Oxygen Free Radicals and Their Biomedical Implications: A Mini Review

R. N. Sharan*[,1], M. M. Odyuo[2] and S. Purkayastha[3]

[1]*Radiation & Molecular Biology Unit Department of Biochemistry North-Eastern Hill University Shillong 793 022, India*

[2]*Department of Biotechnology, St. Anthony's College, Shillong (India)*

[3]*Department of Biotechnology, G. C. College, Silchar (India)*

Abstract: Free radicals (FR) are chemical species of significant importance to biological systems. FRs are generated by endo- as well as exogenous factors. Biological systems are equipped with appropriate metabolic pathways to remove cellular FR as well as repair the damages caused by them. Their cellular/physiological load profoundly influences the metabolism, physiology and overall well being of biological systems. Therefore, they are implicated in cellular degenerative processes and in patho-physiology, including carcinogenesis and ageing. The review shall attempt to give a generalized overview of FR chemistry, especially to the biologically important oxygen free radicals (OFR) and reactive oxygen species (ROS). The review shall also discus the OFR/ROS biology to get an overview of the induced damage at molecular, cellular and organismal levels in order to give a perspective of their influences on the genomic integrity, cellular microenvironment and physiology with special reference to human health.

Keywords: Free radicals, Reactive oxygen species, DNA, Cellular and molecular damage, Diseases.

INTRODUCTION

Free radicals (FR), or simply, radicals are defined as any atom, molecule or ion in an open shell configuration with at least one unpaired electron in its outermost shell, such that the atom is capable of an independent existence [1]. Both the terms are used interchangeably to convey essentially the same meaning. FR is usually abbreviated as a 'dot' immediately following the chemical symbol of the atom or molecule. However, a dot preceding the chemical symbol is also used frequently. It is a common knowledge that atoms are most stable in its 'ground state'. An atom or molecule is considered to be in the ground state when every electron in its outermost shell has a complimentary electron that spins in the opposite direction. A FR is formed when a covalent bond between the entities is broken and one electron each is retained with each newly formed atom, molecule or ion such that the unpaired electron has non-zero spin [1,2]. Such hemolytic cleavage requires input of variable amounts of energy depending on the configuration of the atom or molecule. For example, 435 kJ/mol of bond dissociation energy is required for generating 2 numbers of FR of hydrogen (H^*) by splitting a molecule of H_2 while only 243 kJ/mol is needed to produce two Cl^* species from the molecular Cl_2. The FR which requires higher input of energy is more unstable and, therefore, more reactive than the one requiring less energy. FR may also be formed by single electron oxidation or reduction of an atom or molecule. Professor Gerhard Herzberg, the Nobel Laureate of 1971, suggested a more general definition of FR as 'any transient (chemically unstable) species (atom, molecule or ion)' [3]. FR is usually very unstable and short lived chemical entity. However, some 'stable' FRs are also known. For example, dioxygen radical (O^*_2), α-tocopherol (vitamin E) derived FR and thiazyl radical are remarkably stable FR species. In addition, FR, such as $(KSO_3)_2NO$, R_2NO , melanin FR, etc. are known as 'persistent FR' due to steric crowding around radical center preventing its reaction with other molecules leading to their relatively longer existence.

FRs are highly reactive chemical entities due to the presence of the unpaired electron(s). Numerous atoms, molecules or compounds can form FR in a biological system. However, due to its abundance in and relevance to the living system, the review shall deal with molecular O_2, which is critical to the life process. Any FR involving O_2 is normally referred to as oxygen free radicals (OFR) or reactive oxygen species (ROS). O_2-centeric FR contains two unpaired electrons in the outer shell. When a FR dislodges an electron from a surrounding compound or molecule in order to fill its own deficiency, a new FR is formed. The newly formed FR then attempts to return to its ground state by dislodging electron(s) with anti-parallel spins from other neighboring donor entities or molecules. Thus, a chain reaction or a cascade of events is created, which could be as long as thousands of events [4,5]. Due to these two characteristics, namely, (a) highly reactive nature of FR, and (b) cascading nature of the FR generating reaction, free radicals-mediated oxidative damage have been critical for well being of aerobic cells [6]. Since physiological load of FR, particularly the OFR and ROS, decisively influence the course of cellular physiology and metabolism, mutagenesis, carcinogenesis and other degenerative processes including ageing, it is considered to have played important roles in the origin of life as well as biological evolution process [6,7].

