REVIEW ARTICLE

LIPID PEROXIDATION AND TOTAL ANTIOXIDANT CAPACITY IN HEALTH AND DISEASE - PATHOPHYSIOLOGY AND MARKERS: AN OVERVIEW

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ABSTRACT

Free radicals play a significant role in the pathogenesis of many chronic diseases such as diabetes mellitus, cancer, chronic renal failure etc. Oxidative stress is defined as "a disturbance in the balance between the antioxidants and pro-oxidants (OFR in particular) with increased levels of pro-oxidants leading to potential damage. The knowledge about pathophysiology of lipid peroxidation and the oxidative stress biomarkers will definitely help the researchers to plan for focused study for better management of oxidative stress induced diseases.

KEY-WORDS: Free Radicals; Reactive Oxygen Species (ROS); Reactive Nitrogen Species (RNS); Lipid Peroxidation; Total Antioxidant Capacity; Oxidative Stress

Introduction

Free radicals are defined as the chemical species capable of independent existence that contains one or more unpaired electrons in its outermost orbital as a result, are generally more reactive than non-radicals due to their unpaired electron. A superscripted dot ($^{\cdot}$) is used to denote free radicals. Free radical can be formed in three ways: *(1) By Homolytic Fission:* Radicals can easily be formed when a covalent bond is broken if one electron from each of the pair shared remains with each atom. It requires high energy input from high temperature, ultraviolet light or ionizing radiation. *(2) By Heterolytic Fission:* One atom receives both electrons when a covalent bond breaks. The extra electron gives X a negative charge and Y is left with positive charge. *(3) By Electron Transfer:* Oxidation – loss of electrons by an atom or molecule. Reduction – Gain of electrons by an atom or molecule. Electron transfer is a far more common process in biological systems.[1,2]

Types of Reactive Species

Highly reactive molecules like, Reactive Oxygen Species (ROS) & Reactive Nitrogen Species (RNS).

Reactive Oxygen Species (ROS)

Oxygen molecule (O_2) qualifies as a free radical because it contains two unpaired electrons but is not particularly reactive due to a special electron arrangement that makes the reactions with oxygen spin restricted. However, when oxygen is partly reduced, several different reactive oxygen species (ROS) both radicals and non-radicals are produced. Thus, ROS include a number of chemically reactive molecules derived from oxygen. ROS can arise from: (1) The mitochondrial electron transport chain as part of oxidative phosphorylation; (2) The metabolism of arachidonate by cyclooxygenase or lipoxygenase enzymes to prostaglandins or leukotrienes; (3) Cytochrome p 450 enzymes; (4) Oxidase enzymes like NADPH oxidase and (5) The nitric oxide synthetases. Examples of ROS are hydroxyl radicals ($OH^{\cdot}$), superoxide anion radicals ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl) and singlet oxygen ($^1O_2$).[3]

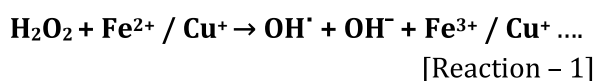
The major forms of ROS are:

1. *Superoxide Radical ($O_2^{\cdot-}$):* The superoxide anion created from molecular oxygen by the addition of an electron is, in spite of being a

free radical, not highly reactive. It lacks the ability to penetrate lipid membranes and is therefore enclosed in the compartment where it was produced. The formation of $O_2^{\cdot-}$ takes place simultaneously, especially in the electron rich aerobic environment in vicinity of the inner mitochondrial membrane with the respiratory chain. Superoxide (as well as H_2O_2) is also produced endogenously by flavo-enzymes. Eg. Xanthine oxidase. $O_2^{\cdot-}$ can act as Bronstead base which in aqueous media shifts the acid base equilibrium resulting in formation of hydroperoxyl radicals ($^{\cdot}HO_2$). In acidic medium $O_2^{\cdot-}$ readily generates H_2O_2. Other superoxide producing enzymes are lipo-oxygenase, cyclo-oxygenase, NADPH-dependent oxidase of phagocytic cells.[4]

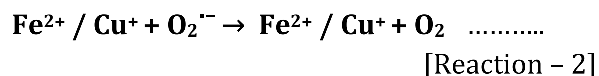
2. *Hydrogen Peroxide (H_2O_2):* H_2O_2 is not a free radical but it is nonetheless highly important much because of its ability to penetrate biological membranes. It plays a radical forming role as an intermediate in the production of more reactive ROS molecules including hypochlorous acid (HOCl**)** by the action of myeloperoxidase, an enzyme present in the phagosomes of neutrophils and most importantly, formation of $OH^{\cdot}$ via oxidation of transition metals.[5] H_2O_2 also plays role as an intracellular signalling molecule. H_2O_2 once produced by the above mechanisms is removed by at least three antioxidant enzyme systems namely- catalases, glutathione peroxidases and peroxi-redoxins.

