Pawan Kumar Maurya · Pranjal Chandra
Editors

Oxidative Stress: Diagnostic Methods and Applications in Medical Science

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Preface

An interdisciplinary approach in science has led to the development of oxidative stress monitoring in various models for real clinical and biomedical applications. Oxidative stress is associated with a diminished capacity of a biological system to overcome an overproduction of reactive oxygen species and other free radicals. Since oxidative stress is a leading cause of many diseases, there is an urgent need for oxidative stress monitoring using analytical approaches and its prevention. There is growing evidence that oxidative stress may cause autism in children. Several biomarkers of oxidative stress have also been identified. The development of methods requires versatile knowledge of biology, chemistry, molecular biology, immunology, and microbiology. The biomedical application of oxidative stress monitoring is extensive, and future influence will be matchless. Monitoring oxidative stress in humans can be done indirectly, by assaying the product of oxidative damage in clinical samples or by investigating the antioxidant potential of an organism, tissue, or body fluids to withstand further oxidation. Recent advances in nanotechnology led to the development of new diagnostic methods for monitoring oxidative stress status in medical science using nanodiagnostics methods. In this book, we will therefore also deal with nanodiagnostics methods for measuring oxidative stress. However, we will focus on the more recent development in this field.

In view of the clinical and healthcare importance of oxidative stress monitoring, this book has been proposed. This book starts with a brief introduction of the role of oxidative stress in major disease conditions and it mainly focuses on the current trends in oxidative stress monitoring using different in vitro and in vivo models which are very much important for on-site clinical applications and can be used in the prevention of diseases.

After numerous deliberations, we came up with the idea to explore the possibility of developing a book on oxidative stress to partially fill the void. Finally, we decided to develop a book by inviting experts in the field who have relevant research experience and an understanding of the intricacies of the subject. We had in mind a book that would help to alleviate most of the worries of both students and instructors. We discussed, argued, and disagreed until we came up with the thought that a resource book would be a reasonable format, as it could provide sufficient information and literature for instructors to teach the subject while providing students with ample information to gain better insight into the subject. Once we formulated these

thoughts to develop a resource book, the ball started rolling, and we identified various experts and convinced them to contribute chapters.

This is our maiden effort to produce a book on oxidative stress and its impact in medical science to help students and instructors. We hope that we will get support from the readers of this book. We are always open to criticism, suggestions, and recommendations that can help to improve the content and presentation of the book. Your suggestions and criticisms will give us an opportunity to explore other aspects of oxidative stress in our future ventures and endeavors.

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About the Editors

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Chemical Biology of Oxidative Stress and Its Role in the Pathophysiology of Neuropsychiatric Disorders

1

Dipti Chourasia and Sumit Sethi

Abbreviations

AB	Antioxidative barrier
BD	Bipolar disorder
CAT	Catalase
ETC	Electron transport chain
GPx	Glutathione peroxidases
GSR	Glutathione reductase
GSH	Glutathione
LPO	Lipid peroxidation
MDD	Major depressive disorder
NAC	N-Acetylcysteine
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SCZ	Schizophrenia
SOD	Superoxide dismutase
TBARS	Thiobarbituric acid reactive substances

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1.1 Introduction

A characteristic feature of living cells in aerobic organisms under physiological conditions is the existence of a dynamic pro-/antioxidative equilibrium. It is a balance derived from the presence of active oxidative agents and those preventing their action that constitutes the so-called antioxidative barrier (AB). The presence of substances with oxidizing potential, which can be divided into direct oxidants and indirect prooxidants, is an unavoidable consequence of the participation of oxidation and reduction processes in the most important life activity, that is, metabolism, especially respiration. Pro-/antioxidative imbalance ensuing in a gradual increase in prooxidants over antioxidants is called oxidative stress (OS).

OS and subsequently formed reactive oxygen species (ROS) and reactive nitrogen species (RNS) affect cellular processes, leading to cellular injury. Furthermore, collecting data suggests that oxidative-free radicals play a significant role in the pathophysiology of a variety of neuropsychiatric disorders, e.g., major depressive disorder (MDD), schizophrenia (SCZ), and bipolar disorder (BD). These studies have led to the discovery of a novel treatment approaches using antioxidants as the adjunctive remedy in the abovementioned neuropsychiatric disorders. This chapter summarizes the current findings on the chemical biology of OS and its role in the pathophysiology of these neuropsychiatric disorders. This chapter also discusses the use of antioxidants as adjunctive remedy in the abovementioned diseased conditions.

1.2 Reactive Species, Cellular Pro-/Antioxidative Balance

The most active oxidizing agents, ROS and RNS, are produced endogenously as by-products in one-electron processes and in processes involved in defense against pathogens and as intracellular and intercellular signaling molecules. Of course, when cells are exposed to a variety of exogenous xenobiotics, ROS and RNS may come directly from the oxidative metabolism of these compounds, or their content increases due to the stimulation of the endogenous production in response to exposure.

Hypothetically, five borderline states can be distinguished based on the relationship between the total sum of ROS and RNS concentrations in the cell and the capacity of the AB, and the impact of these relationships on the resultant pro-/antioxidative equilibrium can be deduced. Figure 1.1 shows schematically these five cases.

When cellular AB balances the supply of ROS and/or RNS, the equilibrium is maintained. This applies both to the physiological condition without any change in the level of both reactive forms and components of AB (Fig. 1.1a), as well as the situation, in which the increase of ROS and/or RNS concentration is accompanied by mobilization of the barrier (Fig. 1.1b). Three other cases concern the changes that shift the pro-/antioxidative balance toward more severe oxidative processes resulting either from an increase in the concentration of ROS and/or RNS at constant capabilities of AB (Fig. 1.1c) or no change in concentrations of ROS and/or RNS but weakening of the barrier (Fig. 1.1d) or finally in the case when the increase in ROS and/or RNS is accompanied by a simultaneous weakening of AB (Fig. 1.1e).

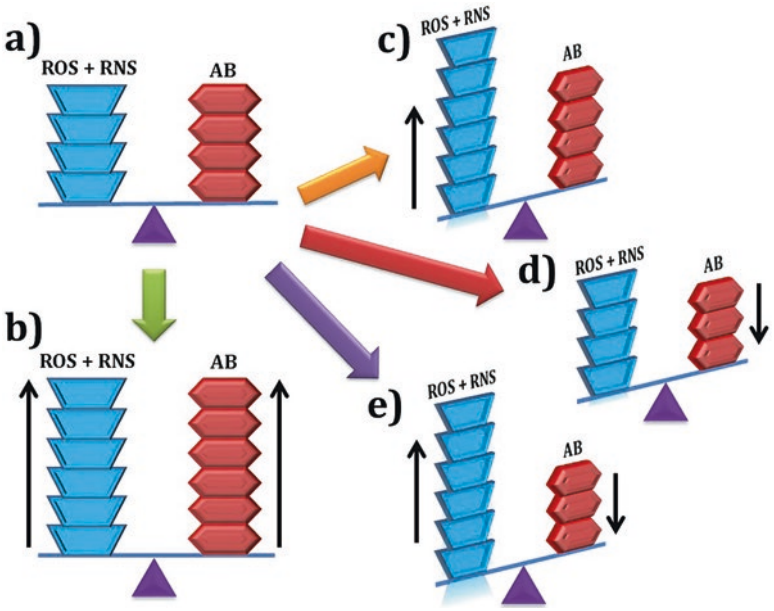

Fig. 1.1 Five hypothetical conditions of pro-/antioxidative balance resulting from the mutual relation between sum of ROS and RNS levels and the capacity of the AB

Possibly, the sixth case can actually exist, when the AB is fortified by natural antioxidants from the diet (e.g., natural polyphenols) in the absence of additional sources of ROS and/or RNS. Such case has no biological significance. The increased inactivation of ROS and/or RNS does not apply to these reactive species that are involved in cell signaling pathways.

ROS and RNS can be regarded as analogous products of two biochemical pathways initiated by the superoxide anion radical ($O_2^{-\cdot}$) and nitric oxide (NO) as precursor molecules. The main pathways of ROS and RNS transformations in biological systems are shown in Fig. 1.2.

1.3 Chemical Biology of ROS and RNS in Brain Environment

Due to the high oxygen demand and high content of lipids, nerve tissue is particularly sensitive to oxidative damage involving ROS and RNS. Critical steps in this regard include intensive aerobic respiration of nerve cells and severe peroxidation of main components of tissue structures. In the cell respiration, one-electron reduction processes of oxygen molecules are the most important endogenous source of $O_2^{-\cdot}$ (Eq. 1.1). They run parallel to four-electron reduction involving cytochrome c oxidase in which there is no formation of ROS (Eq. 1.2).

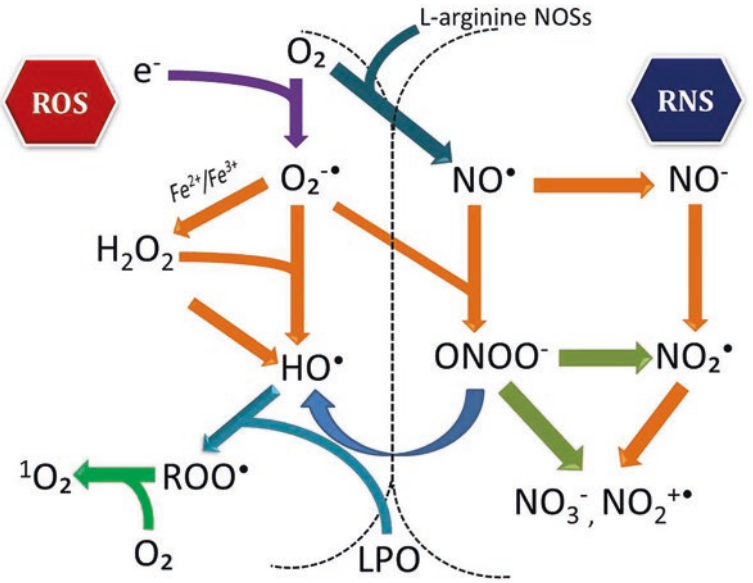

Fig. 1.2 The primary cellular ROS and RNS as well as their biochemical interrelations

$$O_2 \xrightarrow{e^-} O_2^{-\bullet} \tag{1.1}$$

$$O_2 \xrightarrow{4e^-,4H^+} 2H_2O \tag{1.2}$$

The resulting radical anion is a precursor to other ROS (Eq. 1.3) including chemically most active hydroxyl radical ($OH^{\cdot}$).

$$O_2^{-\bullet} \rightarrow HO_2^{\bullet} \xrightarrow{e^-H^+} H_2O_2 \xrightarrow{e^+} HO^{\bullet} + OH^- \tag{1.3}$$

Hydroperoxyl radical ($HO_2^{\cdot}$), which is a protonated form of superoxide anion, although its content in cytosol environment is only 0.3% of the ionic form, has many features which make it preferable in reactions with molecules of biological importance. Interestingly, $HO^{\cdot}$, despite a much lower availability to double bond region in the bilayer than singlet oxygen ($^1O_2$) and $HO_2^{\cdot}$, is the most effective factor that initiates lipid peroxidation (LPO) directly due to the very high chemical reactivity. In relation to lipids, its reactivity is about five orders of magnitude larger than that of the singlet oxygen.

Rate constant of hydroxyl radical reaction with unsaturated fatty acids is 1.0×10^9 [$l \times mol^{-1} \times s^{-1}$], while the corresponding constant for the reaction of superoxide anion with arachidonic acid in alkaline pH is only $10^{-2} - 10^{-1}$ [$l \times mol^{-1} \times s^{-1}$] and for hydroperoxyl radical in acidic pH – 3.1×10^3 [$l \times mol^{-1} \times s^{-1}$] (Table 1.1; referred from Moniczewski et al. 2015). In this situation, the level of the hydroxyl radical formed is the determining factor of the risk of LPO process. Impact on this level is dependent, in addition to the amount of available oxygen, on the presence of iron or

Table 1.1 Characteristics of chemical activity of selected ROS and RNS in biological systems

Name	Structure	Category	$E^{0/}$ [V]		k_{GSH} (M^{-1} s^{-1})	k_{PUFA} (M^{-1} s^{-1})
ROS						
Singlet oxygen	$^1O_2$	Two-electron, non-radical	$^1O_2$/ $O_2^{-\cdot}$	0.65	2.0×10^6	1.0×10^4
Superoxide radical anion	$O_2^{-\cdot}$	One-electron, free radical	$O_2^{-\cdot}$, $2H^+$/ H_2O_2	0.94	~10–10^3	1.0×10^{-1}
Hydroperoxyl radical	$HO_2^{\cdot}$	One-electron, free radical	$HO_2^{\cdot}$, H^+/ H_2O_2	1.06	Non-detectable	1.2–3.0×10^3
Hydrogen peroxide	H_2O_2	Two-electron, non-radical	H_2O_2, $2H^+$/ H_2O	1.77	0.9	nd
Hydroxyl radical	$HO^{\cdot}$	One-electron, free radical	$HO^{\cdot}$, H^+/ H_2O	2.31	1.0×10^{10}	1.0×10^9
Hydroxyl anion	OH^-	Two-electron, non-radical	nd		nd	nd
Peroxyl radical	$ROO^{\cdot}$	One-electron, free radical	$ROO^{\cdot}$, H^+/ ROOH	1.00	nd	nd
Lipid peroxyl radical	$LOO^{\cdot}$	One-electron, free radical	nd		nd	1.3×10^3
Hypochlorous acid	HOCl	Two-electron, non-radical	HOCl, H^+/ Cl^-, H_2O	1.28	1.24×10^8	nd
Hypobromous acid	HOBr	Two-electron, non-radical	HOBr, H^+/ Br^-, H_2O	1.13	nd	nd
Hypothiocyanous acid	HOSCN	Two-electron, non-radical	HOSCN, H^+/SCN^-, H_2O	0.56	nd	nd

(continued)

Table 1.1 (continued)

