

Free Radicals - A New Concept in Medicine

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Introduction

The recognition of organic free radicals by Gomberg in 1900 led inevitably to speculation that free radical species might be involved in living system and some enzymatic reactions involve free radical intermediates (Michaelis). However, it was not until the proposal by Slater in 1966 that the hepatotoxicity of carbontetrachloride was consequence of a free radical reaction that the idea of free radical-mediated processes forming a significant class of reactions in the generation of tissue injury gained a place in the purview of bio chemical pathology.

Free radicals are chemical species possessing an unpaired electron that can be considered as fragments of molecules and which are generally very reactive. They are produced continuously in cells either as accidental product of metabolism or deliverately (e.g. during phagocytosis). In aerobic cells the most important reactants in free radical are oxygen and its radical derivatives (super oxide and hydroxyl radical), hydrogen peroxide and transition metals. Reactive free radicals formed within cells an oxidise bio molecules and lead to cells death and tissue injury. Involvement of free radicals in the pathogenesis of a disease in difficult because of short life span of these species. But the clinical significance of this has been recognized only for the last few years.

Biochemistry of free radicals

Free radicals can be formed in three ways -

- By homolytic cleavage of a covalent bond of a normal molecule, with each fragment retaining on of the paired electron.
- By addition of a single electron to a normal molecule.
- By the loss of a single electron from a normal molecule.

Electron transfer mechanisms are more important and common in biological system than homolytic cleavae. Free radicals can be positively charged, negatively charged or electrically neutral.

Oxygen Free Radicals and Reactive Oxygen Species (ROS)

The most important free radicals in biological systems are radical derivatives of oxygen. Some of the important reactive oxygen species which are involved in pathogenesis of various diseases are listed in (Table-1).

Table 1. Reactive Oxygen Species

Species	Molecule	Species	Molecule
Superoxide ion	O ₂ -	Nitricoxide	NO
Hydroxyl ion	OH	Ferryl ion	FeO ²⁺
Perferryl ion	FeO ₂ ²⁺	Allyl	R
Alkoxy	RO	Peroxy	ROO

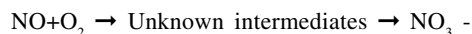
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Generation of Free Radicals in Cells

Respiratory burst : the host defence system against harmful organisms includes neutrophils, monocytes, eosinophils and macrophages which play their protective role by a metabolic event known as respiratory burst, in which a group of powerful oxidising agents like hypochlorous acid, hydrogen peroxide (H₂O₂) and number of oxygen radicals are injected in to phagocytic vacuole. Radical production is thus important for phagocytosis of internalized bacteria. This can be illustrated on patients with chronic granulomatous disease which suffer infections with certain organisms like staphylococcus aureus due to defective NADPH Oxidase system.

Eicosanoid metabolism : During the formation of endoperoxide 9,11- endo peroxy- 15- hydroperoxy prostaglandin (PGG₂) from arachidonic acid, a trace of hydroperoxide is required to react with the Fe(III) haem at the active site of cyclo-oxygenase enzyme to form a peroxy radical. This ROS can stereospecifically abstract a hydrogen atom from arachidonic acid to commence the process of PGG₂ formation. Excess of lipid peroxides can inactivate cyclo-oxygenase activity.

Endothelium-derived relaxing factor (EDRF) : EDRF is produced from vascular endothelium and is an important mediator of vasodilation. EDRF is now been identified as nitricoxide. The endothelium seems to continuously produce small amounts of superoxide which can react with nitricoxide to form nitrate ions, a non-radical product.



It is possible that the impaired endothelium- mediated vasodilation in diabetic patients could be related to increased free radical formation in vivo. NO is also produced by macrophages. In brain, nitric oxide synthetase (NoS) has been localized within neuronal cells with highest activity in neurons of the cerebellum and olfactory bulb.

Controlled leakage in enzymatic reactions : Sources of free radicals within the cells can be derived from inevitable leakage of superoxide anions from the mitochondrial electron transport chain. In addition, many compounds will react with molecular oxygen to from superoxide. Radicals may be generated during metabolism of various drugs by cyt P 450 microsomal oxidation systems, e.g. paracetamol, alcohol. Lastly, some enzymes are known to catalyse the formation of free radicals; for e.g., xanthine oxidase catalyses the conversion of hypoxanthine to xanthine and also oxidizes to uric acid. In both reactions superoxide and hydrogen peroxide are formed.