3. *Hydroxyl Radical ($OH^{\cdot}$):* Due to its strong reactivity with biomolecules, $OH^{\cdot}$ Is probably capable of doing more damage to the biological systems than any other ROS**.** The radical is formed from H_2O_2 in a reaction catalyzed by metal ions (Fe^{2+} or Cu^{+}), often bound in complex with different proteins or other molecules. This is known as *Fenton reaction.*



$$H_2O_2 + Fe^{2+} / Cu^{+} \rightarrow OH^{\cdot} + OH^{-} + Fe^{3+} / Cu^{+} \ldots\text{[Reaction – 1]}$$

Superoxide also plays an important role in connection with reaction-1& 2 by recycling the metal ions:



$$Fe^{2+} / Cu^{+} + O_2^{\cdot-} \rightarrow Fe^{2+} / Cu^{+} + O_2 \ldots\ldots\text{[Reaction – 2]}$$

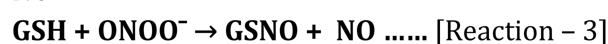
The sum of reactions 1 and 2 is the *Haber-weiss reaction;* Transition metals thus play an important role in the formation of hydroxyl radicals.[1,5]

4. *Singlet Oxygen ($^1O_2$):* $^1O_2$ is the lowest excited state of oxygen and has no unpaired electron in its outermost orbital to strictly qualify as a free radical but is highly reactive towards organic compounds and is included among the ROS. $^1O_2$ arises from H_2O_2 and can react chemically with other molecules or can transfer its excitation energy, returning to the ground state while the other molecule enters the excited state. This phenomenon is called " quenching " of $^1O_2$.[1,2,6] In large amounts $O_2^{\cdot-}$ can quench $^1O_2$ by electron transfer.

5. *Alkoxyl ($RO^{\cdot}$) and Peroxyl Radical ($ROO^{\cdot}$):* Decomposition of organic peroxides (ROOH) can generate $RO^{\cdot}$ and $ROO^{\cdot}$ and both of them are good oxidizing agents. These reactions account for much of the stimulation of lipid peroxidation by transition metal ions in the biological systems. Peroxy radicals seem to be less reactive than alkoxy radicals. In both cases, the formation of radical products will stimulate the reaction of lipid peroxidation by causing more initiation.[1,2]

Reactive Nitrogen Species (RNS)

Reactive nitrogen species are nitric oxide (NO), nitrogen dioxide (NO^2), nitrous acid (HNO^2) and peroxy-nitrite ($ONOO^-$). Nitric oxide and oxidation products of NO like N_2O_3, NO_2^- and NO_3^- can interact with ROS. NO reacts with $O_2^{\cdot-}$ to form peroxy-nitrite. An equal flux of $O_2^{\cdot-}$ and NO can stimulate lipid peroxidation via peroxy-nitrite while a high ratio of NO to $O_2^{\cdot-}$ can decrease lipid peroxidation because of the ability of NO to scavenge peroxyl and alkoxyl radicals. NO/ $O_2^{\cdot-}$ ratio is high in normal vascular endothelium and lowering of NO/ $O_2^{\cdot-}$ ratio favours pro-oxidant effect rather than antioxidant effect of NO. $ONOO^-$ can react with thiols like glutathione to regenerate NO.[7,8]



$$GSH + ONOO^- \rightarrow GSNO + NO \ldots\ldots\text{[Reaction – 3]}$$

Nitric Oxide (NO) is a simple heterodiatomic molecule with broad and diverse effects and was known as endothelium derived relaxing factor (EDRF). NO is synthesized enzymatically from L-arginine by NO synthase (NOS).

L-arginine + O_2 + NADPH → L- citrulline + NO + $NADP^+$ …… [Reaction – 4]

NO synthase is a very complex enzyme and requires five redox cofactors: NADPH, FAD, FMN, heme and BH_4.

There are three isoforms of NOS: (i) neuronal NOS (nNOS); (ii) inducible NOS (iNOS)- present in monocytes, macrophages, smooth muscle cells etc.; (iii) endothelial NOS (eNOS).