Name	Structure	Category	$E^{0/}$ [V]		k_{GSH} (M^{-1} s^{-1})	k_{PUFA} (M^{-1} s^{-1})
RNS						
Nitric oxide	$NO^{\cdot}$	One-electron, free radical	$NO^{\cdot}/NO^{-}$	-0.80	0.08	nd
Nitrogen dioxide	$NO_2^{\cdot}$	One-electron, free radical	$NO_2^{\cdot}/NO_2$	1.04	3.0×10^7	1.0×10^5
Peroxynitrite	$ONOO^{-}$	Two-electron, non-radical	ONOOH, H^+/NO_2^-, H_2O	1.40	6.6×10^2	nd
Nitrite	NO_2^{-}	Two-electron, non-radical	nd		nd	nd
Nitrate	NO_3^{-}	Two-electron, non-radical	nd		2.0-6.0×10^3	nd
Nitronium ion	$NO_2^{+\cdot}$	One-electron, free radical	nd		nd	nd

nd not determined

copper ions and the size of the pool of the nitric oxide (NO). The presence of metal ions catalyzes the pathway known as the Fenton reaction (Eq. 1.4). In this process, the substrate, that is, hydrogen peroxide (H_2O_2), generates an equimolar amount of the hydroxyl radical.

$$O_2^{-\bullet} + H_2O_2 \xrightarrow{Fe^{2+}/Fe^{3}\text{or}Cu^{+}/Cu^{2+}} HO^{\bullet} + OH^{-} + O_2 \tag{1.4}$$

One of the significant sources of iron ions to the Fenton reaction in the nervous system can be supplied by oxidized iron-sulfur clusters in ferredoxin family proteins [4Fe-4S] including, among others, nicotinamide adenine dinucleotide (NADH) dehydrogenase [ubiquinone] iron-sulfur protein 8, in the mitochondrial respiratory chain. $O_2^{-\cdot}$ is a well-known highly reactive agent toward iron-sulfur clusters [13]. Reaction with [4Fe-4S] cluster could be shown as follows (Eq. 1.5):

$$\left[4Fe-4S\right]^{2+} + O_2^{-\bullet} + 2H^{+} \rightarrow H_2O_2 + \left[4Fe-4S\right]^{3+} \rightarrow \left[4Fe-4S\right]^{2+} + Fe^{2+} \tag{1.5}$$

The rate constant of the above reaction is in the range typical of the reaction with the diffusion-controlled rate ($k > 10^9$ M^{-1} s^{-1}).

The supply of H_2O_2 in the cell depends, among other sources, on the amount of superoxide anion produced in the mitochondrial respiratory chain, which can be reduced in one-electron process or may dismutate in an enzymatic process involving superoxide dismutases (SODs). The reaction that competes with Fenton reaction involves decomposition of hydrogen peroxide with the participation of catalase (CAT). Among transformations of superoxide anion, the reaction with NO is kinetically favored ($k = 3.4 \times 10^7$–7×10^9 [$l \times mol^{-1} \times s^{-1}$] (Pryor and Squadrito 1995). The product of this reaction, i.e., peroxynitrite anion, decomposes to the hydroxyl radical and nitrogen dioxide. Nitrogen dioxide more slowly almost by half reacts with unsaturated fatty acids than the hydroxyl radical ($k = 1 \times 10^5$ [$l \times mol^{-1} \times s^{-1}$] (Table 1.1)), but like other RNS is an easily nitrating agent of amino acid residues of proteins, including in particular tyrosyl residues. It is interesting that NO is the only one of the free radicals, which does not promote LPO chain reaction. This is because it reacts very quickly with peroxyl radicals leading to chain termination (Ohara and Sayuri 2012). Within nervous tissue, this situation is very rare, because kinetically competing reactions comprise the reactions of NO with superoxide anion and molecular oxygen, which are in considerable excess in this environment.

Chain LPO process is a generator of the group of hydrocarbon ($R^{\cdot}$), lipid ($L^{\cdot}$), peroxyl ($ROO^{\cdot}$), and lipid peroxide ($LOO^{\cdot}$) radicals. Most of them are able to induce a singlet state of the oxygen molecule ($^1O_2$) by the Russell mechanism (Russell 1957). As Cordeiro (2014) demonstrated, this form of oxygen easily penetrates the phospholipid bilayer and may initiate further peroxidation chains.

1.4 Cellular Source of ROS, RNS, and Free Radicals

ROS and RNS are formed as by-products during physiological processes in cells and may depend on factors related to the lifestyle. Thus, ROS and RNS may be produced in the organism due to the action of endogenous and exogenous factors. There are numerous internal sites of ROS/RNS production. The main locus of reactive species formation, including free radicals, is all the cells of organs and tissues of the body having mitochondria and peroxisomes. Free radicals are produced as a normal part of metabolism within the mitochondria, peroxisomal beta-oxidation, cytochrome P450, and phagocytosis, through phospholipase A2-activating protein leading to an arachidonate cascade, protease-activating enzymes leading to the conversion of xanthine dehydrogenase to xanthine oxidase, NADPH oxidase-activating enzyme complex by hypoxia and ischemia and physical exercise (Dikalov 2011).

1.5 Mitochondrial Sources of Oxidatively Reactive Molecules

It is believed that under physiological conditions, the mitochondrial respiratory chain is the most efficient source of ROS in a mammalian cell. In particular, defective mitochondria release large amounts of ROS. The ROS released by the

mitochondria can be scavenged by cellular antioxidant processes or, through $HO^{\cdot}$ formation, cause oxidative damage to polyunsaturated fatty acids in the biomembranes, proteins, enzymes, and nucleic acids (Table 1.2; referred from Moniczewski et al. 2015). The electron transport chain (ETC) in the inner mitochondrial membrane includes a number of multiprotein electron carriers assembled into four redox complexes (complexes I–IV). There are enzyme carriers: complex I (NADH, ubiquinone reductase, EC 1.6.5.3), complex II (succinate ubiquinone reductase, EC 1.3.99.1), complex III (ubiquinol cytochrome c reductase, EC 1.10.2.2), and complex IV (cytochrome c oxidase, EC 1.9.3.1). Their function is to transport electrons from NADH and $FADH_2$ to molecular oxygen, the terminal electron acceptor. Primary bioenergetic function of ETC is the synthesis of adenosine triphosphate (ATP) by oxidative phosphorylation. The sites of ROS production in the mitochondrial ETC have been confined in the complex I and complex III, although the complex II may also contribute to ROS production.

The primary ROS type formed by mitochondria is $O_2^{-\cdot}$ released primarily from the mitochondrial electron transport by auto-oxidation reactions under normal conditions. The mitochondrial antioxidant manganese superoxide dismutases (Mn-SOD, EC 1.15.1.1) and cytoplasmatic copper-zinc superoxide dismutase (Cu/Zn-SOD) accelerate the dismutation of $O_2^{-\cdot}$, converting it to H_2O_2 and O_2, which results in very low superoxide levels (Morán et al. 2012). Then, CAT (EC 1.11.1.6) and glutathione peroxidases (GPx; EC 1.11.1.9) transform H_2O_2 to water.

A small proportion of O_2 is involved in ROS production, in particular, $O_2^{-\cdot}$, H_2O_2, and the extremely reactive $HO^{\cdot}$. The process of generation of $HO^{\cdot}$ may be the result of H_2O_2 conversion through the Fenton reaction (Eq. 1.4), in which the

Table 1.2 Mitochondrial formation of ROS

Component	Location	ROS
Mitochondrial complex II (succinate dehydrogenase, SDH)	Inner surface/inner mitochondrial membrane	$O_2^{-\cdot}$
Mitochondrial complex I (NADH dehydrogenase)	Inner surface/inner membrane	$O_2^{-\cdot}$; H_2O_2
Mitochondrial complex III (bc1 complex, ubiquinone-cytochrome c reductase)	Inner/inner membrane	$O_2^{-\cdot}$
Mitochondrial cytochrome b5	Outer membrane	$O_2^{-\cdot}$
Monoamine oxidases (MAO-A and MAO-B)	Outer membrane	H_2O_2
Mitochondrial aconitase	Mitochondrial matrix	$O_2^{-\cdot}$; $HO^{\cdot}$
Ketoglutarate dehydrogenase complex (KGDHC, 2-oxoglutarate dehydrogenase)	Inner membrane on the matrix side	$O_2^{-\cdot}$; H_2O_2
Dihydroorotate dehydrogenase (DHOH)	Outer surface of inner membrane	$O_2^{-\cdot}$; H_2O_2
Pyruvate dehydrogenase complex (PGDH)	Mitochondrial matrix	$O_2^{-\cdot}$; H_2O_2
Oxoglutarate dehydrogenase complex (OGDC)	Mitochondrial matrix	$O_2^{-\cdot}$; H_2O_2

passage of H_2O_2 into radical is catalyzed by transition metal ions (Fe^{2+}, Cu^{+}) and results in the observed oxidative damage.

Intact mitochondria produce highly diffusible, membrane permeable NO molecule, which is formed from L-arginine and L-citrulline in a reaction catalyzed by the NO synthase (NOS, EC 1.14.13.39). The formation of NO is catalyzed by three isoforms of NOS that have different tissue distribution and cellular location: Ca^{2+}-dependent activation neuronal NOS (nNOS) which is expressed in glial cells (astrocytes, microglial cells, and macrophages); endothelial NOS (eNOS) expressed in vascular endothelium and Ca^{2+}-independent inducible NOS (iNOS), the expression of which can be induced by bacterial lipopolysaccharide; cytokines; and other agents. NO is an essential physiological messenger being implicated in important functions in the central nervous system, such as the regulation of memory and cerebral blood flow. Additionally, NO plays an important role in the regulation of the immune defense as well as the modulation of cytokine response.

The overproduction of NO leads to an increase nitrosative stress which can direct to nitrosylation reactions that can change the structure and function of DNA, proteins, and lipids and inhibit their normal function. As the half-life of NO is very little, it can be converted to various RNS, such as nitrosonium cation (NO^{+}), nitroxyl anion (NO^{-}), and nitrate (NO^{3-}), to generate peroxynitrite anion ($ONOO^{-}$), a potent oxidant that is an outcome from the reaction between NO and $O_2^{-\cdot}$ produced during mitochondrial respiration. These compounds are known to promote neuronal damage, destroy cellular macromolecules, and can cause oxidative damage, nitration, and S-nitrosylation of biomolecules including proteins, lipids, and DNA. They also play a prominent role in excitotoxic neuronal death (Valko et al. 2007). The enzyme family of monoamine oxidase (MAO; EC 1.4.3.4) which catalyzes the transfer of hydrogen to oxygen to form H_2O or H_2O_2 is another important mitochondrial source of ROS. MAOs are restricted in the outer mitochondrial membrane and are found in both the central nervous system and in the periphery. Two forms of the enzyme (MAO-A and MAO-B, EC 1.4.3.4) catalyze the oxidation of biogenic amine neurotransmitters, such as norepinephrine, dopamine, and 5-hydroxytryptamine (serotonin), but they generate free radicals during their activity causing OS (Sethi and Chaturvedi 2009; Sethi et al. 2010). Catecholamines are oxidized at comparable rates by either enzyme under physiological conditions. Dopamine oxidation process is the most important source of H_2O_2 in the brain through the MAO-induced deamination into the corresponding aldehydes (Meiser et al. 2013).

1.6 Non-mitochondrial Sources of Reactive Molecules and Free Radicals

Alternative potential sources of ROS/RNS are non-mitochondrial and include ROS/RNS released due to respiratory burst of phagocytic cells, microsomal cytochrome P450 enzymatic metabolism of xenobiotic compounds, oxidase and reductase enzymes which catalyze one-electron transfer reactions, uncoupled eNOS and iNOS, and lipoxygenase (LOX).

1.7 OS Biomarkers and Antioxidant Levels in Human

It is not feasible to directly measure the free radicals in a given sample due to their very short half-lives and lower concentration. Alternatively, we can determine OS in a sample indirectly by measuring the metabolites of ROS/RNS, levels of antioxidants, activity of the antioxidant enzymes, and biomarkers of oxidative damage, e.g., DNA damage, protein carboxylation, and LPO, which are more stable than free radicals. In human, the above parameters have been measured in various studies that confirm a link between the presence of OS and disorders related to anxiety, depressive, and alcohol use (Hovatta et al. 2010).

Several reports have measured the role of the free radical nitric oxide (NO), which is known to have a dual role, i.e., can act as a ROS and also as a neuronal second messenger. In the brain, it regulates the release of noradrenaline and dopamine and has roles in learning, memory, wakefulness, and feeding. In a diseased condition, the levels of NO may (increase or decrease) or may not change at all (Hovatta et al. 2010). Thus, NO may modulate the OS via several mechanisms.

Another most studied biomarker as a measure of OS in psychiatric disease is the LPO (Sethi and Brietzke 2017). As measured in biological fluids such as urine or blood, the increased levels of LPO have been informed in patients with obsessive-compulsive disorder (OCD), panic disorder (PD), social phobia (SP), anxiety, depressive, and alcohol dependence conditions (Hovatta et al. 2010). Scarce reports have shown the existence of oxidative DNA damage in the circulating DNA in sera of the patients with MDDs (Forlenza and Miller 2006). Another showed DNA damage in the leukocytes of female patients with anxiety or depression (Irie et al. 2001).

Another strong and best studied biomarker for OS is SOD, whose antioxidant activity is increased in many neuropsychiatric disorders. SOD plays an important role in superoxide radical detoxification pathway as it is one of the first enzymes in this pathway. Other members of this pathway with their increased activity in psychiatric disorders are CAT (Szuster-Ciesielska et al. 2008), glutathione reductase (GSR) (Kodydkova et al. 2009), and GPX (Atmaca et al. 2008). However, other study showed decreased activity of GPX (Ozdemir et al. 2009). Furthermore, several other studies on CAT did not find any change in its activity. The possible reason for varying outcomes of these studies could be due to low sample size, heterogenous study subjects, and variation in methods (Tasduq et al. 2003). Broadly, there is increased activity of antioxidant enzymes in neuropsychiatric disorders.