Nonetheless, other types of FR are also important to the process of life. Among them, notable are reactive nitrogen species (RNS) and FR of fatty acids. Because of a central role of nitric oxide (NO) in signal transduction, nitric oxide radical (NO^*) acquires important roles in the complex cell signaling pathways that regulate the relaxation and proliferation of vascular smooth muscle cells, leukocytes adhesion, platelets aggregation, angiogenesis, thrombosis, vascular tone and homeodynamics to name the most obvious [8]. NO, therefore, also significantly influences the biological metabolism and physiology. Similarly, peroxynitrite (ONOO), a RNS intermediate, also readily interacts with DNA forming many adducts [9,10]. Due to this, both NO^* and peroxynitrite ($ONOO^-$) have lately become molecules of significant concern to human health and physiology [11]. Lipid peroxyl radical (LOO) is another type of FR that affects the cellular integrity by changing the cell, nuclear and other organelle membrane characteristics. As humans are constantly exposed to FR created by various natural or artificial endogenous and exogenous factors, it is important to understand its biological implications.

Oxygen Free Radicals (OFR) and Reactive Oxygen Species (ROS)

As discussed earlier, oxygen is a critical molecule to biological systems. Cells live in an environment fortified with oxygen. Due to various aerobic metabolic processes involving oxygen atom or molecule, oxygen free radicals (OFR) are easily and continuously formed within a cell endogenously. Consequently, OFR and other ROS are produced as by-products of metabolic or physiological and

*Address correspondence to this author at the Radiation & Molecular Biology Unit Department of Biochemistry North-Eastern Hill University Shillong 793 022 India; Tel: +91 (364) 272 2121; Fax: +91 (364) 272 1018; E-mail: rnsharan@nehu.ac.in

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biochemical processes that keep a cell alive [5,12,13]. Apart from this, various exogenous factors also induce formation of OFR and ROS species in a living system. The most common OFR and ROS in a living cell are:

1. Hydrogen peroxide (H_2O_2)

2. Hydroxyl radical (OH^*)

3. Superoxide anion radical (O_2^{*})

4. Singlet oxygen ($^1O_2$).

Hydrogen peroxide (H_2O_2) is a ROS and not strictly an OFR. However, because of its abundance in living cells, characteristic chemical properties and its ability to catalyze downstream reactions, it effectively behaves quite like a typical FR. It is also a short-lived, highly reactive, abundant and one of the most damaging ROS within the body. H_2O_2 is produced *in vivo* under normal physiological condition as a by-product of several metabolic pathways and reactions. The endogenously generated H_2O_2 interacts readily with biologically available and free metal ions, notably, copper and iron, resulting in production of hydroxyl radical ($OH^{\bullet}$), a true FR [14]. This classical and very important physiological reaction was first explained by Fenton and has been referred to as Fenton or Fenton-type reaction [15,16]. The Fenton reaction assumes special significance in biological systems as the substrates required to form OFR by this reaction are found within the body. Therefore, they could easily interact with each other to generate OH^* [17,18]. On the other hand, H_2O_2 can also be metabolized into H_2O by normal physiological processes. Therefore, H_2O_2 becomes a very unique molecule of the biological system in the sense that it can be converted to the highly damaging OH^* or be catalyzed and excreted harmlessly as water. Glutathione peroxidase is essential for the conversion of glutathione to oxidized glutathione, during which H_2O_2 is converted to water [7]. Superoxide anion radicals (O_2^{*}), the most common radical species found in biological systems, are formed when oxygen acquires an additional electron, leaving the molecule with only one unpaired electron. Within the mitochondria, O_2^{*} is continuously generated by the inherent metabolic process. The rate of formation, however, depends on the amount of oxygen flowing through the mitochondria at any given point in time. O_2^{*}, due to its high reactivity and unique properties, which are briefly mentioned below, is capable of inflicting significant damage to living cells *in vivo*. While as a reducing agent it can generate OH^* through a Fe^{2+}-catalyzed Fenton-type reaction, it can also behave as an oxidizing agent and decompose to liberate dioxygen [19]. Under both situations, the resulting reactive species can cause damage to biomolecules in proximity. Other OFR species can also be formed by the interaction of O_2^{*} - with H_2O_2 via the classical Fenton type reaction, which is often referred to as Haber-Weiss reaction [16]. In addition, O_2^{*-} is also a potential source of cellular generation of H_2O_2 via dismutation reaction catalyzed by superoxide dismutase (SOD) enzyme [20].