NO represents an odd member of the free radical family and is similar to $O_2^{\cdot-}$ in several aspects in that it does not readily react with most biomolecules despite its unpaired electron. On the other hand it easily reacts with other free radicals (eg. Peroxyl and alkyl radicals), generating mainly less reactive molecules, thus it functions as a free radical scavenger. Peroxy-nitrite (ROONO) directly or via its reaction products, may oxidize LDL and generally attack tyrosine residues in different proteins. NO has been shown to inhibit lipid peroxidation in cell membranes. It interacts with thiols like glutathione and thiol proteins to form nitrosothiol derivatives (GSNO) by reversible processes and therefore NO can be transported in a stable form. NO binds with certain transition metal ions like iron and copper. It binds with ferrous ion in heme of the enzyme guanylate cyclase and release copper ions by destroying ceruloplasmin.[9]

Transition Metal Ions and Free Radical Reactions

Many transition metals have variable oxidation numbers eg. Iron has Fe^{2+} and Fe^{3+} and copper has Cu^+ and Cu^{2+} ions. Changing between oxidation states involves accepting and donating single electrons. E.g. Transition metal ions are good promoters of free radical reactions. Destruction of cells also leads to liberation of metal ions which in turn catalyses the formation of ROS through Fenton reaction and Haber-weiss reactions.[10]

ROS as a Cause of Oxidative Damage

Oxidative stress constitutes a significant aetiological factor in ageing and in several degenerative diseases such as atherosclerosis, cancer, diabetes, chronic renal failure, ischaemia reperfusion injury and certain nervous system diseases. ROS are due to their high reactivity, prone to cause damage. The targets for ROS damage include all the biomolecules, summarized as follows: Lipids, Proteins and DNA.[11]

Lipid Peroxidation

Lipids are more susceptible to damaging effects of free radicals among all the major classes of biomolecules. Cell membranes are rich sources of polyunsaturated fatty acids (PUFA) which are readily attacked by oxidizing radicals. The oxidative destruction of PUFAs known as lipid peroxidation (LP) is particularly damaging because it proceeds as self-perpetuating chain reaction.

LP is commonly divided into three phases: (1) Initiation; (2) Propagation and (3) Termination.

1. *Initiation Phase:* During this phase, the primary event is the production of carbon centered radical ($R^\cdot$) or a lipid peroxide radical ($ROO^\cdot$) by the interaction of a PUFA with free radicals. The $R^\cdot$ and $ROO^\cdot$ in turn are degraded to form various products including malondialdehyde (MDA) and 4-hydroxy nonenal. MDA is estimated as an indicator of fatty acid breakdown by free radicals.

2. *Propagation Phase:* The carbon cantered radical ($R^\cdot$) reacts with molecular oxygen forming a peroxyl radical ($ROO^\cdot$) which can attack another polyunsaturated lipid molecule. $R^\cdot$ can be converted to a hydroperoxides with consumption can be converted to a hydroperoxide (ROOH) and there is simultaneous conversion of carbon cantered radical to a peroxyl radical, $ROO^\cdot$. This process can lead to continuous production of hydroperoxides with consumption of equimolar quantities of PUFA. One free radical generates another free radical in the neighbouring molecule, "a chain reaction" or propagation is initiated.

3. *Termination Phase:* Peroxidation can be terminated by a number of reactions. Peroxyl radical reacts with another peroxyl radical to form inactive products. Membrane based alpha-tocopherol (∝-TOH) forms more stable tocopherol phenoxy radical that can be recycled by other cellular antioxidants such as ascorbate (vitamin C) or glutathione (GSH). Oxidation of NADPH in enzyme systems and of ascorbate in non-enzymatic reaction regenerates the active-Fe^{2+}. LP decreases the membrane fluidity, changes in the phase properties of the membranes and decreases electrical resistance. Also, cross linking of membrane components restricts mobility of membrane proteins and leads to release of cellular and organelle contents like lysosomal hydrolytic enzymes by rupture of the cells. and inactivation of membrane pumps responsible for maintaining ionic homeostasis. LP has been implicated in a number of diseases and clinical conditions like diabetes mellitus, chronic renal failure, atherosclerosis etc.[11,12]