Another interesting gene expression study in peripheral blood of patients with PTSD showed differential expression of enzymes associated with ROS metabolism as well as downregulation of thioredoxin reductase and SOD (Zieker et al. 2007). This study too showed downregulation of the xc(−) cystine-glutamate exchanger (responsible for the cellular uptake of cysteine), the rate-limiting precursor of glutathione (GSH) synthesis. As we see from the cited studies, there is an increased activity of antioxidant enzymes, most probably due to heightened oxidative defense against high levels of ROS, whereas decreased activity of those enzymes may possibly indicate a situation of excess and continuous oxidative damage, where the enzymes are used up in clearing the ROS.

1.8 OS Biomarkers and Antioxidant Levels in Animal Models

In general, animal models for a neuropsychiatric disease or a condition used in research enable us to perform invasive studies (pertaining to specific brain regions); the use of inbred strains minimizes the variations due to heterogenic samples. Furthermore, we can minimize the effect of environmental factors by performing the experiment under controlled conditions.

Biomarkers of OS and concentrations of antioxidant enzymes have been studied in animal models of anxiety-like and depressive-like behavior (Hovatta et al. 2010). In other neurological conditions such as ethanol-induced acute and chronic models of brain damage, a major role of OS has been implicated.

In animal models of anxiety-like and depression-like behaviors can be created by any of the following parameters, e.g., immobilization stress, swim stress, chronic mild stress, maternal separation, olfactory bulbectomy, and social defeat. Once such a stress is given to the animal (mostly, rodent), we can validate their anxiety-like or depressive-like behavior using behavioral tests, e.g., the elevated plus maze, light/dark box, open field, forced swim, and the tail suspension tests. Both physical stress (e.g., immobilization stress) and psychological distress (e.g., communication box paradigm) have been revealed to enhance oxidative damage in the brain. This stress-induced oxidative damage targets lipids, proteins, and DNA in the brain. Similar effects have been seen in the ethanol-induced brain damage (Hovatta et al. 2010).

As seen in the human studies, the levels of antioxidant enzymes in the brain of animal models of stress- and ethanol-induced damage are also altered but in a little different profile. In rodent models, majority of reports suggest a decrease in the antioxidant enzyme profile, and only a few have reported the increase. These variations might be due to different experimental system, stress model, dosage of ethanol, and route of administration along with the region of the brain used for estimation of enzyme levels. The possible reason for more reports on decreased antioxidant activity in rodent models could be due to extreme oxidative damage (as treated in a controlled environment) and less efficient compensatory antioxidant profile. The most common antioxidant studied in most of the studies is GSH, whose levels were shown to decrease after different stress treatments such as physical (restraint stress, chronic mild stress) or ethanol-induced stress. This explains the corresponding decrease in the activities of GPX and GSR enzymes examined in other studies (Zafir and Banu 2009).

Apart from the abovementioned studies on specific biomarkers for OS, a more detailed study on the array of proteins and genes involved in the OS in the brain has been done, which suggests a connection between OS and anxiety (Sethi et al. 2015). Another approach to find the functional role of a gene is the use of knockout mouse models of the genes involved in OS pathway. Knockout mice of the life span determinant Shc1 gene, a controller of mitochondrial ROS metabolism and apoptosis pathway, show reduced levels of intracellular ROS and OS markers and also exhibit reduced anxiety-like behavior (Berry et al. 2007). Another study on the knockout mice lacking the phospholipid transfer protein (PLTP), a transfer factor for vitamin (vitamin E), an

antioxidant, has reduced levels of vitamin E in the brain and increased levels of OS markers with higher anxiety-like behavior (Desrumaux et al. 2005).

Hence, in animal models of psychological and physical stress, there are enlarged levels of free radicals and reduced levels of antioxidant enzymes and their activities, causing more oxidative damage in the brain. Furthermore, global gene and protein expression profiling also suggests a relationship between OS and anxiety-like behavior. The outcomes of these have identified specific pathways involved in OS, and further genetic and pharmacological work on these identified markers can help in better understanding of the oxidative damage mechanism.

1.9 OS and Accelerated Telomere Shortening

Telomere shortening is a phenomenon observed with aging. Telomeres, the terminal ends of chromosomes, are shortened after each cell division, and, hence, telomere shortening is used as a biomarker for aging. Interestingly, OS may also lead to accelerated telomere shortening in neuropsychiatric disorders. In germ cells and stem cells, the length of telomeres is maintained by the activity of RNA-dependent enzyme telomerase. Whereas normal mitotic cells have minimal telomerase activity, OS affects the telomeres more adversely, and the damaged DNA cannot be repaired efficiently. Hence, OS contributes to the accelerated loss of telomeres. Accelerated telomere shortening in leukocytes has been seen in many complex diseases, including mood and anxiety disorders. Mechanisms that contribute to accelerated telomere shortening in vivo remain mostly unidentified. OS shortens telomeres in vitro, as shown by mild stress induced in different ways as well as chronic hyperoxia, treatment with homocysteine, and low doses of tert-butyl hydroperoxide or hydrogen peroxide (von Zglinicki 2002).

Importantly, action of antioxidant vitamin C and the free radical scavenger α-phenyl-*t*-butylnitrone can reverse the OS-induced telomere shortening in vitro. Most probable reason for accelerated telomere shortening due to OS could be that, resultant unrepaired or damaged nucleotide will interfere with the replication machinery at telomeres (von Zglinicki 2002).

1.10 Cellular Balance Between Oxidative Stress and Antioxidant Processes and DNA Damage

DNA damage, whether it is due to OS or cytogenetic damage, would lead to alteration in coding properties and, hence, function of DNA. This DNA damage could be due to various exogenous factors (ultraviolet rays, ionizing radiations, or toxic chemicals) or endogenous factors (OS leading to generation of free radicals, ROS, and RNS) (Kryston et al. 2011). As mentioned before, these free radicals can assault all cellular vital components (DNA, proteins, lipids, and even mitochondrial DNA damage) and can obstruct genome replication and transcription, leading to mutations or genome aberrations which could be lethal too. Further, the DNA damage

could be of various types such as small base chemical modification, helix distorting abrasion, and single-strand and double-strand breaks, all of which can be a challenging task for cellular repair processes. One of the earliest events of DNA damage in actively dividing cells triggers activation of cell cycle checkpoints for DNA repair or undergo programmed cell death (apoptosis), if the damage could not be repaired. DNA damage also turns on signaling pathways that provide to arrest the cell cycle when DNA repair takes place. Hence, in every cell, DNA damage is constantly synchronously followed by DNA repair, involving the recognition, repair, and restitution of the damaged DNA. Four DNA repair pathways are active in the brain pertaining to a specific kind of DNA damage:

1. Base excision repair for correction of base modifications in DNA due to ROS.
2. Nucleotide excision repair eliminates DNA lesions which deform its helical structure and generate cross-links.
3. Mismatch repair plays a key role in postreplication repair of misincorporated bases.
4. Single-strand and double-strand break repairs for renovating DNA strand breaks, either by nonhomologous end joining (NHEJ) or homologous recombination (HR) repair.

Equilibrium between oxidative and reductive processes supplies to the neuronal function. The reductive processes comprise antioxidant enzymes such as CAT, SOD, GPx, and GSR. This built-in clearance system gives defense against free radicals and ROS. Both exogenous and endogenous antioxidants lead to ROS inhibition, binding the metal ions required for ROS generation and eliminating ROS and its precursors. Conversely, in metabolic stress, the intracellular milieu may face accumulation of these free radicals after the antioxidant mechanisms get exhausted. This imbalance in the OS and reductive (antioxidative) processes is responsible for the cellular damage and inefficient DNA repair mechanisms, leading to many biological disorders (Fig. 1.1). In case of neural disorders also, the neuronal damage can be due to the imbalance of OS and reductive processes. These DNA damage and DNA repair imperfections can lead to a diversity of consequences, like impaired DNA replication and transcription processes, which certainly contributes to aging and neurodegenerative diseases (Coppede and Migliore 2015). Furthermore, OS has been concerned as a potential pathophysiological factor in various neuropsychiatric disorders (Pandya et al. 2013). There is a coexistence of DNA damage and impaired DNA repair in these diseases, though still a promising research area.

1.11 OS in Neuropsychiatric Disorders

The brain is regarded as principally susceptible to oxidative damage due to high oxygen consumption and, hence, will generate more free radicals and will have inadequate antioxidant defense mechanisms, elevated lipid content, and excitotoxicity. This turbulence of antioxidant defense mechanisms may lead to a broad

diversity of neuropsychiatric disorders. The role of free radicals and antioxidants in the pathophysiology of SCZ, BD, and MDD are discussed below.

1.11.1 OS in SCZ

Various factors have been implicated in the pathophysiology of SCZ, such as neural maldevelopment, impaired neurotransmission, viral infections, and environmental and genetic factors (Sethi et al. 2016; Tasic et al. 2016). However, mitochondrial dysfunction and OS have been identified as one of the crucial factors in the pathophysiology of SCZ (Maurya et al. 2016). Mitochondrial electron transfer chain is the main source of ROS generation. These ROS are increased along with altered antioxidant defenses, increase in lipid peroxidases, and increased levels of pro-apoptotic biomarkers in patients with neuropsychiatric disorders.

1.11.1.1 Role of Nonenzymatic Antioxidants in the Pathophysiology of SCZ

TAS (total antioxidant status) terminology indicates the combined actions of all the antioxidants in a system. Decreased plasma TAS was seen in a recent study in first-episode drug-naïve patients with SCZ (Li et al. 2011). Furthermore, TAS was less in erythrocytes of children and adolescents with a first psychotic episode than healthy controls (HCs) as well (Mico et al. 2011).

Patients with SCZ were found to have lower levels of plasma antioxidants, albumin, bilirubin, and uric acid. Furthermore, reduced levels of total and reduced GSH, together with modulated antioxidant enzyme activities, have been stated in plasma of patients with drug-naïve first-episode (Raffa et al. 2011). Another study showed the decreased levels of reduced, oxidized, and total GSH in patients with SCZ than the control group in the postmortem brain sample (Gawryluk et al. 2011).

1.11.1.2 Role of Enzymatic Antioxidants in the Pathophysiology of SCZ

Several reports have examined the role of antioxidant enzymes in SCZ, but outcome are not consistent. Reports carry out in patients with neuroleptic-naïve first-episode schizophreniform and SCZ demonstrated both improved and reduced SOD activity, possibly because with series of the illness, the SOD levels increase as a compensatory response to OS. Another study showed that the activity of SOD was considerably lesser in RBCs from patients with SCZ and their impassive siblings than controls. Meta-analysis of the OS markers predicting its risk in SCZ has found that SOD activity was considerably lower in the incompetent type of SCZ patients than HCs (Zhang et al. 2010). Studies using postmortem brain samples also designate distorted antioxidant levels in SCZ patients.

Increased plasma GPx activity (a key enzyme for clearance of H_2O_2 and lipid peroxidases) was found in drug-naïve first-episode SCZ patients as compared to controls (Raffa et al. 2011). However, GPx activity was lower as compared to the controls in neuroleptic-treated chronic SCZ patients, in drug-free female SCZ

patients, and in neuroleptic-naïve psychotic children. However, reports carried out in skin fibroblasts did not demonstrate any alteration in GPx activity in SCZ patients than normal controls. The abovementioned reports signify that alterations in GPx activity in SCZ patients could be related to secondary compensatory phenomenon rather than governed genetically.

Studies on the role of CAT have shown increased activity in erythrocytes but no change in leukocytes of SCZ patients. Furthermore, reduced plasma CAT activity was found in drug-naïve first-episode SCZ patients than controls (Raffa et al. 2011).

1.11.2 OS in BD

BD is a major mood disorder distressing an estimated 1–3% of the population (Sethi and Brietzke 2016; Sethi et al. 2017a). While the pathophysiology of BD is inadequately unstated, the role of OS has been concerned in BD. Several reports have shown that patients with BD have major modulations in antioxidant enzymes, LPO, and NO levels; however, other studies could not reproduce these findings (Andreazza et al. 2008). Andreazza et al. (2008) showed that patients with BD have increased LPO and NO levels, but the compiled data they analyzed found no statistically significance as reported in the original data. They were not succeeding to uncover lowering of GPx activity in patients with BD.

Gergerlioglu et al. (2007) has shown the role of NO on the generation of illusions in patients with BD. Concomitantly, another study also shown higher OS parameters and activated antioxidant defenses in initial manic episodes (Machado-Vieira et al. 2007). Andreazza et al. (2008) reported considerable increases in thiobarbituric acid reactive substances (TBARS) and NO activity in patients with BD with a large effect size for TBARS and a modest effect size for increase in NO. Conversely, no considerable effect sizes were seen for the antioxidant enzymes such as SOD, CAT, and GPx. Yet an additional study observed considerably increased levels of NO and SOD in patients with the serum samples of BD than in controls, with an association seen between the number of the manic episodes and NO levels, but not with SOD levels (Savas et al. 2006).

1.11.3 OS in MDD

MDD is distinguished by considerably reduced plasma levels of important antioxidants, such as vitamin E, zinc, and coenzyme Q10, along with reduced antioxidant enzyme activity by GPx (Maurya et al. 2016). Antioxidants such as N-acetylcysteine (NAC), compounds that imitate GPx activity, and zinc were shown to have antidepressive effects (Maurya et al. 2016).

There is an important relationship between depression and genetic polymorphisms of genes engaged in oxidative pathways, as they affect enzymatic activity in Mn-SOD and CAT (Maurya et al. 2016). Galecki and colleagues reported an increase in CAT activity during acute episodes of depression. Several studies found

considerable decreased activity in GPx enzymes; others found conflicting or no alteration in GPx (Galecki et al. 2009). Maes and co-workers noticed low GPx activity from whole blood of MDD (Maes et al. (2010). Gawryluk and colleagues reported that GPx levels were decreased in postmortem prefrontal cortex samples of MDD and SCZ (Gawryluk et al. 2011).

Even though there are a few discrepancies in the findings, a huge body of literature supports the modulation of OS and antioxidant defense mechanisms in SCZ, BD, and MDD. It would be interesting to investigate antioxidant supplementation in effective attenuation of the disease progression in the abovementioned disorders.