If H_2O_2 is not converted into water, it is also likely that it will form a singlet oxygen ($^1O_2$). $^1O_2$ is, like H_2O_2, a ROS that can behave as a FR during radical reactions and also support cascade of further reactions. $^1O_2$ violates Hund's rule of electron filling. It has eight outer electrons existing in pairs leaving one orbital of the same energy level empty. When oxygen is energetically excited, one of the electrons can jump to empty orbital creating unpaired electrons [21]. $^1O_2$ can then transfer the energy to a new molecule and act as a catalyst for FR formation. The excited molecule can also interact with other molecules leading to the formation of new FRs.

OFR, such as hydroxyl radical (OH^*) and superoxide anion radical (O_2^{*-}), and/or ROS, such as hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$) are primarily produced in the body as a consequence of aerobic metabolism(s) [4,5,13]. Therefore, they are mainly produced in the organelles where oxidative metabolisms occur, such as the mitochondria, lysosomes, peroxysomes, endoplasmic reticulum (ER), cytoplasm, and nuclear, organelle and cellular membranes. However, the main site of the production of cellular FR is the interior of the mitochondria, where electron transport chain resides. Superoxide anion radicals (O_2^{*}) are formed when molecular O_2 acquires an additional electron from the electron transport chain, leaving the molecule with only one unpaired electron. Occasionally, atmospheric O_2 might exhibit two radical centers. In this case it exists as triplet oxygen, which is essentially a diradical in its ground state. The diradical state of O_2 confers high reactivity and paramagnetic characteristics to atmospheric O_2, which may act as a catalyst for the downstream cascade of reactions.

FR Generation in a Living Cell

FR is produced in a living cell as a by-product of endogenous metabolic processes. In addition, hydrolysis of cellular H_2O catalyzed by exogenous factors also generates FR [22]. As is obvious, the major FR load of metabolically active cell comes from the oxidative metabolic reactions [23, 24]. Exogenous factors, such as, radiation and natural or artificial chemicals, categorized as xenobiotics, to which humans may be exposed, also contribute significantly to FR generation in a living cell. Among them, radiation of different qualities and doses, especially ionizing radiation, constitute the major exogenous sources of FR production in living cells besides the xenobiotics [25]. Approximately, 70% of the radiation induced cellular damage is estimated to be caused by the highly reactive FR produced in the cell due to hydrolysis of H_2O caused by radiation [26]. Likewise, living cells or organisms are continuously exposed to a large number of xenobiotics which populate the environment. Many more xenobiotics get added to different human population groups due to social and cultural legacies, and lifestyle factors prevalent in different societies. The overall estimated load of oxidative damages of different kinds induced in a metabolically active cell exposed to all FR generating systems is put at between 1×10^6 and 1.5×10^6 [23].