Other sources : Apart from these, free radicals can also be generated from toxic environmental pollutant; ionizing radiations; gases like Ozone, nitrogenoxide; heavy metals like Hg, Pb; cigarette smoking; alcohol; emotional stress and many more.

Damaging Reactions of Free Radicals

All classes of biomolecules may be attacked by free radicals but lipids are the most susceptible. Cell membrances are rich source of polyunsaturated fatty acids (PUFAs), which are readily attacked by oxidising radicals. The oxidative destruction of PUFAs known

as lipid peroxidation is damaging because it proceeds as a self-perpetuating chain reaction. Lipid peroxidation is of particular significance as a damaging reaction consequent to free radical production in cells because : (i) it is a very likely occurrence, given the availability and susceptibility of PUFA in membrane; and (ii) is very destructive chain reaction that can directly damage the structure of membrane and indirectly damage other cell components by reactive aldehyde production. Proteins, nucleic acids and carbohydrates are less susceptible. Random attack of radicals on proteins is unlikely to be very damaging unless very extensive. On proteins, it causes inactivation of enzymes. It damages nucleic acid by causing break in DNA strands leading to abnormal cell multiplication.

Assessment of Free Radicals

Direct detection of free radicals within biological systems is difficult because of their low concentrations and their extremely short life-span. Currently, **three principal approaches** are used to ascertain whether oxidative stress has occurred : (a) **by exogenous spin traps and free radical indicators**; (b) **by detection of free radical products**; (c) **by antioxidant status**

Exogenous spin traps and free radical indicators : The spin trap phenyl-Nt-butyl nitron reacts rapidly with free radicals to form a relatively stable spin adduct which can then be analysed by electron spin resonance. Hydroxyl radicals generated within biological fluids can be trapped by salicylate to form a 2, 3-dihydroxybenzoate, which can then be quantiated by high-performance liquid chromatography (HPLC).

Detection of free radical products - Numerous methods for detection of reaction products have been published but unfortunately they are nonspecific.

Protein oxidation : Schiff's bases are produced when an aldehyde reacts with amino groups. This can be detectable by Fluorescence spectroscopy and is considered to be a highly significant marker of free radical generation.

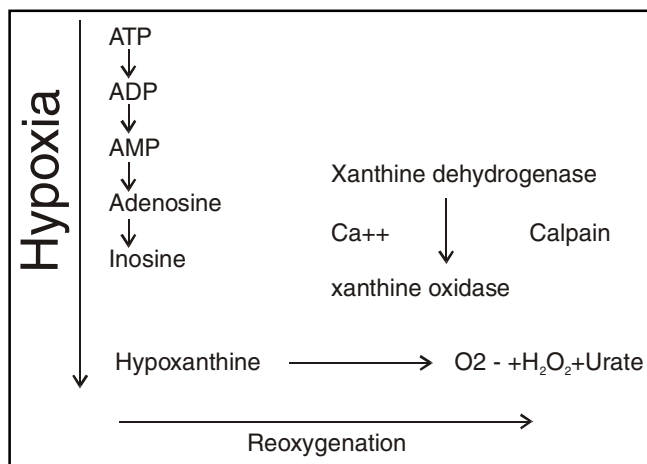
Lipid peroxidation : The detection of products of lipid peroxidation in biological materials fhas provided the basis for several popular assays for reactive oxygen species. Different methods used are Diene conjugates, Thiobarbituric acid test and Volatile hydrocarbons measurement.

Antioxidant status : Recently, biological fluids have been investigated for their antioxidant status either by direct measurement of the cytoprotective enzyme activities and antioxidant content or by the capacity of the sample to quench an invivo generating system.

Role of Free Radicals in Disease Pathogenesis

In Ischaemic reperfusion damage : one of the more recent advances in free radical research has been the realization that reactive oxygen species may be generated during the reperfusion of ischaemic tissue. This is important in organ transplanation. There is considerable evidence that injury to ischaemic tissue occurs exclusively during reperfusion phase. Such a process is reliant on two important constituents, xanthine oxidase and a trace amount of catalytic iron such damage is also clearly relevant in patients of acute myocardial infarction and cerebrovascular accidents. Reperfusion injury may even play a role in the exacerbation of damage in patients with rheumatoid arthritis (Box).