Damage to Proteins

It can occur either by direct attack of ROS / RNS on proteins or by end products of lipid peroxidation such as MDA. Oxidative damage to cellular proteins includes formation of cross linked aggregates, modified proteins and peptide fragments leading to damage of receptors, enzymes, signal transduction pathways and transport proteins. The damage also occurs to Ca^{2+} ATPase and Ca^{2+}–Na^{+} exchange systems that helps to maintain lower intracellular Ca^{2+} concentration under physiological conditions. The Na^{+}–K^{+} ATPase system in plasma membrane has thiol group on its active site. The thiol group is susceptible to oxidative damage and is readily inactivated during lipid peroxidation. Reactive carbonyl groups (ketones and aldehydes) are formed as a result of protein oxidations. Carbonyl groups can be the product of a reaction between amino acid side groups (usually Lys, Arg, Pro, or Thr) and hydroxyl radicals. They can also result when the products of the reaction between ROS and lipids (4-HNE and MDA) or carbohydrates (ketoamines and ketoaldehydes) react with amino acid side chains. A ferric (or cupric) ion is reduced by superoxide to form a ferrous ion that binds to a cation binding site on a protein where one residue is a Lys. The bound metal then reacts with H_2O_2 to form a hydroxyl radical that in turn leads to the production of a carbon radical. This radical can lead to cleavage of the polypeptide backbone, cross-linking of two polypeptide chains, or, hydrolysis of the ε-amino group to leave an aldehyde.[13,14]

Damage to DNA

DNA damage can be caused by ROS / RNS by direct chemical modification of DNA. A number of alterations involving deoxyribose sugar and / or alteration of purine and pyrimidine bases, cleavage of DNA, DNA-protein cross links etc are due to reactions with ROS, especially $OH^{\cdot}$. Indirect mechanisms of damage include impaired replication and repair of DNA, increased mitochondrial permeability, released cytochrome C, increased intracellular Ca^{2+} and thus leading to apoptosis and eventually cell death.[15]

Antioxidant Systems

Antioxidants are defined as "any substance present at low concentrations in comparison to the oxidizable substrate which significantly delays or prevents oxidation of that substrate". They render free radicals harmless and stop the chain reaction formation of new free radicals. They may be compartmentalised in subcellular organelles within the cell to provide maximum protection. The cellular antioxidant systems can be divided into two major groups[16]:

1. *Enzymatic Antioxidant System:* These include superoxide dismutase (SOD), catalase, glutathione redox cycle enzymes like glutathione peroxidase (GPx) and glutathione reductase (GR)
2. *Non-Enzymatic Low Molecular Weight Antioxidant System:* These include:
    a. Water soluble compounds – vitamin C, uric acid, glutathione, albumin, thiols, ferritin, ceruloplasmin, transferrin etc.
    b. Fat soluble compounds – vitamin E, β carotene, bilirubin etc.

Total antioxidant capacity (TAC) parameter summarizes the overall activity of antioxidants and antioxidant enzymes.[17]

Antioxidant Scavenging Enzymes

1. *Superoxide Dismutase (SOD):* SOD was the first genuine ROS metabolizing enzymes discovered. Dismutation of $O_2^{\cdot-}$ to H_2O_2 by SOD is called the primary defense for it prevents further generation of free radicals. In the reaction catalyzed by SOD, two molecules of superoxide form H_2O_2 and molecular O_2 and are thereby the source of cellular H_2O_2.[18] SOD is specific for catalytic removal of $O_2^{\cdot-}$. SOD is classified into three distinct classes: [A] Copper – Zinc SOD (Cu-Zn-SOD): dimer found in cytosolic compartment of the cell. It contains two protein subunits, each of which bears an active site containing one copper ion and one zinc ion. The copper ions appear to function in the dismutation reaction by undergoing alternate oxidation and reduction. i.e. The zinc ion(Zn^{2+}) does not function in the catalytic cycle but it appears to stabilize the enzyme. [B] Manganese SOD (Mn-SOD): is a tetramer and contains at its active site in Mn (III) state in the resting enzyme. Mn-SOD is located in the mitochondrial matrix. Mn-SOD catalyzes similar reaction as Cu Zn SOD. [C] Iron SOD (Fe-SOD): contain two protein subunits containing 1 or 2 moles of iron per mole of enzyme. The iron in the resting state is Fe (III) and it probably oscillates between Fe (III) and Fe (II) states during the catalytic cycle.[18]

2. *Catalases:* Catalases are mainly heme containing enzymes. They consist of four protein subunits each of which contains a heme [Fe(III)-protoporphyrin] group bound to its active site. The predominant subcellular localization in mammalian cells is in peroxisomes, where catalase catalyses the dismutation of hydrogen peroxide to water and molecular oxygen. Catalase also has functions in detoxifying different substrates eg. Phenols and alcohols, via coupled reduction from H_2O_2: Catalase lowers the risk of hydroxyl radical formation from H_2O_2 via the Fenton-reaction catalyzed by copper or iron ions. Catalase binds NADPH, which protects the enzyme from inactivation and increases its efficiency.[1,18]