Intracellularly, the major site of FR generation is mitochondria, as stated earlier. The electron transport chain, housed in a mitochondrion, facilitates metabolic transfer of electrons from one intermediate molecule to another until it reaches its terminal acceptor, the molecular O_2. In this process, eventually O_2^{*-} is generated. Under normal physiological condition, up to 3% of O_2 molecules are estimated to be converted into O_2^{*} in the mitochondria [27,28]. Likewise, the cytochrome oxidase of the mitochondrial electron transport chain, which uses O_2 to oxidize NADH and $FADH_2$, may add up to 4 electrons on a molecule of dioxygen in a series of reduction reactions. A large number of O_2^{*-} is generated during this process [29]. Some other organelles also contribute to cellular FR generation. Among them, prominent are microsomes, peroxysomes and endoplasmic reticulum (ER). While microsomes mainly generate H_2O_2, peroxisomes contribute to O_2^{*-} production [30,31]. Peroxysomal oxidation of fatty acid, especially during starvation, may also generate H_2O_2. Sizeable amount of cellular FR is also generated in the ER, which hosts monoxygenase system (MOS) responsible for detoxification of foreign toxic compounds, in most mammalian systems. The terminal component of the MOS, the cytochrome P-450, facilitates oxidation and/or hydroxylation of toxic compounds in order to detoxify them. During this process, some of the electrons leak out to molecular O_2, thereby, generating O_2^{*-} [32]. Many membrane bound oxidases, especially on blood lymphocytes and phagocytes, perform detoxification by a cascade of reactions leading to oxidation of foreign toxic or undesirable particles. In the process O_2^{*-} are generated [33]. Therefore, it may be a valid generalization that all organs, which are usually hyperoxygenated under physiological conditions and are rich in microsomes and peroxisomes, contribute to generation of cellular OFR and ROS. Liver is a critical organ that falls under this category. It is estimated

that liver might be contributing to generation of over 50% of H_2O_2 in a mammal.

OFR-induced Damage at Molecular Level

The FRs produced *in vivo*, either by the inherent, endogenous mechanisms or due to exogenous factors, being highly reactive chemical entities, readily interact with biomolecules. Notable among the biomolecules are the nucleic acid, lipids and proteins [34,35]. The interaction of FR with different biomolecules causes a variety of chemical changes in these biomolecules. In the changed chemical status, the biomolecules may acquire different properties, which could disturb or influence the metabolic processes. Therefore, all such changes and alterations that affect normal metabolic processes causing deviation from the 'normal' are considered to be damages. When FR is generated in excessive quantities, the resulting metabolic and cellular damage load becomes significant and detrimental to normal life process.

For obvious reasons, any alteration and/or damage to the genetic material, DNA, is categorized as 'critical' in a living cell. All ROS, OFR and other FR, on the other hand, have high potentials to interact with all constituents of DNA, including bases or nucleotides (NT), sugar moieties and the deoxyribosyl phosphate backbone of DNA. The interaction induces a variety of changes in them. Consequently, FR interaction produces damaged NTs, strand breaks in the DNA double helix and chromosomal aberrations of different types [23,31,36]. As described earlier, the major load of cellular FR comes from the ROS. Therefore, oxidative damage to DNA becomes very critical to the life process [23]. OFR can also oxidize lipids or proteins, thus, generating intermediates that can also react with DNA and result in the formation of adducts on the genetic material [37]. Some oxidative DNA adducts and other lesions are pro-mutagenic. Therefore, the oxidative damage is proposed to play decisive roles in ageing, cellular degenerative processes and development of pathophysiological conditions, including certain cancers [38]. NT damage by OH^* mediated oxidation includes oxidized bases, apurinic (AP) sites, DNA strand breaks, DNA-DNA intra- and inter-strand crosslinks, and DNA-protein crosslinks [39-44]. Radical attack on the NTs generally involves addition of OH to the electron rich double bonds, particularly the purine N-7, C-8 bonds and the pyrimidine 5, 6 bonds and hydrogen abstraction from thymine-methyl groups [45]. Among the oxidized purines, fomamidopyrimidines and 7, 8-dihydro-8-oxoguanine (8-oxo-G), and among the pyrimidine, thymine glycol and its spontaneous hydrolysis products predominate. Therefore, they have been subjects of intensive studies. It is estimated that 8-oxo-G conversion occurs in one out of 10^5 guanidine residues per cell under normal physiological conditions [46]. Detailed investigations suggest that presence of excessive amount of 8-oxo-G in a cell or an organism, indicating high cellular FR activity, might become indicative of such degenerative process as carcinogenesis [47].