In Carcinogenesis : Exposure to active oxygen species in aerobic organisms is continues and unavoidable. Cancer in humans and animals is a multistep process. The complex series of cellular and molecular changes that occur through the development of cancer can be mediated by diversity of exogenous and endogenous stimuli.



Mechanism for free radical injury induced by anoxia-reoxygenation

Active oxygen species and other free radicals have long been known to be mutagenic; further, these agents have more recently emerged as mediators of the other phenotypic and genotypic changes that lead from mutation to neoplasia.

In Shock related cell injury : Shock related organ failure evolves from a variety of starting points - ischaemia, reperfusion, nonbacterial or bacterial inflammation - several mechanisms are involved. In addition to the effects of xanthine oxidase after ischaemia/reperfusion, toxic oxygen species form phagocytes that accumulate in both intra and extravascular tissue spaces are of central importance. Damage of membranes by lipid peroxidation and by exposure to mediators leads to increased permeability, tissue edema and organ dysfunction.

In Atherosclerosis : It is hypothesized that it is initiated by damage to the vascular endothelium. Endothelial cells are known to be sensitive to damage by ROS and lipid hydroperoxides. Macrophages play an important role in the development of atherosclerotic lesion. Activated monocytes and macrophages could injure neighbouring endothelial cells by secreting superoxide, H_2O_2 and hydrolytic enzymes, while factors released by macrophages can stimulate smooth muscle cell proliferation. Macrophages possess receptors for LDLs, but if LDL has already undergone lipid peroxidation it is recognized by a separate class of receptors known as Scavenger receptors. These modified LDL are taken up with greater efficiency, leading to rapid accumulation of cholesterol in macrophages and their conversion to foam cells, characteristic of atheromatous lesion.

In Inflammation : In man, free radicals play a role in a variety of normal regulatory systems, the deregulation of which may play an important role in inflammation. As examples, the second messenger roles of : NO in the regulation of vascular tone, O_2^- in the fibroblast proliferation and H_2O_2 in the activation of transcription factors such as NFkB. At the site of inflammation, increased free radical activity is associated with activation of the neutrophil NADPH oxidase and the uncoupling of a variety of redox system including endothelial cell xanthine dehydrogenase. Although free radicals thus produced, have the capacity to mediate tissue destruction, disturbances in the second messenger and regulatory activities of free radicals may also contribute significantly to inflammatory process.

Free radicals primarily attacks on PUFAs. The overall effect of peroxidation is to decrease membrane fluidity, destabilising membrane receptors. Lipid peroxidation products can inhibit protein synthesis, block macrophage action and cause changes in chemotaxis and enzymic activity.

In Neurological disorders : Reactive oxygen metabolites; namely

superoxide and hydroxyl radical and H_2O_2 , are produced as a consequence of the physiological metabolic reactions and functioning of the CNS. Evidences suggest that in several neurological diseases, for example Parkinson's disease, Huntington's disease, Alzheimer's disease and multiple sclerosis, there is iron accumulation secondary to the initial toxic lesion. The reason is uncertain but such accumulates exacerbates the initial lesion by generation of reactive oxygen species (ROS).

Diabetes mellitus (DM) : Diabetes mellitus is a syndrome initially characterized by a loss of glucose homeostasis. The disease is progressive and is associated with high risk of athero-sclerosis, renal and nerve damage as well as blindness. Abnormalities in the regulation of peroxide and transition metal metabolism are postulated to result in establishment of the disease. DM is associated with oxidative reaction, particularly those which are catalysed by decompartmentalized transition metals but their causative significance in diabetic tissue damage remains to be established.

In prematurity : In recent years increasing experimental and clinical data have provided a number of evidence for the involvement of oxygen free radicals in three main disorders of prematurity-chronic lung disease, retinopathy and intraventricular haemorrhage. Infants born prior to 30th week gestation or weighing less than 1500gm. at birth appears to be at risk. The basis for free radical involvement in these disorders is that oxygen centred radicals and related reactive oxygen metabolites are formed too rapidly to be detoxified by the antioxidant defence mechanism. In case of chronic lung disease, evidence currently favours excess oxygen as the cause of greater oxygen free radical production, whereas in retinopathy and intraventricular haemorrhage it is proposed that low oxygen tension followed by periods of reoxygenation is the more likely stimulus for excess radical formation.