1.12 Antioxidants as a Potential Therapeutic Agent for Neuropsychiatric Disorders

Preceding studies have shown a strong association in the activity of free radicals and antioxidants in the pathophysiology of several neuropsychiatric disorders. Yet, the exact underlying mechanism in the pathophysiology of these disorders is still unclear due to varying study outcomes of the clinical subjects. The standard pharmaceutical treatments for SCZ or mood disorders have restrictions in the long-term management of the abovementioned disorders. Alternatively, researchers have started with antioxidant treatment as adjunct remedy for psychiatric disorders. Data from clinical, preclinical, and epidemiological studies indicates an advantage of using antioxidant compounds that promote neuroprotection and should be regarded as adjunctive remedy in the abovementioned patients.

Numerous antioxidants that could be employed as likely salutary are vitamin E, vitamin C, omega-3 fatty acid, coenzyme Q10, NAC, GSH, melatonin, hydroxytyrosol, resveratrol, quercetin, and lycopene. Apart from them, metal ions such as Zn and Mn are also valuable via advanced antioxidant defense. Here, we discuss the most common antioxidants studied as adjunctive remedies in SCZ, BD, and MDD (Table 1.3; referred from Pandya et al. 2013).

1.12.1 SCZ

Vitamin C (ascorbic acid) is a recognized co-substrate for many enzymes that helped to stimulate antioxidants and increase the effects of other compounds, such as vitamin E. Vitamin E is considered the first line of defense against LPO, protecting cell membranes from free radical damage. Vitamins C and E work mutually by having both hydrophilic and hydrophobic properties which provide total antioxidant defense.

NAC is recognized to reinstate the key endogenous antioxidant GSH and retain the oxidative equilibrium in the cell. Besides this, NAC has directly been revealed to scavenge oxidants, mainly the reduction of the hydroxyl radical, ˙OH, and hypochlorous acid. Increasing data propose the prospective of NAC as an adjunctive

Table 1.3 Adjunctive antioxidant therapy in neuropsychiatric disorders

	Treatment	Trial type	Findings
Schizophrenia			
Vitamins	Vitamins E, C (400 IU:500 mg) along with EPA/DHA	Adjunct therapy for 4 months	Decrease in BPRS and PANSS
	Vitamin C (500 mg/day) with atypical antipsychotics	8-week, double-blind, placebo-controlled, noncrossover trial	Decrease in BPRS and oxidative stress, increase in ascorbic acid levels
N-Acetylcysteine (NAC)	2 g/day	60-day, double-blind, randomized, placebo-controlled trial	EEG synchronization
	1 g orally twice daily	24-week, randomized, multicenter, double-blind, placebo-controlled study	Improved in PANSS total, PANSS negative, PANSS general, CGI severity, and CGI improvement scores
Ethyl eicosapentaenoic acid (EPA)	3 g/day	16-week, double-blind supplementation	No change in symptoms
	1, 2 or 4 g/day	Adjunct therapy for 12 weeks	Improvements in PANSS at 2 g/day
	EPA/DHA (180:120 mg) along with vitamins	Adjunct therapy for 4 month	Clinical significance of improvement remained after EPUFAs normalized to baseline with washout
Bipolar disorder			
Vitamins	12 g of inositol or D-glucose as placebo (stable doses of lithium, valproate, or carbamazepine)	6 weeks, controlled study	No significant effect between groups
	Inositol 5–20 g/day in divided doses to mood stabilizer treatment	6-week, double-blind, placebo-controlled trial	No significant effect between groups
N-Acetylcysteine (NAC)	1 g twice daily	2-month, open-label phase of a randomized placebo-controlled clinical trial	Reduced Bipolar Depression Rating Scale (BDRS)
	1 g twice daily	Randomized, double-blind, multicenter, placebo-controlled study, 24 weeks, with a 4-week washout	Significant improvement on the Montgomery-Asberg Depression Rating Scale (MADRS)
	2 g/day	24-week placebo-controlled randomized clinical trial	Moderated functional outcomes but not depression

(continued)

Table 1.3 (continued)

	Treatment	Trial type	Findings
Ethyl eicosapentaenoic acid (EPA)	1.5-2 g/day	6 months, open-label study	Significant reduction of Hamilton Depression Scale Score
	1-2 g/day ethyl-EPA	12-week, randomized, double-blind, placebo-controlled study	Significant improvement in the HRSD and the CGI scores
	EPA-DHA (360:1560 mg/day)	6 weeks, open-label study	Lower depression and mania improved functionality
Major depressive disorder			
EPA/DHA	1 g doses twice a day for a total of 2 g/day	4-week, parallel-group, double-blind addition of either placebo or E-EPA to ongoing antidepressant therapy	Significant reduction of Hamilton Depression Scale Score
	1 g/d	EPA or placebo for 8 weeks, a double-blind, randomized, controlled pilot study	EPA demonstrated an advantage over placebo in 17-item Hamilton Depression Rating Scale (HDRS-17) but not statistically significant
	Two 500 mg or one 1000 mg capsule daily (400 mg EPA and 200 mg DHA per 1000 mg capsule; 190 mg EPA and 90 mg DHA per 500 mg capsule)	8.16-week, controlled, double-blind pilot study	Significant effects of omega-3 on symptoms using the CDRS, CDI, and CGI
	1.9 g/day (1.1grams of EPA and 0.8 g of DHA)	9.8-week, randomized placebo-controlled study 10	No significant effect on symptom scores
	3.4 g/d (total daily dose of 2.2 g EPA and 1.2 g DHA)	11.8-week, double-blind, placebo-controlled trial	Significantly lower HAMD scores

action in SCZ. Collectively, NAC appears to be a secure, efficient, supportable, and reasonable adjunctive antioxidant molecule for the action of SCZ.

Polyunsaturated fatty acids (PUFAs) contain ω-3 and ω-6 fatty acids. ω-3 Fatty acids such as EPA and DHA are necessary for normal brain development. The probable relations between PUFA and neuropsychiatric disorders have been examined for more than two decades (Sethi et al. 2016, 2017b). PUFAs are necessary components in cell membranes and are considered to affect signal transduction pathways. They are recognized to hinder phospholipase A-2 and cyclooxygenase and considered to alter OS. Increasing data specify a diversity of membrane scarcity in SCZ (Sethi et al. 2016). Hence, improving the lower levels of membrane phospholipid-EPUFAs, mainly AA (20:4n-6, ω 6-EPUFA) and DHA (22:6n-3, ω 3-EPUFA), is a good strategy to defend the membrane from injury in SCZ.

It is recognized that ω-3-fatty acids have antioxidant properties. Supplementation of endothelial cells with ω-3 fatty acids resulted in decreased ROS when compared with ω-6 fatty acids (Sethi et al. 2016). Further research has revealed that n-6 fatty acid-derived eicosanoids such as arachidonic acid (AA) are recognized to have pro-inflammatory roles, while n-3 fatty acids demonstrate the property of anti-inflammation. It has been described that n-3 fatty acids slow down transcriptional activity of NF-κB resulting hindering pro-inflammatory cytokine production. Though, saturated fatty acids increase the activity of NF-κB in macrophages and dendritic cells.

1.12.2 BD

Several researches have shown that LPO and important changes in antioxidant enzymes survive in BD (Andreazza et al. 2008). Thus, it is likely that antioxidant properties of compounds could advance symptoms and should be investigated as probable adjunct remedy. Many studies have demonstrated the potential of inositol, a member of vitamin B family in BD. Though, no major variation in depression scores between bipolar and control group was found. NAC has been widely used as adjunctive remedy for BD. Berk and colleagues carried out a randomized, double-blind, multicenter, placebo-controlled study in BD patients and found that NAC treatment causes a major enhancement on the Montgomery–Asberg Depression Rating Scale (MADRS) and most secondary scales (Berk et al. 2008).

1.12.3 MDD

Research was done by using NAC supplementation to test the improvement of depression. Besides antioxidants, Zn has been revealed to play a significant role in MDD (Maurya et al. 2016). Serum sample of MDD have considerably reduced zinc levels compared to the control. Zn transportation to the brain occurs by crossing the blood-brain and blood-cerebrospinal fluid barriers. Chronic treatment of higher Zn dose is essential to enhance transcriptional and translational activity of BDNF in the frontal cortex, whereas BDNF expression enlarged with lower, more acute doses of Zn in the hippocampus.

1.13 Conclusions

There is increasing evidence that OS is concerned in the pathophysiology of most important neuropsychiatric disorders. Confirmation from postmortem and peripheral tissues indicates changes in both free radicals and antioxidant defense mechanisms in neuropsychiatric disorders. Approaches to improve oxidative injury and thus progress clinical symptoms are of substantial significance. Antioxidants as supplements have provided potential outcomes in the treatment of neuropsychiatric disorders. These works propose that antioxidants should be attempted as

stand-alone intervention or as adjunct to conventional medications. Typically, antioxidants could be more useful as compared to the invented drugs with low risk.

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Oxidative Stress: Diagnostic Methods and Application in Medical Science

2

Vikram Dalal, Narendra Kumar Sharma, and Sagarika Biswas

Abbreviations

4-HNE	4-Hydroxynonenal
8-oxodG	8-Oxo-7-hydroxydeoxyguanosine
A1BG	α-1-β-glycoprotein
AGE	Advanced glycation end
AGP	Alpha-1 acid glycoprotein
AHSG	Alpha 2 HS glycoprotein
AOPP	Advanced oxidation of protein products
AR	Amplex Red
BMPO	5-Tert butoxycarbonyl-5-methyl-1-pyrroline N-oxide
CAT	Catalase activity
CT	3-Chlorotyrosine
DAF-2DA	Diaminofluorescein diacetate
DCF	Dichlorofluorescein
DCFDA	Dichlorofluorescein diacetate
DCFH	Dicholofluorescin
DECPO	5,5-Diethylcarbonyl-1-pyrroline N-oxide
DEPMPO	5 Diethoxyphosphoryl-5-methyl-1-pyrroline N-oxide

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DHR	Dihydrorhodamine 123
DMPO	5,5-Dimethyl-1-pyrroline N-oxide
ELISA	Enzyme-linked immunosorbent assay
EPPN	N-2-(2-Ethoxycarbonyl-propyl)-a-phenylnitrone
ESR	Electron spin resonance
GC-MS	Gas chromatography
GFAP	Glial fibrillary acidic protein
GSH	Glutathionine
GSSG	Oxidized glutathione
H_2O_2	Hydrogen peroxide
Hp	Haptoglobin
HPLC	High-performance liquid chromatography
IEM	Immune electron microscopy
IHC	Immunohistochemistry
INT	2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride
MAA	Malondialdehyde-acetaldehyde
MALDI	Matrix-assisted laser desorption ionization
MBL	Mannose-binding lectin
MDA	Malondialdehyde
NBT	Nitroblue tetrazolium
NQO1	Quinine oxidoreductase 1
NQO2	Quinine oxidoreductase 2
PBN	N-tert-butyl-a-phenylnitrone
PC	Protein carbonyls
PCOOH	Phosphatidylcholine
PEOOH	Phosphatidylethanolamine
RA	Rheumatoid arthritis
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
RS	Reactive species
SLE	Systemic lupus erythematosus
SOD	Superoxide dismutase
TAC	Total antioxidant capacity
TBARS	Thiobarbituric acid reactive substances
TNF	Tumor necrosis factor
TTR	Transthyretin
YFP	Yellow fluorescent protein

2.1 Oxidative Stress

Oxidative stress is an imbalance between production of free radicals and biological system's ability to counteract or detoxify the reactive intermediates through neutralization of antioxidants. The production of peroxides and free radicals can cause toxic effects on the components of cells like proteins, lipids, and DNA.

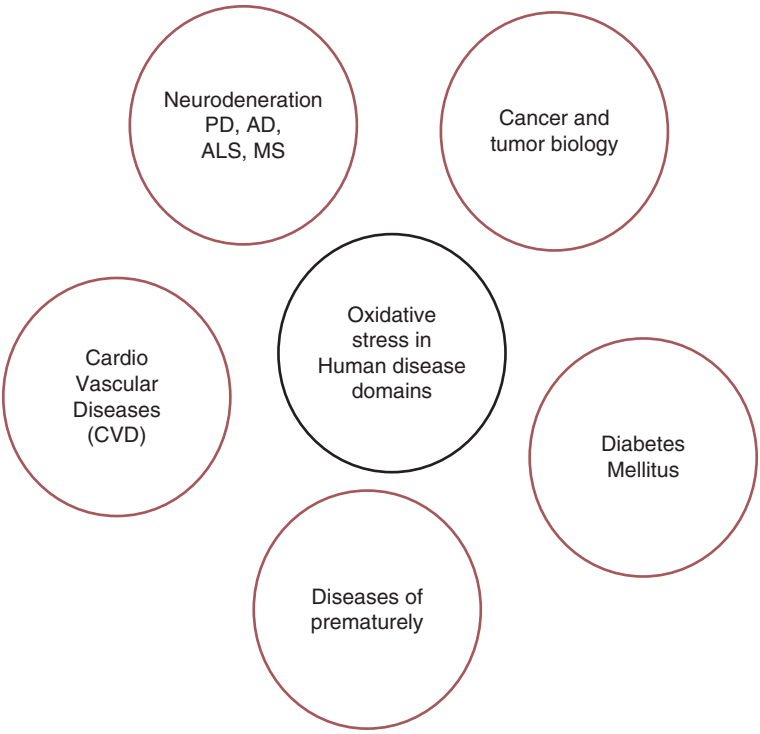

Fig. 2.1 Oxidative stress causing human diseases

Reactive oxygen species like O_2^- (superoxide radical), OH (hydroxyl radical), and H_2O_2 (hydrogen peroxide) can cause base damage. Oxidative stress can cause several diseases (Fig. 2.1) like Asperger syndrome, cancer, rheumatoid arthritis, Parkinson's diseases, Alzheimer's diseases, vitiligo, myocardial infractions, chronic fatigue, and depression (Valko et al. 2007).

H_2O_2 has been found in freshly voided human (Long et al. 1999). Drinking coffee may increase H_2O_2 levels in urine because hydroxyhydroquinone from coffee absorbed into the body and auto-oxidized to produce H_2O_2. Cancer patients show a rise in H_2O_2 level in urine.