Oxidative damage to DNA by FR, therefore, results in point mutations and chromosomal aberrations of different types [23,26,38,48]. The reactive OH^* interact with NT and cause deamination, normally under physiological conditions via a hydrolytic reaction, producing a host of entities depending on the affected NT, such as uracil, 5-hydroxyuracil, 5-hydroxymethyluracil, hypoxanthine, xanthine, deoxyinosine, deoxyxanthosine, etc. [49,50]. In the presence of H_2O_2 also, DNA shows increase in the amount of xanthine [51]. All these events lead to induction of point and other mutations at different sites on DNA, either immediately due to mis-repair or during the following cycle of DNA replication. γ-radiation, which predominantly interacts with DNA via OFR-mediated indirect pathway [25,26], was used as a source of OFR in the genomic DNA of *E. coli* to follow up the strand-breaking and mutation-inducing abilities of OFR using the four microsatellite regions, e.g. TNT1, ANTW, INTG and NCGT [52]. The unpublished results show that while the OFR was able to induce dose-dependent strand breaks, no mutation was induced in the selected microsatellite regions up to 250 Gy [53]. The results point to significant ability of the *E. coli* genomic DNA to repair oxidative stress induced mutations while the strand breaks were not repaired so efficiently.

While a lot of attention is focused on FR-induced damage to DNA, ROS and OFR also readily interact with other important components of cells. Among them, cell membrane, which is responsible for the maintenance of organeller and cellular microenvironments, is a major target of FR injury. Due to its critical roles, membrane damage has serious biological consequences [54]. OFR, ROS and other FR species oxidize membrane phospholipids containing polyunsaturated fatty acids (PUFAs), which are abundant in cellular membranes and in low density lipoproteins (LDL) [55]. PUFAs generate hydroperoxides (HPs) upon oxidation by OFR. The HPs are short lived chemical moieties and react with cellular metals to produce several reactive and cytotoxic products, such as saturated aldehydes and epoxides [56]. One of the most common aldehyde products of lipid peroxidation found in cells is malondialdehyde (MDA). MDA can further react with NTs like guanine, adenine and cytosine to produce their adducts [57]. Some of these adducts have been shown to be electophilic in nature. Due to this, they might contribute to DNA-DNA cross linking process [58]. The high susceptibility of PUFA to FR-induced oxidation is due to the presence of doubly allylic methylene groups on PUFA.

Pentadienyl radical formed via oxidation of lipids can potentially abstract H atoms from the additional polyunsaturated fatty acyl groups generating new pentadienyl radicals and hydroperoxides. For example, linoleic acid (LA), one of the most common animal fatty acids, can produce 9-hydroperoxy-10, 12-octadienoic acid (9-HPODE) and 13-hydroperoxy-9,11-octadienoic acid (13-HPODE) via this non-specific FR pathway or by the action of lipoxygenases. Fragmentations of hydroperoxides produce numerous reactive compounds, including γ-hydroxy-α-alkenals, such as 4-hydroxy-2-nonenal (HNE) and 9-hydroxy-12-oxo-10 (E)-dodecenoic acid (HODA). γ-Hydroxy-α-alkenals are toxic and can react with proteins producing pyrrole adducts [59,60]. It is known that the PUFAs are critical to maintenance of normal membrane fluidity, and, therefore, for the overall functionality of the cellular membrane. Oxidation of PUFAs due to ROS, OFR and other FR species, would alter these membrane characteristics. Consequently, the homeostasis of cellular microenvironment is disturbed. This significantly affects the overall well being of the cell. In addition, peroxidation of PUFAs on the membrane may also result in breakdown of various aldehydes, thereby, indirectly contributing to the formation of DNA adducts [61]. It is reported that the protein bound aldehydes in rats correlate well with ageing [62], thereby, suggesting that such metabolic products do contribute to various cellular degenerative processes. OFR and ROS may also cause modifications of proteins. Oxidative attack of the polypeptide backbone is initiated by the OH-dependent abstraction of the α-hydrogen atom of an amino acid residue to form a carbon-centered radical [63]. Practically all amino acids can serve as targets for oxidative attack by OFR and/or ROS, although some amino acids, such as tryptophan, tyrosine, histidine, and cysteine are particularly sensitive to ROS and OFR [64-66].