In aging : Recent data available on free radical theory of aging confirm that aging is associated with an impaired control of oxygen homeostases. The presence of a system of antioxidant defence in all aerobic organisms indicates that the involvement of oxygen in the preservation of anaerobic life necessarily involves the production of activated oxygen species. The question is therefore, whether the loss of control of oxygen homeostasis acts in concert with other factors to trigger the inevitable process of aging or whether oxidative stress is an inevitable consequence of other molecular events. The failure to extend maximal life span by the attempt to maintain oxygen homeostasis appears to be inconsistent with the free radical theory of aging. If oxidative stress were to be recognized as a major factor which causes aging, the question then arises how the imbalance between pro and antioxidants is established. It can be said with certainty that oxidative stress is able to promote the process of aging and also to increase the probability of age specific diseases where free radicals are implicated.

Prospects for treatment of free radical-mediated tissue injury

Toxic metabolites of oxygen have emerged as a major final common pathway of tissue injury in a wide variety of disparate disease processes. Consequently, free radical ablation offers a substantial potential for the treatment of human diseases. This is because many constituents of the cell are potentially subject to free radical injury. The progression from free radical generation to tissue injury yields many levels for potential interventions. These can be classified into five major levels-

- (i) blockade of initial generation of toxic oxidants,
- (ii) scavenging oxidants e.g., superoxide dismutase (SOD)
- (iii) blocking the chain of toxic oxidants e.g., α -tocopherol

- (iv) enhancing endogenous antioxidant capability of the target
- (v) blocking secondary generation of toxic metabolites and/or mediators

The endogenous antioxidant system provides an important defence mechanism that allows the organism to cope with daily attacks of oxidative stress. Cellular compartmentalization is probably the most important endogenous mechanism of defence. Mitochondria, lysosomes, peroxisomes they are provide separate microenvironments in which free radicals are generated and coupled immediately to adjacent antioxidants defence system. Some of these endogenous antioxidants are summarized in Table-2. An important part of antioxidant defence system in side cells are the antioxidant enzymes, e.g., superoxide dismutase (SOD) which scavenges superoxide anion. It was recently discovered that the gene for SOD is defective in patients of Amyotrophic Lateral Sclerosis (ALS) also known as Lou Gehrig's disease. Other enzymes like catalase, glutathione peroxidase, ALA etc. have their role in antioxidant defence.

Table 2. Endogenous Antioxidants

Antioxidants	Comments
Enzymatic	
Cytochrome oxidase system	Detoxifies 95-99% of O_2 in cell
Catalase	Detoxifies H_2O_2
Peroxidase	Detoxifies H_2O_2
Superoxide dismutase (SOD)	Detoxifies superoxide anion
Glutathione peroxidase	Detoxifies H_2O_2
Non-Enzymatic	
Lipid soluble	
a-tocopherol	Vitamin E
b-carotene	Vitamin A precursor
Water Soluble	
Glutathione	---
Ascorbic acid	Vitamin C
Urate	Scavenges $O_2^{\cdot -}$, OH
Cysteine	Scavenges $O_2^{\cdot -}$, OH
Albumin	Scavenges LOOH, HOCl
Bilirubin	Scavenges $O_2^{\cdot -}$, OH
Ceruloplasmin	Detoxifies superoxide anion
Transferrin	Binds circulating iron
Lactoferrin	Binds circulating iron
Ferritin	Binds tissue iron
Haemopexin	Binds haem and prevents it from decomposing lipid peroxides

In addition to the antioxidant enzymes, there are small endogenous molecule antioxidants that play a role in defence mechanism. These molecules of antioxidants are important particularly in blood and fluids present in extracellular compartment, where antioxidant enzymes are absent or present in very minute quantities. These small molecules are lipid soluble and water soluble antioxidants summarized in Table-II. However, in many pathophysiological conditions, local endogenous antioxidants become incapable to overcome the tissue injury caused by free radicals. In such cases, the administration of exogenous antioxidants, as listed in Table-3, may be salutary.