2.1.1 Oxidants

However, oxidative stress is used by the immune system to kill the pathogens. Moderate oxidative stress can cause apoptosis and severe oxidation which can result in cell death (Lennon et al. 1991). Different oxidants are given in Table 2.1.

Leakage of mitochondrial activated oxygen during oxidative phosphorylation is the main source of reactive oxygen radicals. It has been found that other enzymes contribute to the oxidants in *E. coli* (Rice-Evans and Gopinathan 1995). Multiple redox-active flavoproteins may play a vital role in the overall

Table 2.1 Different oxidants

$•O_2^-$, superoxide anion	It is one-electron reduction state of O_2, formed during electron transport chain and several auto-oxidation reactions. It can form H_2O_2.
H_2O_2, hydrogen peroxide	It is a two-electron reduction state, formed by dismutation of O_2^-, and it is lipid soluble, so it can cross the plasma membrane.
•OH, hydroxyl radical	It is three-electron reduction state, formed by Fenton reaction and decomposition of peroxynitrite. It is extremely reactive.
ROOH, organic hydroperoxide	It is formed by radical reactions with cellular components like lipids and nucelobases.
ONOO-, peroxynitrite	It is formed in the reaction between $•O^{-2}$ and NO•

production of oxidants (Messner and Imlay 2002). Some enzymes, which can produce superoxides, are xanthine oxidase, cytochrome P450, and NADPH oxidases, while oxidases produce the hydrogen peroxide. Four endogenous sources of oxidants are produced by cells. (a) In aerobic respiration, O_2 reduces to produce O_2, H_2O_2, and –OH. (b) Bacteria or virus-infected cells are destroyed by phagocytosis and produce the nitric oxide (NO), O_2^-, H_2O_2, and OCl. (c) Peroxisome produces H_2O_2 during fatty acid production. (d) Animal cytochrome P450 enzyme may produce some oxidative by-products that can damage DNA. Vitamins C and E, β-carotene, and coenzyme Q are some common antioxidants of diet. Plants may consist of a wide variety of free radical scavenging molecules such as phenolic compounds (phenolic acids, flavonoids, lignans, tannins, quinones, etc.), nitrogen compounds (amines, alkaloids, etc.), vitamins, and terpenoids (Velioglu et al. 1998).

Monitoring of biomarkers like reactive oxygen species and nitrogen species shows that production of these biomarkers may be involved in pathogenesis of Alzheimer's disease and schizophrenia, while cumulative oxidative stress with mitochondrial damage and disrupted respiration is related to Alzheimer's disease, Parkinson's disease, and other neurodegenerative diseases (Ramalingam and Kim 2012). Oxidation of low-density lipoproteins in the vascular endothelium is a precursor to plaque formation, so oxidative stress may link to certain cardiovascular disease. Oxidative stress can cause tissue injury, hyperoxia, and diabetes. It may be involved in age-related development cancer. Reactive species are mutagenic, cause direct or indirect damage to DNA, and may also suppress apoptosis and promote metastasis, invasiveness, and proliferation. High doses of synthetic beta-carotene enhance the rate of lung cancer in smokers. Excess NO combines with tyrosine, which is essential for enzyme ribonucleoside diphosphate. Excess vascular O_2 production may cause the hypertension and vasopasm (Lepoivre et al. 1994). Reactive oxygen species (ROS) attack glial cells and neurons and lead to neuronal damage (Gilgun-Sherki et al. 2001). It has been found that the deleterious effects of ROS on human cells may cause oxidative injury which can lead to apoptosis (Salganik 2001).

2.1.2 Antioxidant

Antioxidant compounds have anti-inflammatory, antiatherosclerotic, antitumor, antimutagenic, anticarcinogenic, antiviral, and antibacterial activities (Owen et al. 2000). Natural antioxidants decrease the risk of cancer, diabetes, and cardiovascular diseases and protect humans from infection, but they can cause oxidative damage and mutation to DNA and cancer. Antioxidant can be exogenous (natural or synthetic) or endogenous; both types of antioxidants can remove the free radicals, scavenge ROS, and bind metal ions which are necessary for catalysis of ROS generation (Gilgun-Sherki et al. 2001). Natural antioxidants are classified into two groups, enzymatic and nonenzymatic shown in Fig. 2.2.

Different applications of antioxidants are shown in Fig. 2.3. Enzymatic antioxidants consist of a few proteins such as catalase and glutathione peroxidase. Nonenzymatic antioxidants like ascorbic acid, lipoic acid, polyphenols, carotenoids, etc. comprise direct-acting antioxidants, which play a vital role in defense against oxygen species. Indirect-acting antioxidants consist of chelating agents. They bind to redox metals and stop the free radical generation (Gilgun-Sherki et al. 2001).

Activated phagocytes produce reactive oxygen and nitrogen species, used by the immune system to kill the pathogens. It has advantages that it damages almost every part of the target cell and prevents the escaping of the pathogen by mutation of single molecular target (Rice-Evans and Gopinathan 1995).

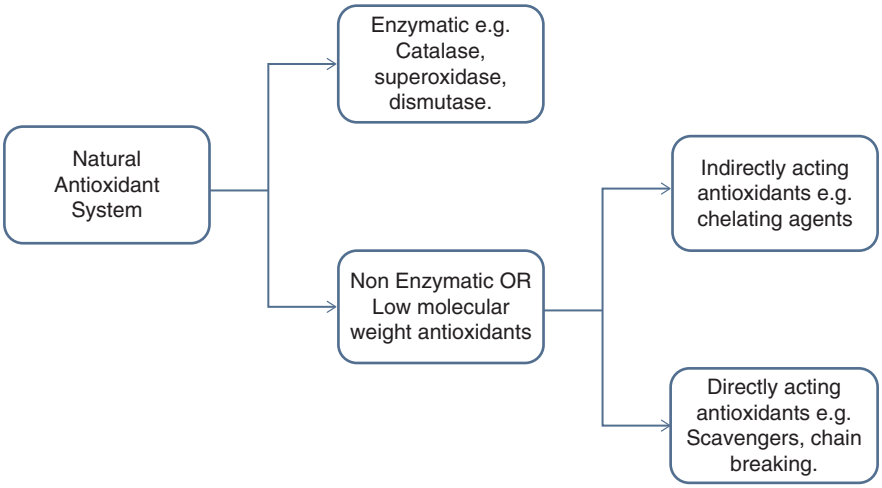

Fig. 2.2 Categorization of natural antioxidants

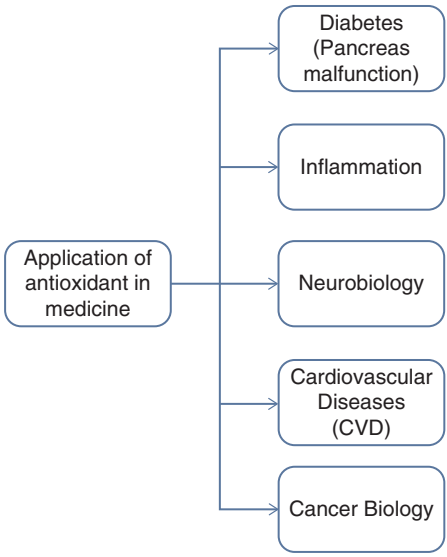

Fig. 2.3 Applications of antioxidants in medicine

2.2 Measurement of RS

Several techniques which have been used to detect the principal markers of DNA oxidative damage are given in Table 2.2. Principal markers of lipids and protein oxidative damage and their detection techniques are given in Tables 2.3 and 2.4, respectively.

2.2.1 Measuring RS In Vivo

Techniques like magnetic resonance imaging spin and L-band electron spin resonance with nitroxy probes may be used to measure RS directly, in whole animals, but no probes are available for human use (Berliner et al. 2001). Most RS cannot be measured directly because they persist only a short time in vivo. However, some RS can be measured in vivo like H_2O_2 and $NO^{\cdot}$. There are two methods to detect the transient RS: (1) measure the level of trapped species and (2) measure the damage level of reactive species that is the amount of oxidative damage. Other approaches like falling in the measurement of erythrocytes defense and total antioxidant activity of body fluids can be considered as oxidative stress. The plasma or serum total antioxidant capacity (TAC) is usually due to ascorbate, urate, and albumin – SH groups – however different methods measure different things (Prior and Cao 1999). For example, rise in urate level will raise TAC, and reduction in the plasma albumin level will reduce the TAC. Consumption of certain ingredients may change in plasma ascorbate or urate levels, so diet can also influence the TAC.

Table 2.2 Principal markers of DNA oxidative damage

Markers	Techniques	Matrices
8-Oxoguanine	HPLC-ECD	Urine, DNA
	GC-MS	DNA
8-Hydroxy-guanine	GC-MS	Urine, DNA
8-Hydroxy-deoxy-guanosine	HPLC-ECD	Urine, DNA
5-(Hydroxymethyl) uracil	GC	DNA, synthesized oligonucleotides
8-Oxo-2′-deoxyguanosine	HPLC-ECD	Urine, DNA

Table 2.3 Principal markers of lipid oxidative damage and their detection techniques

Markers	Techniques	Matrices
Hydroperoxides	Enzymatic methods	Plasma
	GC-MS	Cellular membranes
	Iodometric methods	Plasma, cellular membranes
	HPLC-MS	Plasma
	HPLC-ECD	Plasma cells
	HPLC-CL	Tissue, plasma, cellular membranes
Malondialdehyde	TBA test	Plasma, serum, tissue
	HPLC	Plasma
	GC-MS	Plasma, serum, tissue
Conjugate dienes	Second derivative spectrophotometry	Plasma tissue
Isoprostanes	GC-MS	Plasma, tissue, urine
	Immunoassay	Urine
	Radioimmunoassay	Plasma, urine
4-Hydroxynonenal	GC-MS	Plasma, tissue, urine
	HPLC	Plasma, tissue
Total aldehydes	UV spectroscopy	Plasma, tissue

2.2.1.1 Trapping of RS

Electron spin resonance (ESR) is the only technique which can detect the free radicals directly because it detects the unpaired electrons. However, ESR cannot detect the reactive radicals because they do not accumulate to enough level to be measured. This problem has been sorted out by adding probes or trap agents that can react with unreactive radicals and form them stable reactive radicals which can be detected by the ESR. ESR techniques have been used for animals, but they cannot be applied to humans due to the lack of human safety data on probes (Berliner et al. 2001). Different traps have tested like N-tert-butyl-a-phenylnitrone (PBN), 5,5-dimethyl-1-pyrroline N-oxide (DMPO), 5,5-diethylcarbonyl-1-pyrroline N-oxide (DECPO), N-2-(2-ethoxycarbonyl-propyl)-a-phenylnitrone (EPPN), 5 diethoxyphosphoryl-5-methyl-1-pyrroline N-oxide (DEPMPO), and 5-tert butoxycarbonyl-5-methyl-1-pyrroline N-oxide (BMPO). One common problem of ESR detection is that reaction products which give the ESR signal can be rapidly

Table 2.4 Principal markers of protein oxidative damage and their detection techniques

Markers	Techniques	Matrices
2-Oxohistidine	HPLC-ECD	Experimental model
	Ion exchange chromatography	Experimental model
Carbonyl groups	Spectrophotometry	Tissue, serum, cerebrospinal fluid
Hydroperoxides	Iodometric methods	Tissue, cell membranes
	HPLC	Tissue
Dityrosine	HPLC-FL	Cells, cerebrospinal fluid
Valine hydroperoxides and hydrooxides	HPLC	Plasma, cells
Protein-bound DOPA	HPLC-FL	Tissue
	HPLC-ECD	Tissue
5-Hydroxy-2-animovaleric acid	GC-MS	Tissue
3-Nitrotyrosine	HPLC-UV	Tissue cells
	HPLC-FL	Plasma, tissue, cells
	HPLC-ECD	Plasma peritoneal exudates
	GC-MS	Tissue
m-,o-tyrosine	GC-MS	Tissue
Leucine hydroperoxides and hydroxides	HPLC	Tissue

eliminated in cultured cells or in vivo by direct reduction or enzymatic metabolism. For example, ascorbate can reduce the DMPO-OH during the trapping of $OH^{\cdot}$ by DMPO which leads to no signal in ESR.

2.2.2 Ex Vivo Measurement

2.2.2.1 Spin Traps

Spin traps cannot be used directly to humans because unknown toxicity is required to trap the endogenous free radicals. However, traps can be used on tissue samples and body fluids. DMPO and hydroxylamine probes have been used to measure free radicals in the skin and liver biopsies, respectively (Haywood et al. 1999). ESR detects the secondary radicals like lipid-derived radicals (alkoxy, peroxyl, etc.) and protein radicals produced due to reaction of RS with biomolecules. Ascorbic acid (vitamin C) can react with different RS, and one of its oxidation products is semidehydroascorbate radical which can be easily detected by ESR. Free radical production in the organs, skin, and blood plasma can be identified by the measurement of semidehydroascorbate (Haywood et al. 1999).

2.2.2.2 Aromatic Traps

Aromatic traps are more useful than spin traps and can be used for human consumption, including salicyclate and phenylalanine. $OH^{\cdot}$ hydroxylates the salicyclate and L or D phenylalanine to produce 2-3 dihydroxybenzoate and ortho- and meta-tyrosines, respectively (Ingelman-Sundberg et al. 1991). Phenylalanine and salicyclate

can be used to measure ex vivo radical formation in humans exposed to ozone or blood from rheumatoid arthritis patients (Liu et al. 1997). Phenylalanine has been used to measure the $OH^{\cdot}$ production in the saliva. Salicyclate has been used to detect the $OH^{\cdot}$ in diabetes studies, myocardial infraction, and alcoholism.

