides, proanthocyanidins, tea catechin-gallates, and anthocyanins[73]. The small amounts may suffice to meet the demands for the selected functions they perform. Boosting blood levels of an antioxidant, thereby the 'bioavailability', derives from the perception that higher the concentration, higher the effect, with hardly any evidence on how much is effective.

The example of curcumin underscores this view. The medicinal properties of turmeric, a standard spice used in cooking, are recorded in 'alternative' medical systems for treating wounds and burns externally, and a number of conditions and diseases given internally. Curcumin is the main bioactive ingredient, a phenolic compound, diferuloylmethane. Circulating level in blood, monitored after oral injestion increased 2-fold in humans and 20-fold in rats on supplementing piperine, a component of black pepper, also a common culinary spice. Piperine is known to inhibit glucuronidation, a preparative step in the liver, necessary for excretion of exogenous compounds in urine. Preventing glucuronidation of curcumin will increase its stay in the body longer[74,75].

This backing up of a compound in the blood is taken as increased bioavailability subsuming that it improves in its bioefficacy. This could also be due to defect in the use or excretion, as in the case of glucose in hyperglycemia.

True functions of oxidants and antioxidants

The hypothesis that oxidative damage of tissues is inflicted by ROS, and by quenching them antioxidants give protection has now become a dogma. Each part is happening. Damages by oxidations are seen in tissues. Increased ROS are produced. Antioxidants can quench them. But the expected protection by antioxidants in diseases has little support in many clinical studies. The oxidation of cellular components, proteins, nucleic acids and lipids, and the associated inflammation in cardiac injury on introducing oxygen under ischemia-reperfusion can hardly be reversed by antioxidants[76,77].

'Oxidative stress' is a favorite expression in most articles in this field and is accepted, nay welcomed, by authors and editors as the basis of diseases with no questions asked. This article used it sparingly. New questions seem to arise. Does the oxidant activity represent stress? Are the antioxidant and oxidant activities in the cells counter each other to maintain an undefined balance? Or are they functionally independent and occur to meet some yet unknown requirement? Don't the cells know how to take care of the small number of damaged molecules by repair or exclusion? Mistakes in protein synthesis and damages nucleic acids, and equally corrections thereof, are part of normal cellular activity.

The concept of oxidative stress is powerful, lively and satisfying. It is inclusive of oxidants and radicals for oxidation of cell constituents, and glutathione redox buffer and antioxidants for restoring them. The underlying reactions can be demonstrated with isolated systems, but not translated as benefits of tissue recovery in the clinical trials. Tissue injury may be unrelated to unspecific oxidative modifications occurring naturally. Oxygen radical quenching actions of antioxidants may serve other roles. This calls for change in the hypothesis and seeking new perceptions.

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