The **beneficial effects** of various antioxidants in different clinical conditions are detailed below -

Role in Atherosclerosis and cardiovascular diseases :

- in endothelium, e.g., inhibition of lipoxigenase and thus of oxidative LDL modification, prevention of cellular transitions, reduction of monocyte adhesion.
- in smooth muscle cells, e.g., inhibition of proliferation and of the signal transducing protein kinase.
- in blood platelets, e.g. inhibition of platelet adhesion.

Table 3. Exogenous (Pharmacological) Antioxidants

Class of Agent	Specific Agent	Mechanism of action
Xanthine oxidase inhibitors	Allopurinol Oxyprurinol Folic acid tungsten Pterin aldehyde	Inhibit superoxidation by Xanthine oxidase
Protease inhibitors	Soyabean trypsin inhibitor Other serine protease inhibitors	Block proteolytic activation of Xanthine oxidase from Xanthine dehydrogenase
NADPH oxidase inhibitors	Adenosine Local anaesthetics Ca++ channel blockers NSAIDs Cetiedil Diphenylene iodonium Monoclonal antibodies to NADPH oxidase	Inhibit superoxide generation by NADPH oxidase in neutrophils and macrophages
Superoxide dismutase (SOD)	Native SOD Polyethylene glycol-SOD Liposome-encapsulated SOD	Catalyse $O_2^- + 2H_+ \rightarrow H_2O_2$
Catalases	Native catalase PEG-catalase	Catalyse $O_2^- + 2H_+ \rightarrow H_2O_2$
NADPH oxidase inhibitors	Adenosine Local anaesthetics Ca++ channel blockers NSAIDs Cetiedil Diphenylene iodonium Monoclonal antibodies to NADPH oxidase	Inhibit superoxide generation by NADPH oxidase in neutrophils and macrophages
Superoxide dismutase (SOD)	Native SOD Polyethylene glycol-SOD Liposome-encapsulated SOD	Catalyse $O_2^- + 2H_+ \rightarrow H_2O_2$
Catalases	Native catalase PEG-catalase Lipase encapsulated capsule	Catalyse $O_2^- + 2H_+ \rightarrow H_2O_2$
Non-Enzymatic free radical scavengers	Mannitol Albumin Dimethyl sulfoxide Glutathione Urate Bilirubin Lazaroids	Scavenges OH Scavenges LOOH, HOCl Scavenges OH Scavenges H_2O_2 , OH Scavenges O_2^- , OH Scavenges free radicals Scavenges LOOH, O ₂ -
Inhibitors of iron redox cycling	Desferoxamine Apotransferrin Ceruleplasmin	Bind free Fe ³⁺
Substances that augment endogenous antioxidants	Ebselen Oltipraz Glutathione Acetylcysteine	Augment endogenous glutathione peroxidase activity
Antineutrophil agents	Antineutrophil serum Antiadhesion agents - Monoclonal antibodies to CD11/CD18 - Soluble GMP140 Platelet activating factor antagonists - BN52021 - WEB2086	Depletes circulating Neutrophils Inhibit neutrophil adhesion to endothelia Inhibit neutrophil adhesion and extravasation

- improves immunoresponses
- in macrophages; modulation of their migration into subintimal space, reduction of LDL-modifying 'respiratory burst' of radicals, diminishing foam cells formation by improving the catabolism of modified LDL, reduced production of cytokines like IL-1.

Glutathione, a sulphur containing naturally occurring amino acid function to decrease the formation of LDL which plays an important role in development of athero-sclerosis.

Role of antioxidants in radiation injury : Irradiation produces a cascade of free radicals. Antioxidants have been used to treat irradiation damages. These compounds also decrease the harmful effects caused by cancer chemotherapy. Submucous fibrosis, leucoplakia and photoradiation have been shown to be greatly benefited.

Role in ischemia reperfusion injury : Reperfusion of a previously ischaemic area has been shown to promote the release of free radicals. The rapid free radical activity is due to transition metal ions released by cells with subsequent damage of sarcoplasmic reticulum and membranes leading to impaired handling of calcium and other ions. SOD, catalase, tocopherol, allopurinol, desferrioxamine and glutathione improves the out come.