2.2.3 Measuring Variation in Blood Pressure

O_2 can antagonize the role of vasodilation of free radical $NO^{\cdot}$. Increased oxidative stress can decrease the bioactivity of $NO^{\cdot}$ (Kojda and Harrison 1999). Vascular endothelium, fibroblasts, lymphocytes, phagocytes, and enzyme xanthine oxidase are the major sources of $O_2^{\cdot}$ inside the vessel wall. The upregulation of O_2 can generate NAD(P)H oxidases, which may play a vital role in hypertension and vascular tension. High level of antioxidant can scavenge ROS, restore $NO,^{\cdot}$ and cause human endothelial dysfunction. So, localized scavenging of RS in the blood vessel walls by antioxidants can be predicted by examination of short-term vascular effects which can be measured by blood pressure.

2.2.4 Fingerprinting of RS

The combination of RS with biological molecule gives a unique chemical fingerprint which can be used to confirm the RS (Halliwell and Gutteridge 2015). All methods of direct measurement of RS cannot be used for humans, so clinical studies measure the oxidative damage caused by reactive species. For example, highly reactive $OH^{\cdot}$ radicals may react with DNA (cause strand breakage or mutagenesis), key proteins, or lipids. Criteria for ideal biomarker for oxidative damage are:

1. Core criterion: Biomarker is productive for later development of diseases.
2. Technical criterion:
   (a) Biomarker should detect a minor part of total oxidative damage in vivo.
   (b) There should be the very less difference between difference sample assay of same sample.
   (c) Its level should not vary widely in the same subjects under the same physical conditions at different times.
   (d) It should not be confounded by diet.
   (e) It should be ideally stable in storage.

RS can oxidize, nitrate, and chlorinate the lipids (Halliwell and Gutteridge 2015). Oxidative DNA damage increases the risk of cancer development later in life. Oxidative damage may modify the nitrogenous bases and sugars, which can be measured by HPLC, liquid chromatography (LC-MS), gas chromatography (GC)-MS, and antibody-based techniques. 8-Hydroxy-2-deoxyguanosine (8-OHdG) in the urine can be measured by HPLC, ELISA, and MS techniques, which can be used to determine the rates of oxidative damage in the body.

Oxidative damage may affect the proteins (alter the function of receptors, enzymes, transport proteins, etc.), and it may generate the new antigens that can cause immune responses (Halliwell 1978). Oxidative damage is more complex; 20 different amino acids can be attacked in different ways by RS rather than just related with four bases and one sugar of DNA (Headlam and Davies 2003). Free radicals can form the amino acid radicals from the proteins. Peroxyl radicals can be generated by the cross-linking of amino acid radicals. These peroxyl radicals can generate more free radicals and produce protein peroxides, which can decompose and trigger the formation of yet more radicals (Headlam and Davies 2003).

2.2.5 Measurement of RS in Cells

Aromatic compounds and spin traps can be used to measure the RS production in cells, while oxidative damage is measured by the assay of end products like oxidized DNA bases, 3-nitrotyrosine, and protein carbonyls.

2.2.5.1 Dichlorofluorescein Diacetate (DCFDA)

DCFDA is commonly used to detect the cellular peroxidases although it reacts very slowly with H_2O_2 or lipid peroxidases (Ischiropoulos et al. 1998). It can enter the cells and accumulate in the cytosol. Esterases deacetylate the DCFDA to dicholofluorescin (DCFH) and RS and convert DCFH into dichlorofluorescein (DCF). DCF can be visualized at 525 nm after excitation it at 488 nm, and unreacted DCFDA, DCFH, and DCF can diffuse out during washing of the cell. So, light emission measurement in the plate reader may be a measurement of the medium as well as from the cells.

2.2.5.2 Dihydrorhodamine 123 (DHR)

DHR has been used to detect $OH^{\cdot}$, $ONOO^{-}$, and $NO2^{\cdot}$, but it is poorly responsive to $O_2^{\cdot-}$, H_2O_2, and $NO^{\cdot}$ (Buxser et al. 1999). It is more sensitive than DCFDA for detection of HOCl (Buxser et al. 1999). DHR gets oxidized into rhodamine 123, which is fluorescent after excitation at 536 nm. Rhodamine is positively charged and lipophilic. It can accumulate in mitochondria and can detect the singlet O_2 in the mitochondria and cause NAD(P)H oxidation.

2.2.5.3 Dihydroethidium (Hydroehidine)

It can detect the $O_2^{\cdot-}$ and oxidized to a fluorescent product which can fluoresce at 600 nm when excited at 500–530 nm. It is usually thought to be ethidium (Fig. 2.4) which can intercalate into nuclear DNA. However, it has been found that the end product is not ethidium (Zhao et al. 2003).

2.2.5.4 Luminol

Luminol can detect the production of RS by activating phagocytosis (Faulkner and Fridovich 1993). Luminol cannot react directly with $O2^{\cdot-}$ but oxidized it, and the resulting luminal can react with $O2^{\cdot-}$ to generate the light-emitting product. Luminol is not a reliable probe because luminol radical can also reduce O_2 to produce O_2 (Faulkner and Fridovich 1993).

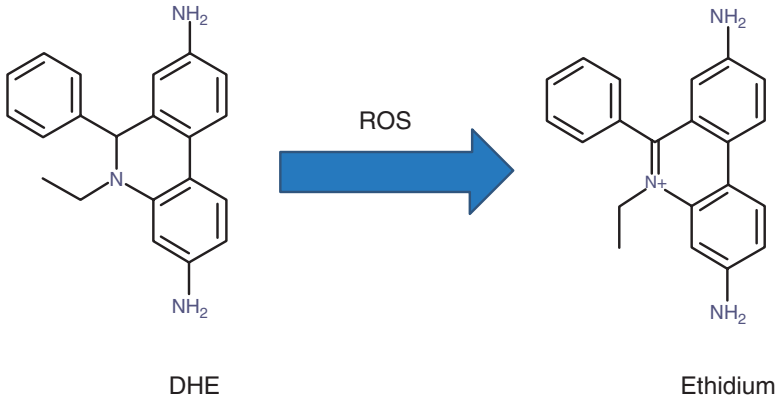

Fig. 2.4 Conversion of DHE to Ethidium

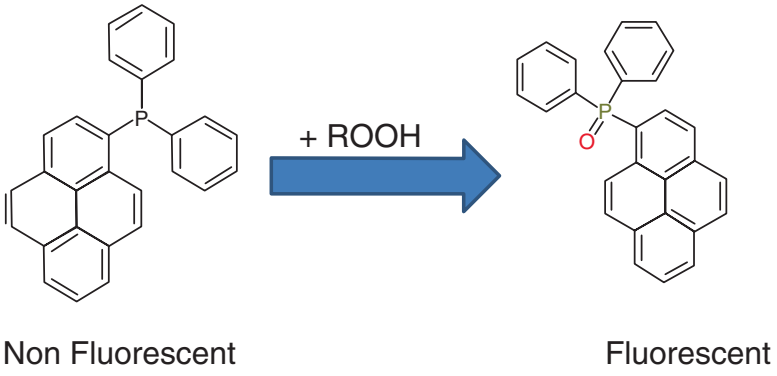

Fig. 2.5 Conversion of diphenyl-1-pyrenylphosphine to a fluorescent product by peroxides

2.2.5.5 Diphenyl-1-pyrenylphosphine

Diphenyl-1-pyrenylphosphine can react with peroxides and produce the fluorescent product which can be detected at 380 nm when excited at 351 nm (Fig. 2.5) (Takahashi et al. 2001). Lipid-soluble hydroxide like methyl linoleate hydroperoxide reacts with DPPP, while hydrogen peroxides located in the cell membrance did not react with DPPP. The fluorescent product of DPPP is quite stable in living cells and stayed up to 2 days, while other little effects on cell proliferation, cell morphology, or cell viability stayed up to 3 days.

2.2.5.6 Cis-Parinaric Acid

It can rapidly oxidize and lose its fluorescence (emission at 413 nm and excitation at 324 nm), when incorporated into peroxidized lipids (Ritov et al. 1996). It is a highly susceptible to nonspecific oxidation because it is a polyunsaturated structure, so it should be stored in the dark under the N_2 atmosphere.

2.2.6 Methods to Measure the Output of Probes

2.2.6.1 Fluorescence Microplate Reader

It is the simplest technique which shows the decreases or increases in relative fluorescence. But the sensitivity and quality of machine vary tremendously, and addition of extra excitation and emission filters makes it expensive. Cells are to be in suspension for the measurement of top reading fluorescence. While bottom reading is advantageous because cells can be measured in situ without the addition of trypsinization which generates cellular oxidative stress (Halliwell 2003).

2.2.6.2 Flow Cytometry

It offers the advantage to measure the intracellular fluorescence of cells in the culture. Quantitative data on the numbers of cells emitting fluorescence can be obtained rather than relative fluorescence units. But it has disadvantages that it required the addition of trypsin which can induce oxidative stress. It gives data with certain cells like murine primary at room temperature rather than at 37 °C. Control experiments may be run to optimize the assay conditions which can be used to limit the disadvantages.

2.2.6.3 Confocal Microscopy

It is a powerful tool, and the cells have to load with fluorescent dyes and viewed in real time in situ in the culture chamber at 37 °C. Counter stains such as Milto tracker, ER tracker, or LystoTracker dyes can be used to visualize the role of mitochondria, endoplasmic reticulum, or lysosomal events in oxidative stress and the intracellular location of RS. Different probes are available to study different cellular events like pH changes and ion movements.

2.2.7 Other Techniques

2.2.7.1 HPLC

The level of phosphatidylcholine (PCOOH) and phosphatidylethanolamine (PEOOH) in the liver of a mouse model has been measured to determine the extent of lipid peroxidation by CL-HPLC (Miyazawa et al. 1987). Lipid peroxidation produces hydroperoxides, endoperoxides, end products of malondialdehyde, pentane, and ethane, so it is the most reliable marker of ROS activity in vivo. Determination of PCOOH or PEOOH is one of the most reliable methods for analyzing the lipid peroxidation (Miyazawa et al. 1987). An increase in the production of ROS may induce the mtDNA deletion in the livers of transgenic mice because mtDNA is 10–15 times more sensitive to oxidative damage than nuclear DNA.

2.2.7.2 Multiphoton Microscopy and Amplex Red

Multiphoton microscopy and Amplex Red (AR) can be used to evaluate the therapeutic approaches which are based on antioxidant treatment that can decrease the levels of oxidative stress and focal pathological neuronal alterations in Alzhiemer mouse models (Garcia-Alloza et al. 2006). A screen is developed to determine the

series of natural antioxidants, which could decrease the plaque-associated reactive oxygen species formation in ex vivo. But not all antioxidants are effective to reduce the plaque-associated oxidative stress. The effective antioxidants have been determined, which can cross the blood-brain barrier to reduce the reactive oxygen species production in the animals.

2.2.7.3 Biosensors

H_2O_2-specific genetically encoded biosensor "HyPer" has been developed by fusing yellow fluorescent protein (YFP) with the regulatory domain of OxyR to determine the biological roles of hydrogen peroxide (Belousov et al. 2006). The regulatory domain of OxyR detects H_2O_2 via oxidation of cysteine residues. This process causes fluorescence of YFP at 516 nm. HyPer gives a fluorescent signal even for submicromolar concentrations of H_2O_2. This HyPer sensor has been used to find the role of H_2O_2 in the early wound responses of zebra fish (Niethammer et al. 2009).

2.3 Effects of Oxidative Stress on Diseases

Reactive oxygen species are formed continuously in the cells due to metabolic reactions and external factors (Ames 1989). Antioxidant system damaged by ROS is one of the main causes of cancer and degenerative disease. ROS can damage DNA and cell division and even can interfer with cell signaling and growth (Cerutti et al. 1994).

2.3.1 Autoimmune Diseases

Lymphocytes of autoimmune disease patients are found to be deficient in repair of 6-methylguanine which is a DNA alkylation product, mutagenic and carcinogenic in nature (Harris et al. 1982). Reactive oxygen species are released from the phagocytic cells at the tissue injury site in the rheumatoid arthritis and systemic lupus erythematosus (SLE). These RO intermediates may cross the cell membrane and react the nuclear DNA. The reaction of hydroxyl radical at the C8 position of deoxyguanosine produces the 8-oxo-7-hydroxydeoxyguanosine (8-oxodG), regulated by the mutagenic, carcinogenic, and cytotoxic compounds. The 8-oxodG level was found to be greater in the DNA of lymphocyte of SLE, RA, and vasculitis than the normal healty person (Bashir et al. 1993). RA patients have more DNA unwinding in the blood mononuclear cells than the healthy controls which show that there may be a deficiency of DNA repair in autoimmune disease. In SLE and RA, the production of superoxide anion radical ($O_2^{\cdot}$) is increased in the inflammatory cells like polymorphonuclear leukocytes. The increment in chromosomal aberrations and sister chromatid exchange found in the mitogen-stimulated lymphocytes may be due to the induction of the DNA damage in RA and SLE. In RA and SLE, the lymphocytes are found to be more hypersensitive to cytotoxic effects of H_2O_2 than from healthy controls (Bashir et al. 1993).

Vitiligo patients have highly reactive oxygen species (ROS), hydrogen peroxide (H_2O_2), and peroxynitrite (Schallreuter et al. 1991). The reduction of antioxidants like catalase, glutathione peroxidase, superoxide dismutase, vitamins C and E, and glucose-6-phosphate dehydrogenase superoxide dismutase shows the systemic redox defect in the vitiligo (Schallreuter et al. 1991). Vitiligo patients have high frequencies of melanocyte-reactive cytotoxic T cells, which can release perforin, IFNγ, and type B granzyme.

Highly reactive aldehydes like 4-hydroxynonenal (4-HNE) and malondialdehyde (MDA) formed by the lipid peroxidation (oxidative degeneration of polyunsaturated fatty acids by ROS) can bind and cause structural modifications of proteins which lead to antigenic profile and enhance the antigenicity of proteins (Grune et al. 1997).