3. *Glutathione Peroxidase (GPx):* There are four different GPx, all of them containing selenocysteine. GPx1 and GPx4 are both cytosolic enzymes abundant in most tissues. GPx2 and GPx3 are mainly expressed in gastrointestinal tract and kidney respectively. All glutathione peroxidases catalyse the reduction of H_2O_2 using glutathione (GSH) as substrate. The enzyme is specific for GSH as a hydrogen donor but will accept other peroxides as well as hydrogen peroxide. The catalytic mechanism proposed for reduction of hydroperoxide by GPx involves oxidation of the active site selenolate (Se^-) to selenelic acid (SeOH). Upon addition of one molecule of GSH, the SeOH is transformed to a selenyl sulfide adduct with glutathione (Se-SG), which can be regenerated to the active selenolate and glutathione disulfide (GSSG) by the addition of a second molecule of GSH.[1,18]

4. *Glutathione Reductase (GR) :* The ratios of GSH/ GSSG in normal cells are kept high for reducing GSSG back to GSH. This is achieved by glutathione reductase (GR) enzymes, which catalyse the reaction: The NADPH required is mainly provided by oxidative pentose-phosphate pathway. The rate at which the pentose phosphate pathway operates is controlled by the supply of $NADP^+$ to glucose-6-phosphate-dehydrogenase. As GR operates and reduces the NADPH / $NADP^+$ ratio, the pentose phosphate pathway speeds up to replace NADPH. GR contains two protein subunits, each with FAD at its active site. Apparently the NADPH reduces the FAD, which then passes its electron onto a disulfide bridge (-S-S-) between two cysteine residues in the protein. The two –SH groups so formed then interact with GSSG and reduce it to 2GSH, reforming the protein disulfide.[1,12,18]

Low-Molecular Weight Antioxidant Compounds

1. *Vitamin C OR Ascorbic Acid :* It is water soluble and has been shown to be a major antioxidant in human plasma as well as in cell membranes. It reduces α- tocopherol as well as peroxides and ROS such as superoxide. The vitamin serves mainly to prevent lipid

hydroperoxide formation in plasma lipoproteins, e.g. LDL, by reducing α-tocopherol radicals formed upon reaction with lipid peroxyl radicals. Ascorbate also protects lipids in cell membranes by this mechanism. Intracellularly, in aqueous phase, ascorbate and GSH act in concert to protect the cell from oxidative damage. Ascorbate recycling is studied in RBC. Glutathione is believed to be the major reductant of dehydroascorbic acid (DHA) in erythrocytes.[19,20]

2. *Vitamin E:* Vitamin E or tocopherol (α-TOH) is a lipid soluble vitamin present in biological membranes. It contains a hydroxyl group by which it reacts with unpaired electrons and can reduce e.g. peroxyl radicals. Vitamin E acts as a free radical quencher. Attached to the hydrophobic structure of α-TOH is an –OH group whose hydrogen atom is easily removed. Hence peroxyl and alkoxyl radicals generated during lipid peroxidation preferentially combine with the antioxidant, instead of with an adjacent fatty acid chain. This therefore terminates the chain reaction. Hence, the term chain-breaking antioxidant. The new radical tocopherol-$O^{\cdot}$ is poorly reactive and unable to attack adjacent fatty acid side chains consequently stopping the chain reaction. The tocopherol radical can migrate to the membrane surface and reconvert to α-TOH by reaction with ascorbic acid. Some thiol compounds, such as GSH, might also be involved in regenerating α-TOH from its radical.[20,21]

3. *β - Carotene and vitamin A:* Both vitamin A and β-carotene are effective antioxidants for destroying singlet oxygen ($^1O_2$) while β-carotene is more effective than vitamin A for superoxide free radical ($O_2^{\cdot -}$) and peroxyl radical ($ROO^{\cdot}$). β-carotene and tocopherols act as lipid antioxidants, the former inactivates free radicals in relatively hypoxic or aerobic conditions and the latter at relatively high O_2 concentrations. Organic peroxyl free radicals ($ROO^{\cdot}$) are mopped up from tissues by getting bound to the conjugated alkyl molecular chain of β-carotene as resonance stabilized carbon-cantered substituents.[22]