OFR-induced Damage at Cellular Level

ROS can damage mitochondrial macromolecules either at or near the site of their formation. Therefore, in addition to serving as a platform of generation of OFR and/or ROS, the mitochondria themselves can be damaged by OFR and/or ROS [67,68]. It has been reported that OFR induces mitochondrial DNA (mtDNA) damage as well as a decline in the mitochondrial RNA (mtRNA) transcripts, protein synthesis and mitochondrial function. mtDNA is more susceptible to oxidative attack than nuclear DNA, due to various reasons, including (a) its proximity to the respiratory chain in

the mitochondrial inner membrane, (b) lack of protective histone-like proteins, and (c) poor repair activity found in mitochondrion [69]. mtDNA mutations may lead to prevention of its replication or expression and the mitochondrial ROS may result in the progressive destruction of the mtDNA. Such mtDNA damage can lead to a decline of mtRNA transcription and a loss of function [70]. Studies have shown OFR and ROS-mediated mtDNA damage, alterations of gene expression and mitochondrial dysfunction in cultured vascular endothelial and smooth muscle cells *in vitro* [71]. It has been postulated that OFR and ROS induced mtDNA damage, which leads to defects of mtDNA-encoded gene expression and respiratory chain complex enzymes, may contribute to the progression of left ventricular remodeling and failure after myocardial infarction [72]. Similarly, confocal microscopy reveals gross structural changes that are induced in different organelles by γ-radiation in mouse bone marrow and spleen cells. They include swelling and blebbing of nuclear membrane leading to increase in the size of the nucleus, irregular nuclear and cellular contours, stretched out perinuclear regions and enlarged nucleoli [53].

OFR-induced Damage at Organism Level

FR-induced oxidative damage is believed to constitute the very first step of cellular damage that can induce metabolic and physiological degenerative processes, including mutagenesis, acute as well as chronic inflammatory disorders, atherosclerosis, myocardial infarction, cardiovascular disorders, rheumatoid, malignancies and ageing. The list is only suggestive and not exhaustive, which clearly indicates the deep influence of oxidative damage to the physiology and the overall well being of a human being. The FR-induced damage is also postulated to be involved in Alzheimer's and Parkinson's diseases [73]. Since the major source of FR load is inherent oxidative metabolic processes, it appears that cellular degenerative processes are dependent on the status of the metabolism. As exogenous factors also increase the metabolic load of FR, it would be logical to think that different exogenous factors would enhance the pace of cellular degeneration, pathophysiology and aging. These hypotheses get support from the observed fact that the cellular concentration of OFR and ROS, particularly OH^*, correlates rather well with various pathophysiological conditions [74-76]. Similarly, under conditions of high cellular 8-oxo-G, which is indicative of extensive oxidative damage to cellular PUFA, inflammatory disorders increase [77,78]. These observations suggest that the cellular microenvironment is greatly influenced by the cellular FR load. However, in an interesting study that needs to be followed up carefully, alcohol seemed to reverse the oxidative load of 8-oxo-G in human lymphocytes [79]. The quality and quantity of alcohol also inversely correlated with the FR load. In contrast, cigarette smoking, an exogenous factor that is usually greatly implicated in increased oxidative stress, showed lower than expected impact upon pathways of DNA damage induction and repair using several parameters [35,80]. Further studies seem necessary to clearly unravel the effects. Nonetheless, these observations may indicate that physiological interventions may enhance or reverse pace of oxidative cellular damage.

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