2.3.2 Rheumatoid Arthritis

2.3.2.1 Introduction

Rheumatoid arthritis is a chronic inflammatory disorder which causes joint disease. It has evolved during the last decade. It involves genetic risk factors, environmental factors, and activation of autoimmune response. RA is characterized by inflammation (signs are redness, heat, swelling, and pain) and loss of function of connection of one or more body parts. It causes synovial cell proliferation and destruction of cartilage. The presence of autoantibodies in the patient serum is the main characteristic of this disorder. Rheumatoid factor is the most known autoantibody for the immunologic detection of RA; however, it is absent in one-third of RA patients. Anti-citrullinated protein antibodies like anti-filaggrin antibodies, anti-keratin, and anti-Sa have been used for early diagnosis of RA, but these antibodies have very less diagnostic importance over rheumatoid factor alone. Several other antibodies have been described like antibodies against heat shock proteins (Hsp65, Hsp90, DnaJ), immunoglobulin-binding protein (BiP), heterogenous nuclear RNPs, mannose-binding lectin (MBL), and elongation factor human cartilage gp39 (Biswas et al. 2013). Antibodies against various antigens like citrullinated vimentin, alpha-enolase, and fibrinogen have been found in high titers in RA patients, but their presence in synovial fluid is less characterized, and detection of these antibodies is not effective (Aletaha et al. 2010). RA is still diagnosed by special specific clinical parameters, radiographic evidence of joint destruction, C-reactive protein, complete blood count, and the presence of anti-CCP/rheumatoid factor/anti-MBL.

2.3.2.2 Biomarkers

Fourteen autoantigens have been identified in synovial fluid of RA patients. The expression profile of five important proteins, vimentin, gelsolin, alpha 2 HS glycoprotein (AHSG), glial fibrillary acidic protein (GFAP), and α-1-β-glycoprotein (A1BG), was found to be high compared to control by Western blot analysis in RA synovial fluid as shown in Fig. 2.6 (Biswas et al. 2013). GFAP and A1BG were further validated by ELISA and thus may be used as biomarkers for the diagnosis of RA (Biswas et al. 2013). From plasma of RA patients, TTR has been identified as

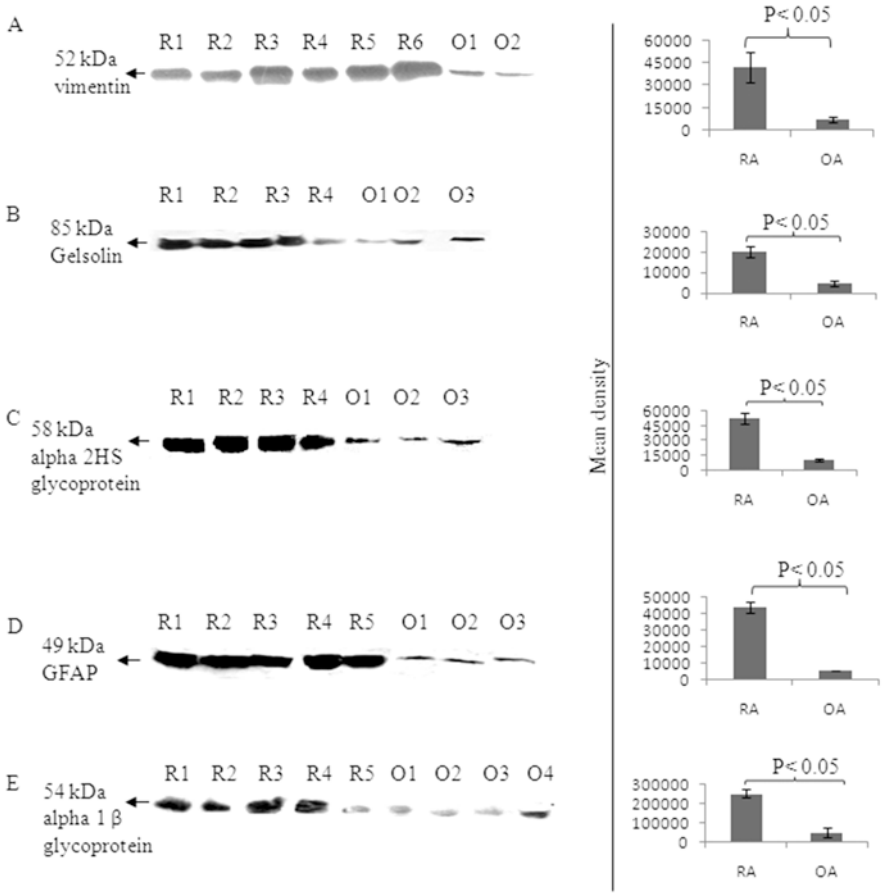

Fig. 2.6 The level of expression of four different autoantigens (vimentin, gelsolin, AHSG, GFAP, and A1BG) by Western blotting. Synovial fluid of various RA (R1–R6) and OA (O1–O4) patients were differentiated on SDS-PAGE and Western blotted with (**a**) anti-vimentin, (**b**) anti-gelsolin, (**c**) anti-alpha 2HS glycoprotein, (**d**) anti-glial fibrillary acidic protein, and (**e**) anti-alpha 1-B glycol protein antibody. The densitometry analysis is shown as bar diagram in the right panel, and the OA patients synovial fluids served as control sample (Source: Biswas, Sagarika, et al. 2013)

autoantigen and has been validated by Western, ELISA, IHC, and IEM studies from the plasma of the RA patients (Sharma et al. 2014).

Two most common acute-phase glycoproteins alpha-1 acid glycoprotein (AGP) and haptoglobin (Hp) expressed 2.5 and 1.6 times in the RA plasma as compared to healthy control (Saroha et al. 2011). The diagnostic accuracy of RA may be improved by combining unique monosaccharide pattern of AGP and Hp along with the diagnostic method using autoantibody against mannose-binding lectin (MBL) (Das et al. 2010). The use of lectins as an enrichment tools or lectin-based fractionation strategies may be used to find the biomarker in the RA.

The expression of ficolin3 was found to be higher in the plasma of the RA patients (Roy et al. 2013). Ficolin3 or H-ficolin, like MBL, plays an important role in innate immunity because it activates the complement system via lectin pathway. The level of aglycosylated form of IgG increased in the serum of RA patients. The alteration of ficolin3 was found to be associated with pathogensis of RA, so this glycoprotein may be used as protential biomarker for the diagnosis of the RA.

Nine proteins were differentially expressed in RA patients as compared to normal healthy controls. Out of these nine proteins, some like apolipoproteins, albumin, haptoglobin beta chain are known to be involved in the development of RA. Transthyretin (TTR) is expressed significantly in high level ($p > 0.05$) in RA as compared to healthy control (Sharma et al. 2014). It has been found that TTR is differentially expressed in the synovium, synovial fluid, and plasma of the RA patients. The expression level of TTR is associated with the progression of severity of the diseases. Autoantibodies against TTR may be used for the diagnosis of RA along with the other tests (Sharma et al. 2014).

Baccaurea sapida has been found to be a rich source of bioactive compounds and can be used to relieve the symptoms of inflammation in RA (Mann et al. 2015). Quercetin has been found the most potent anti-inflammatory compound by comparative docking analysis along with no toxicity and carcinogenicity. It interferes the surface antigen epitope of staphylococcal protein A and inhibits the protein.

2.3.2.3 Oxidative Stress and RA

Several studies found that oxidative stress can cause tissue damage observed in RA patients (Ozkan et al. 2007). Although several antioxidants like metallothioneins, thioredoxin reductase, GSH reductase, etc. are present in the synovial tissues of RA patients, they do not fully counterbalance local oxidative stress . The increase in the level of NO in the synovial fluid of RA patients can regulate many different cell functions at the site of inflammations like signal production, apoptosis, mitochondrial functions, and cytokine production. The study of redox gene knockout mouse supports the role for redox imbalance in the pathogenesis of RA. For instance, mice knocked out for NAD(P)H: quinine oxidoreductase 2 (NQO2), quinine oxidoreductase 1 (NQO1), and cytosolic enzymes that catalyze metabolic reduction of quinones and derivatives showed increased susceptibility to collagen-induced arthritis (Iskander et al. 2006). Thioredoxin is over expressed in the synovial tissues of RA patients, co-stimulates the TNF-α-induced synthesis of IL-6 and IL-8 by synovial fibroblast-like cells, and activates the NF-jB pathway. Rise in blood malondialdehyde (MDA) levels and lower levels of blood concentrations of total glutathione (GSH), vitamin C, and thiols are found in the RA patients as compared with controls. The level of expression of ROS, HO^-, and O_2^- raised in neutrophils, i.e., sourced from peripheral blood and synovial infiltrate (Kundu et al. 2012). The generation of ROS in the neutrophils is positively correlated with RA, so evaluation of ROS is useful as indirect measure of the degree of inflammation of RA patients (Kundu et al. 2012). The ROS/RNS generation is increased by the persistence and infiltration of hematopoietic immune cells within RA joints, while induction of redox-sensitive signaling pathways causes migration of several abnormal expressed adhesives

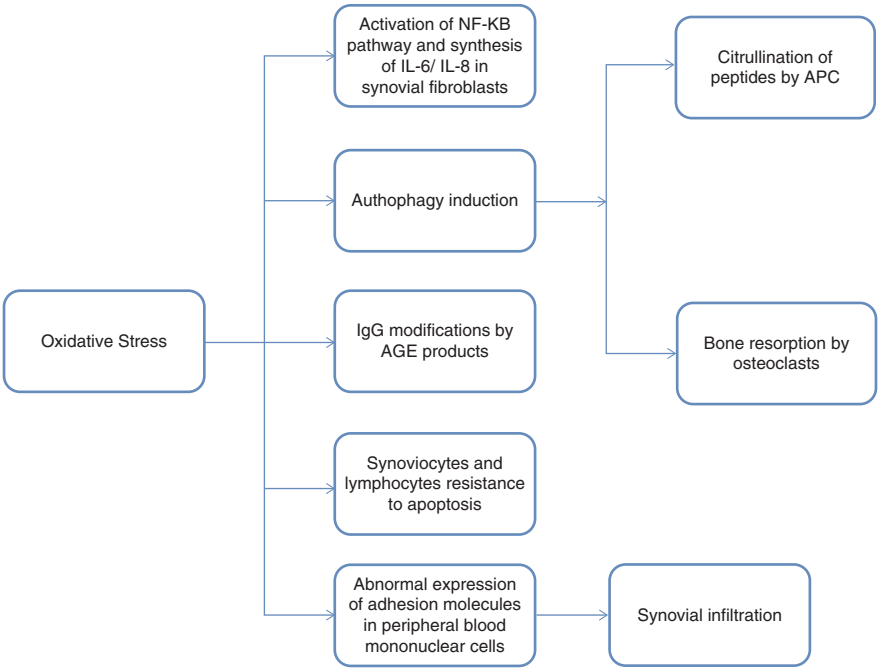

Fig. 2.7 Model showing the oxidative stress involvement in the pathogenesis of rheumatoid arthritis. *AGE*, advanced glycation end, *APC*, antigen-presenting cells

molecules on lymphocytes and monocytes into the RA synovium. Oxidative stress involved in the pathogenesis of rheumatoid arthritis is shown in Fig. 2.7.

Oxidative stress causes the IgG modification in RA in which advanced glycation end (AGE) IgG has been described (Newkirk et al. 2003). Nonenzymatic glycation of proteins results in the AGE, and AGE-modified IgG has been found directly related to RA disease activity (Kurien and Scofield 2008).

Reduction in apoptosis in RA synoviocytes results in massive synovial hyperplasia. NO-inhibited capase 3 activations have been observed in the rheumatoid synovial cells (Migita et al. 2001). The resistance in the apoptotic mechanism in synovial tissue has been found (Xu et al. 2013). TNF-α activates the autophagy in RA which regulates the osteoclast differentiation and bone resorption.

The levels of superoxide anion radical and hydrogen peroxide increase in the plasma of RA patients (Veselinovic et al. 2014). Superoxide anion radicals may be dismutated to produce hydrogen peroxide by superoxide dismutase (SOD) activity in the plasma of RA; however catalase activity (CAT) or glutathione cannot detoxify hydrogen peroxide. Iron may convert hydrogen peroxide into hydroxyl radicals due to the lower transferrin level, which might lead rise in the serum lipid peroxidation in RA patients (Filippin et al. 2008). The levels of TBARS, O_2, and H_2O_2 are found higher in RA patients than in controls (4.08 ± 0.31 vs. 2.39 ± 0.13 nmol/l, p\0.01; 8.90 ± 1.28 vs. 3.04 ± 0.38 nmol/l, p\0.01, 3.65 ± 0.55 vs. 1.06 ± 0.17 lmol/l,

p\0.01). SOD activity increased in the RA patients than healthy controls (2918.24 ± 477.14 vs. 643.46 ± 200.63UgHbx103, p\ 0.001).

Reactive oxygen species and lipid peroxides may play a vital role in RA, whereas reduction in the concentration of antioxidants in the blood increases the probability of occurrence of RA. RA patients are found to be more prone to lipid peroxidation. The calcium/phosphorus ratio is very important in the formation of the bones. The generation of ROS may be an important factor for the restoration of the bone in the inflammatory process (Bijlsma and Jacobs 2000). Hypoxic conditions may disrupt an intracellular ionic environment and vary calcium and phosphorus level (Cheeseman and Slater 1993). Increased oxidative stress is caused by increased lipid peroxidation in peripheral blood of RA patients (Walwadkar et al. 2006). MDA is the product of lipid peroxidation, which can react with lysine residues in protein to produce immunogenic molecules. Lipid peroxide ($p < 0.001$) and nitric oxide ($p < 0.001$) levels are found to be higher in RA patients as compared to controls. Whereas vitamin E ($p < 0.001$) and calcium/phosphorus ($p < 0.001$) are found to be decreased in the RA patients as compared to controls. The positive correlation was found between vitamin E and calcium as well as between lipid peroxides and nitric oxides. While nitric oxide and lipid peroxides were found to be negatively correlated with vitamin E, the negative correlation was found between MDA and calcium/phosphorus ratio in RA patients. Increased oxidative threat in rheumatoid arthritis is confirmed by the rise in the level of lipid peroxides and nitric oxide and decreased vitamin E and calcium/phosphorus ratio.