4. *Proteins:* Since iron and copper ions are powerful promoters of free radical damage, accelerating lipid peroxidation and causing formation of hydroxyl radicals, iron is rarely allowed to be free by a complex system of transport and storage proteins: (a) Transferrin: is the plasma iron transport protein and iron bound to transferrin cannot catalyze free radical damage. (b) Ferritin: is the storage form of iron. Iron within ferritin will not stimulate free radical reactions. (c) Ceruloplasmin: is a safe transport form of copper and it assists loading of iron onto transferrin. (d) Albumin: which has one sulphydryl group per molecule and is present at about 0.5 mmol/L in plasma, can scavenge several radicals and binds copper ions.[13,16]

5. *Urate:* is present at about 0.5 mmol/L in body fluids and is the end product of purine metabolism. It scavenges several free radicals.[13,16]

6. *Ubiquinone (Coenzyme Q/Q 10) :* exerts its main natural function in mitochondria as a part of the electron transport chain, but it is also present in low concentrations in plasma and in cell membranes where it functions as an antioxidant by preventing lipid peroxidation.[1,13,16]

Detection of Free Radicals

Free radicals are highly unstable with a half-life of only few seconds. Direct method of detection of free radicals is by electron spin resonance, which can also be used to monitor changes in the chemical forms of the oxidizable transition metal ions. But again the sensitivity is low. To overcome this sensitivity issue, short--lived radicals are allowed to react with a trap molecule to produce a stable radical or with a detector molecule to yield a stable product, which can be further evaluated. These methods require rigorous sampling procedures and reflect current exposure to oxidative stress species. Superoxide can be detected by Electron spin resonance assays based on it's ability to reduce cytochrome c, nitroblue tetrazolium (NBT), and other compounds, or with chemiluminescence or fluorescence-based methods (i.e. lucigenin assay). Samples when

treated with NBT leads to the formation of a dark blue insoluble formazen compound that can be detected microscopically to localize superoxide generation in tissues.[23]

Horseradish peroxidase assays rely on H2O2-dependent oxidation of a nonfluorescent substrate to form a fluorescent product that can be easily detected. A variety of substrates, including 4-aminoantipyrine, scopoletin, Amplex Red (N-acetyl- 3,7-dihydrophenoxazine), dichlorofluor-escin diacetate (DCFDA), and homo-vanillic acid, are employed in these assays. Peroxidase assays are simple and easy to perform and kits are readily available enabling the estimation of H_2O_2 levels in isolated subcellular fractions. The advantage of Histochemical staining methods is that they allow for subcellular localization of H_2O_2. 3,3-diaminobenzidine (DAB) is also used to localize H_2O_2. DAB reacts rapidly with H_2O_2 in the presence of peroxidase, forming a brown polymerization product. Another widely used cytochemical assay is based on the H_2O_2 reaction with cerium chloride to produce electron-dense precipitates of cerium perhydroxides. As with superoxide detection, many methods commonly used to measure H_2O_2, including DAB and DCFDA assays, suffer from low specificity and therefore often do not measure a particular ROS but rather generalized oxidative stress in the cell.[24]

Assay for Lipid Peroxidation

ROS cause peroxidation of polyunsaturated fatty acids, producing α,β-unsaturated aldehydes such as 4-hydroxynonenal (4-HNE) and malondialdehyde (MDA). These aldehydic secondary products of lipid peroxidation are generally accepted markers of oxidative stress. Several analytical techniques can be used to assay for lipid peroxidation. Continuous monitoring of copper-induced formation of conjugated dienes in isolated LDL by UV radiation absorption at 234 nm is widely accepted. A popular thiobarbituric acid (TBA) assay for MDA is based on its reaction with TBA followed by measuring A532. The TBA assay and its modifications were used to measure lipid peroxidation. Lipid peroxidation can be measured by serum MDA estimation according to the colorimetric method of Satoh.k. In this method, Lipoproteins will be precipitated from the specimen by adding trichloroacetic acid. 0.05 M sulphuric acid and 0.67% thiobarbituric acid (TBA) in 2 M sodium sulphate are added to this precipitate and the coupling of lipid peroxide with TBA is carried out by heating in a boiling waterbath for 30 minutes. The resulting chromogen will be extracted in n-butanol, which is measured colorimetrically at 530 nm.[25,26]

Mass spectrometry (MS) based methods are more accurate and sensitive for 4-HNE, MDA, and other lipid peroxidation products. A method developed by Deighton et al. (1997) is based on the highly sensitive liquid chromatography-MS detection of the 2,4-dinitrophenyl (DNP) hydrazine (DNPH) derivatives of 4-HNE and MDA. An advantage of MS-based methods is the ability to identify individual lipid species targeted by ROS and to detect the various oxidative products formed which can demonstrate the molecular mechanisms of lipid peroxidation and to identify new oxidative stress markers.[27,28]