Role in cancer : Smokers have defective antioxidant protection. Tobacco smoke is rich in free radicals which damages the DNA causing lung cancer. Antioxidants block oxidative DNA damage resulting from carcinogen induced generation of free radical. Vit C and E inhibit nitrosamine formation and fecapentene production. N-acetyl L-cysteine reduces the occurrence of experimental tumours of lungs, colon and liver.

Antioxidants in diabetes mellitus : There is a major role of free radicals in pathophysiology of IDDM, retinopathy and nephropathy. Glucose combines with serum proteins and lipoproteins in a non-enzymatic glycation reaction and may auto-oxidise generating free radicals.

Antioxidants are shown to inhibit glucose auto-oxidation and reduce covalent linking of glucose to serum proteins and inhibit the glycation of serum proteins providing a promising future for the treatment of diabetic complications.

Role in organ transplantation : Studies show that antioxidants can decrease endothelial damage and prolong graft survival. Human recombinant SOD when given to recipients of renal transplants had reduced the rejection events and improved the graft survival.

Antioxidants and neurological disease : Brain is very vulnerable to free radical injury after any trauma and/or hypoxia because of high lipid and poor iron binding capacity. Clinical trials have shown the neuroprotective role of 'lazaroids', which are inhibitor of iron dependent lipid peroxidation. IN Parkinsonism, oxidative metabolism of dopamine is probably involved in production of oxidants. Increased Fe and Al and reduced glutathione has been bound thus clearing the way for antioxidants. MAO inhibitors like selegiline possibly decrease radical formation, thus slowing the disease progression. Antioxidant in brain are glutathione (in astrocytes) and vit. C (neurons).

Role in AIDS : Reports indicate that AIDS patients have less glutathione level. Thiol donating compound have been shown to inhibit viral replication and potentiate the effects of zidovudine.

Role in prematurity : Tocopherol has shown to reduce the incidence of retrolental fibroplasia, intraventricular haemorrhage and respiratory distress syndrome in neonates.

Role in cystic fibrosis (CF) : CF is associated with severe oxidative stress. Due to pancreatic involvement there is failure to absorption of fat soluble antioxidants. Supplementation of antioxidants have thus an attractive role.

Antioxidants in pregnancy and eclampsia : It has been shown that lipid peroxidation products are high and total serum antioxidants level are low in such patients, thereby implicating increased levels of fibronectin and endothelial damage. Role of antioxidants in such cases are on trial.

Role in inflammatory diseases : Antioxidants have a role in treatment of rheumatoid arthritis, Crohn's disease and ulcerative colitis.

Role in skin diseases : Human skin presents the first line of defence and the surface of skin must be equipped to deal the free radical damage. Depressed levels of antioxidants like glutathione is observed in patients with psoriasis, eczema, atopic dermatitis, vasculitis and mycosis fungoides. Therefore supplementation have shown an encouraging result.

Role in ophthalmology : Various epidemiological studies have shown that use of antioxidants reduces cataract possibly because of the role of free radical generating UV-B radiations in cataract formation. Lutein and Zeaxanthin are protective against macular degeneration.

Role in other diseases : Antioxidants defence is also seen in congestive cardiac failure, chronic renal failure, liver cirrhosis, systemic sclerosis, malaria, pancreatitis, shock etc.

Conclusion

Free radicals are constantly being generated in biological system either accidentally or deliberately. These free radicals which are highly reactive species react with biomolecules like lipids, proteins, nucleic acids etc. causing tissue injury and cell death. To limit their damage and to prevent free radical formation our body has developed a comprehensive array of antioxidant defence system. These include both enzymatic (like SOD, glutathione, peroxidase, ALA) and non-enzymatic (like ascorbic acid, tocopherol etc.) antioxidants which act by various mechanisms. Apart from this there are large number of exogenous antioxidants taken in the

form of dietary or pharmacological products which helps to contribute to natural antioxidant defence mechanism and may protect us from certain age related degenerative processes, various cancer and viral and other diseases. Although the choice of antioxidants may be important, the therapeutic time frame of the insult appears to play an even more critical role in the treatment of free radical mediated human diseases.

Recommended Reading

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