Assays for Nucleic Acid Oxidation

ROS can cause DNA strand breaks or modification to deoxyribose sugar and bases. Several common DNA adducts resulting from oxidative damage have been characterized. Formation of the 8-hydroxy-2′-deoxyguanosine, 8-hydroxyguanine, and DNA-MDA adducts are the most characteristic features of DNA oxidation. These metabolic markers form the basis for the majority of assays aimed at measuring oxidative DNA damage.[29,30]

Assays for Protein Oxidation

Total protein oxidation levels can be determined spectrophotometrically in whole organ extracts. The DNPH system, coupled with the commercially available anti-DNP antibodies will allow both immunodetection and immunoenrichment to be applied to protein oxidation studies. Antibody-based ELISA method and Western blot is more sensitive. Combining the anti-DNP antibody with one- or two-dimensional gel electrophoresis is more qualitative using a commercial kit.[31- 33]

Assays for Total Antioxidant Capacity

Although antioxidant research continues to thrive, determining the total antioxidant capacity (TAC)

via one or more assays of an individual compound or extract remains challenging, in part because of the inherent differences between the in vitro assay and the complexity of biological systems. Tests which measure the combined antioxidant effect in biological fluids may be useful in providing an index of ability to resist oxidative damage. Depletion of antioxidants is denoted by a change in signal, such as rate of oxygen utilization or chemiluminescence. Measurement of these signals requires specialized equipment, and such tests can be time-consuming as well as technically demanding. This limits the availability of such tests and, furthermore, makes clinical evaluative studies difficult. This review focuses on the ferric reducing antioxidant power (FRAP) & combined FRAP with ascorbic acid concentration (FRASC) assays as a simple & putative index of antioxidant, or reducing, potential of biological fluids within the technological reach of every laboratory and researcher interested inoxidative stress and its effects.[34]

Reaction Mechanisms

On the basis of the chemical reactions involved, major TAC assays can be divided roughly into two categories: (1) hydrogen atom transfer (HAT) and (2) single-electron transfer (SET) reaction–based assays. These two mechanisms yield identical results, but they differ in terms of kinetics and the potential for side reactions to occur.

HAT-based procedures measure the classical ability of an antioxidant to quench free radicals by hydrogen donation. TAC or reactivity measurements of HAT assays are based on competition kinetics. HAT reactions are solvent- and pH-independent and usually are quite rapid—typically they are completed in seconds to minutes.[35] AOC Methods Using HAT are:

1. The *Oxygen Radical Absorbance Capacity Assay (ORAC)* has been broadly applied in academe and in the food and dietary supplement industries as a method of choice to quantify AOC. Disadvantages of the ORAC assay are: It is limited to measurement of hydrophilic chain–breaking antioxidant capacity against only peroxyl radicals. Fluorescent markers, although sensitive, require detection by fluorometers, which may not be routinely available in analytical laboratories. The long analysis time (~1 h) also has been a drawback. Temperature-control problems can lead to intra-assay variability and diminished reproducibility.[36]

2. The *Total Reactive Antioxidant Potential (TRAP) Assay* is often used for measurements of in vivo antioxidant capacity in serum or plasma because it measures nonenzymatic antioxidants such as glutathione, ascorbic acid, α-tocopherol, and ß-carotene. The TRAP assay is relatively complex and time consuming and requires a high degree of expertise and experience.[37]

SET-based methods detect the ability of a potential antioxidant to transfer one electron to reduce a species, including metals, carbonyls, and radicals. The relative reactivity in SET methods is based primarily on deprotonation and ionization potential. SET assays are very sensitive to ascorbic and uric acid, which are important in maintaining plasma redox tone.[34] AOC Methods Using SET are:

1. The *Trolox Equivalent AOC Assay (TEAC)* can be used in multiple media to determine both hydrophilic and lipophilic antioxidant capacities of extracts and body fluids. Disadvantages are: The ABTS [2,2'-azinobis(3-ethylbenzothiazoline- 6)-sulfonic acid] radical used in TEAC assays is not found in mammalian biology and thus represents a nonphysiological radical. The evaluation of antioxidant capacity using TEAC can be troublesome or even impossible.[38]

2. The *Folin-Ciocalteu (F-C) Procedure* is an electron transfer– based assay is useful for botanical samples. The reaction is slow, lacks specificity & the assay is not standardized. Moreover it suffers from a number of interfering substances (sugars, aromatic amines, sulphur dioxides, ascorbic acid & many inorganic substances).[39]

3. *Ferric Reducing Antioxidant Power (FRAP) Assay* is another method of wide suitability for assay of antioxidants in vitro as well as in

organisms. In some literature the FRAP method is referred to as the ferric reducing ability of plasma.

Principle: At low pH, reduction of ferric tripyridyl triazine (Fe III TPTZ) complex to ferrous (Fe II TPTZ) form (which has an intense blue colour) can be monitored by measuring the change in absorption at 593nm. The reaction is non-specific, in that any half reaction that has lower redox potential, under reaction conditions, than that of ferric ferrous half reaction, will drive the ferrous (Fe III to Fe II) ion formation. The change in absorbance is therefore, directly related to the combined or "total" reducing capacity of the electron donating antioxidants present in the reaction mixture. FRAP directly analyses total low molecular weight antioxidants. Measurement is based on the ability of antioxidants in the sample to inhibit the oxidative effects of reactive species purposefully generated in the reaction mixture. Ferrozine, a compound closely related to TPTZ, has also been widely used, with excess ascorbic acid, to measure iron.[40]

Advantages of FRAP assay: In contrast to other tests of total antioxidant capacity, the FRAP assay is inexpensive, reagents are simple to prepare, results are highly reproducible, the procedure is straightforward and speedy and does not require specialized equipment. The FRAP assay can be performed using automated, semiautomated, or manual methods. TAC activity in FRAP assay is not concentration-dependent. The 0-to 4-min reaction time window, therefore, is suitable for the measurement of bilirubin's contribution to FRAP, as well as that of the other antioxidants tested. In FRAP assay no interaction is observed between the antioxidants tested that either enhanced or dampened antioxidant activity in this system.

Disadvantages: FRAP cannot detect thiols, such as glutathione, and proteins. Some polyphenols react more slowly and require longer reaction times for detection, e.g., 30 min. The order of reactivity of a series of antioxidants can vary considerably and even invert, depending on the analysis time.[41]

4. *Ferric Reducing & Ascorbic Acid Concentration (FRASC) Assay:* A recent modification of the FRAP assay, known as FRASC, enables the simultaneous measurement of total antioxidant activity and ascorbic acid concentration in the sample. It is based on the same principle of FRAP assay & ascorbic acid is selectively destroyed in one of a pair of samples by ascorbic acid oxidase. The difference between paired samples in terms of their absorbances at 593 nm after a 1-minute reaction time is due to ascorbic acid.

   Advantages of FRASC assay: FRASC is a modified version of the ferric reducing (antioxidant) power (FRAP) assay and is an objective and reproducible measuring tool to assess the combined in vitro ability of scavenging, electron donating antioxidants in biological samples. Unlike HPLC, the FRASC assay takes only a few minutes, is relatively inexpensive, and provides two indices of antioxidant status. This makes FRASC a potentially valuable tool for use with fluids, such as tears, where both measurements are desirable and where sample volumes are small. The speed of FRASC is of particular benefit in measuring unstable antioxidants, such as ascorbic acid, which are destroyed rapidly ex vivo.

Clinical Significance of Frap & Frasc Assays: FRAP assay has been used as a novel method for assessing total antioxidant capacity in various conditions like chronic renal failure, cancer, infectious diseases, cardiovascular diseases, stroke, obesity, aging etc. FRASC assay has been used for measuring total antioxidant activity and ascorbic acid in human tears. Commercially available analytical kits for the estimation of TAC are expensive & time-consuming. Hence, FRAP & FRASC assays offer simple, fast, cost-effective & putative indices of measuring TAC along with ascorbic acid which reduces labor time, material cost & sample volume. However, as with all in vitro tools the clinical utility of the results obtained can only be determined by extensive clinical study.[42]

Ability to Localize Oxidative Stress

Knowledge about free radicals (e.g. H_2O_2 can cross cell membranes, superoxides and hydroxide radicals cannot) is useful in localizing the site & source of free radicals. This type of spatial information can be obtained in several ways. Isolation of Organelles and determination of oxidized proteins by conventional biochemical techniques or using proteomic techniques the subcellular localization of a protein from a whole cell extract can be achieved.[43]

Conclusion

Evaluating oxidative stress by measuring lipid peroxidation and TAC can lead to a better understanding of free radical damage in oxidative stress related diseases which would be useful to identify the patients with increased risk of progression of the disease and also for monitoring and optimization of antioxidant therapy.

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