ROS production in whole blood and monocytes increased by fivefold in RA patients as compared to healthy subjects suggests that oxidative stress is a pathogenic hallmark in RA. Free radicals are secondary messengers in inflammatory and immunological cellular response in RA. The exposure of T cell to increased oxidative stress may regulate several stimuli which may cause variation in growth, death, and immune responses (Hassan et al. 2011). Free radicals can inhibit the synthesis of joint cartilage or directly degrade by attacking its proteoglycan (Hadjigogos 2003). Genotoxic events induced by ROS cause mutation of p53 in RA-derived fibroblast like synoviocytes. An increase in the intra-articular pressure in RA joints shows the chronic oxidative stress in RA synovium; it increases the ROS production in cellular oxidative phosphorylation and further induces repetitive cycles of hypoxia. Inflammatory responses induce the cellular proliferation which can cause the hypoxia in RA joints.

Thirty different oxidants and antioxidant markers were identified in different studies of RA. These were classified in the seven groups as (a) lipid peroxidation (4 markers: thiobarbituric acid reactive substances [TBARS], malondialdehyde [MDA], malondialdehyde-acetaldehyde [MAA], isoprostane [F2-I], adducts), (b) protein oxidation (4 markers: 3-chlorotyrosine [CT], protein carbonyls [PC], nitrosothiols [RSNO], and advanced oxidation of protein products [AOPP]), (c) DNA damage (2 markers: micronucleus [MN] and DNA stand breaks [DNA sb]), (d) urate oxidation (1 marker: allantoin [ALLA]), (e) enzymatic activity (7 markers: SOD, GPx, myeloperoxidas [MPO], GR, CAT, NADPH oxidase [NADPH ox], and arylesterase [AE]), (f) antioxidants (6

markers: GSH, β-carotene [βC], vitamin E [VE], oxidized glutathione [GSSG], SH group and total antioxidant capacity [anti-cap]), and (g) free radical/anions (6 markers: reactive oxygen metabolites (ROM), H_2O_2, total ROS, $O2^{-}\bullet$, •OH, and NO•) (Quiñonez-Flores et al. 2016).

2.3.2.4 Measurement of RS in RA

Lipid Oxidation

Ten lipid biomarkers measured the MDA blood levels in RA patients. The levels of MDA in the synovial fluid are correlated with levels of OH and ROS radicals (Datta et al. 2014). The levels of blood TBARS increase in the RA patients (Veselinovic et al. 2014).

Protein Oxidation

Protein oxidation is evaluated by different biomarkers (CT, RC, AOPP, and RSNO) in five different studies (Datta et al. 2014; Nzeusseu Toukap et al. 2014; Stamp et al. 2012; Tetik et al. 2010). Three studies show that protein carbonylation is higher in plasma of RA patients than healthy controls (Stamp et al. 2012; Tetik et al. 2010). RNS, AOPP protein carbonylation, and PC are found in the synovial fluid of RA patients (Datta et al. 2014). CT level is found higher in RA patients than controls (Nzeusseu Toukap et al. 2014).

Lipid Peroxidation

Lipid peroxidized decomposition results in different end products including malondialdehyde (MDA). The elevated level of MDA is found in the serum and synovial fluid of RA patients (Gambhir et al. 1997).

GSH-Px, Catalase, and GSH Assay

GSH-Px activity of plasma can be measured sphectrophotometrically at 37 °C and 412 nm (Gambhir et al. 1997). The yellow complex of molybdate and hydrogen peroxide can be measured at 405 nm against blank by using spectrophotometer (Gambhir et al. 1997).

SOD Activity

The generation of superoxide radicals by xanthine and xanthine oxidase which can react with 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (INT) to form a red formazan dye is used for the measurement of erythrocytes SOD activity (McCord and Fridovich 1969). One unit of SOD is defined as the amount of an enzyme necessary to produce 50% inhibition in the INT reduction rate.

GSH-Px Activity

GSH-Px catalyzes the oxidation of glutathione by cumene hydroperoxide (Paglia and Valentine 1967). The oxidized glutathione is converted into the reduced form with the oxidation of NADPH to NADP+ in the presence of glutathione reductase and NADPH.

CAT Activity

0.2 ml erythrocyte hemolyase incubated in 1.0 ml substrate at 37 °C for 60 s. 32.4 mmol/L ammonium molybdate ($(NH_4)_6\,Mo_7O_{24}I_4H_2O$) used to stop the reaction and molybdate yellow complex and H_2O_2 measured at 405 nm against blank (Goth 1991).

Determination of Plasma MDA Levels

The reaction between MDA and thiobarbituric acid can be measured by fluorometric method, and it can be used to observe the plasma lipid peroxidation level (Conti et al. 1991).

Measurment of Total Antioxidative Capacity

Automated calorimetric measurement method can be used to measure the plasma total antioxidant levels (Erel 2004).

Measurent of NADPH Oxidase Activity

Nitroblue tetrazolium (NBT) can be used as a substrate to measure the NADPH oxidase activity in plasma and SF (Dong et al. 2011).

Measurement of Intracellular Nitric Oxide (NO)

Diaminofluorescein diacetate (DAF-2DA) is a nonfluorescent dye which fluoresces on reaction with NO can be used to measure the intracellular NO (Sarkar et al. 2011).

2.4 Conclusion

The imbalance between oxidative system and antioxidant system which can disrupt protein, lipids, membranes, and genes is called the oxidative stress. Reactive oxygen species like superoxide anion, peroxynitrite, hydroxyl radical, hydrogen peroxide, etc. can cause several diseases like Alzheimer's disease, Parkinson's diseases, neurodegenerative disease, automimmune disease (SLE, RA, etc.), and even cancer. Several enzymes like xanthine oxidase, cytochrome P450, and NADPH oxidase can produce the superoxides, while oxidases can produce the hydrogen peroxides.

RS can be measured either by the level of trapped species or the damage level of reactive species. Magnetic imaging spin can be used to measure RS directly in the animal cells; however no probe is available for humans. ESR detects the unpaired electrons. Different traps like N-tert-butyl-a-phenylnitrone (PBN), 5,5-dimethyl-1-pyrroline N-oxide (DMPO), 5,5-diethylcarbonyl-1-pyrroline N-oxide (DECPO), and N-2-(2-ethoxycarbonyl-propyl)- a-phenylnitrone (EPPN) have been tested. DMPO and hydroxylamine have been used to measure the free radicals in the skin and liver biopsies, respectively. Phenylalanine and salicyclate have been used to measure ex vivo radical formation in rheumatoid arthritis patients. Localized scavenging of RS in the blood vessel walls by antioxidants may be predicted by short examination of vascular effects which can be measured by blood pressure. Oxidative damage may modify the sugars and nitrogenouses bases which can be measured by HPLC, LC-MS,

GC-MS, and immunotechniques. Reactive species in the cells can be measured by various compounds like dichlorofluorescein diacetate (DCFDA), dihydrorhodamine 123 (DHR), dihydroethidium, luminol, diphenyl-1-pyrenylphosphine, cis-parinaric acid, etc. Various techniques like fluorescence microplate reader, confocal microscopy, flow cytometry, etc. can be used to measure the output of the probes in oxidative stress. PCOOH or PEOOH is one of the most reliable methods to analyze lipid peroxidation which can produce hydroperoxides, endoperoxide pentane, etc., so it is used to measure the ROS activity. A biosensor HyPer has been used to determine the role of H_2O_2 in early wound response of zebra fish.

ROS are released from the phagocytic cells at the infection site in the autoimmune diseases. RO intermediates may cross the cell membrane and react the nuclear DNA and can produce the 8-oxo-7-hydroxydeoxyguanosine (8-oxodG). Oxidative species like ROS, H_2O_2, and peroxynitrite are expressed highly in the vitiligo patients, while the expression of antioxidants like catalase, glutathione, peroxidase, superoxide mutase, and vitamin C decreases.

Rheumatoid arthritis is a long-lasting autoimmune disease which affects joints. It can be characterized by synovial cell proliferation, destruction of cartilage, and inflammatory cell infiltration. Some proteins like apolipoproteins, albumin, haptoglobin beta chain are known to be involved in the development of RA. The generation of ROS in neutrophils is positively correlated with the RA. RA patients are found to be more prone to lipid peroxidation. Lipid peroxides and nitric oxides are found to be higher in the RA patients as compared to controls. Oxidative stress is a pathogenic hallmark in RA because ROS production in monocytes and whole blood increases by fivefold in RA patients as compared to healthy ones. Thirty different oxidants and antioxidants have been identified in RA, and these are classified into seven major groups like lipid peroxidation, protein oxidation, DNA damage, urate oxidation, free radicals, etc. RS in RA patients can be measured by lipid oxidation, protein oxidation, lipid peroxidation, SOD activity, GSH assay, CAT activity, plasma MDA levels, TAC, NADPH oxidase activity, intracellular nitric oxide, etc.

Research in the last decade has been focused on the identification of ROS, RNS, or free radicals in vivo or ex vivo. Several techniques and biomarkers have been identified which can detect the ROS. New probes or sensors are required, which can detect the ROS inside the human cell. There is the need of development of molecules which can inhibit the radical species or activate the antioxidants.

It is required to develop more advanced techniques for the detection of the oxidative stress. New biomarkers can be found in the RA to diagnose the rheumatoid arthritis in the early stages of pathogenesis by proteomics.

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Nanomaterials in Antioxidant Research

3

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3.1 Introduction

Oxidative stress is proposed as leading event in the deterioration of health and basic biological processes. Ever since the Harman's theory of aging was proposed based on the ill effects of oxidative in the body, the pace of oxidative stress research became rapid. The antioxidants were proposed as putative therapeutic and prophylactic agents for the prevention of oxidative damage and its aftermath. Despite the escalating research publications in the domain of oxidative stress and antioxidant therapy, apparent clinical transitions are fairly low. Perhaps, this should not be looked as question on the studies which were performed on the antioxidants, rather our poor understanding of cross talk of antioxidants and oxidants in the cells and its downstream effects. It seems that decision of considering antioxidants as miracle drugs for aging and similar condition was too early. There is lot more to be explored in this domain, and as we move deeper, we realize that oxidative stress and antioxidant interplay is one of the most complicated biological events that has several fold more complexity than basic cellular processes and metabolism. The scientific questions such as how much antioxidant dose is optimal and which antioxidant is most suitable can only be answered in a context-specific manner. The several anomalies and unfruitful clinical translations of antioxidants have led to the continuation and intensification of antioxidant research. With the advent of a new domain of science named nanotechnology, few exciting possibilities have emerged in the antioxidant

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researches which are likely to answer some of the issues of conventional antioxidants. This chapter is aimed to discuss the emerging trends in nano-antioxidants with a special focus on much-studied antioxidant *nanoceria.*

The chapter is divided into six main sections in which we will discuss about the unique features of one of the most studied antioxidant nanomaterials called cerium oxide nanoparticles, including the in vitro and in vivo evidences showing the benefits. In the later part of this chapter, we will additionally focus on the diagnostic abilities of nanoceria in antioxidant research.

3.2 The Process of Oxidative Stress and Need for Antioxidants

Any molecule or chemical entity that can accept electrons is an oxidant or oxidizing agent (Prior and Cao 1999), and the process is known as oxidation; one such potential oxidizing agent is oxygen itself. In biology, the process of oxidation is always accompanied by reduction, and these reactions are known as redox reactions. Redox reactions are central to several biochemical pathways including biosynthesis, regulation of metabolism, biological oxidation, and radical/antioxidant effect. Although oxidant and reductant are purely chemical terms, they are termed as prooxidant and antioxidant, respectively, in biological context (Kohen and Nyska 2002). Prooxidant includes several radical and nonradical species (Halliwell 2006). Some of the commonly known radical and nonradical oxidants are enlisted in Table 3.1.

Environmental stressors tend to increase the level of oxidants that sometime exceeds beyond the capacity of antioxidant defense in the body leading to progressive increase in cellular pool of oxidants causing further damage to biomolecules. This delicate imbalance between oxidants and antioxidants in the body is defined as oxidative stress (Halliwell 2006) (Kalyanaraman 2013). Such conditions are known to either initiate the pathology or fasten its progression, and therefore several key molecules associated with oxidative stress are linked to human diseases as biomarkers (Dalle-Donne et al. 2003). Hypoxia impairs the oxygen flux in mitochondria and therefore promotes reactive oxygen species via mitochondrial route, while stressors such as UV radiations directly induce the radicals by homolytic cleavage of covalent bonds in various biomolecules.

3.2.1 Mechanisms of Oxidant Generation

Most of the biological systems constantly get exposed to reactive oxidants originating either endogenously or exogenously; this is how metabolizing oxygen remains in continuous interconversion (Winterbourn 2008). ROS can be generated by several means in living cells, either intracellular or extracellular, but mitochondrial route is most significant (Beal et al. 1997; Novo and Parola 2008). About 5% of electrons circulating through the electron transport chain can get diverted to beyond the conventional route and lead to the formation of $O_2^{\bullet -}$. Generally this leakage

Table 3.1 Some reactive species

Free radical species	Nonradical species
Reactive oxygen species	*Reactive oxygen species*
Superoxide, $O_2^{\bullet -}$	H_2O_2
Hydroperoxyl, $HO_2^{\bullet}$ (protonated superoxide)	Hypobromous acid, HOBr[a]
Hydroxyl, $OH^{\bullet}$	Hypochlorous acid, HOCl[b]
Carbonate, $CO_3^{\bullet -}$	Ozone, O_3[c]
Peroxyl, $RO_2^{\bullet}$	Singlet oxygen ($O_2{}^1\Delta g$)
Alcoxyl, $RO^{\bullet}$	Organic peroxides, ROOH
Carbon dioxide radical, $CO_2^{\bullet -}$	Peroxynitrite, $ONOO^-$[d]
Singlet $O_2{}^1\Sigma g^+$	Peroxynitrate, O_2NOO^-[d]
	Peroxynitrous acid, ONOOH[d]
	Peroxomonocarbonate, $HOOCO_2^-$
Reactive chlorine species	*Reactive chlorine species*
Atomic chlorine, $Cl^{\bullet}$	Hypochlorous acid, HOCl[b]
	Nitryl chloride, NO_2Cl[e]
	Chloramines
	Chlorine gas (Cl_2)
	Bromine chloride (BrCl)[a]
	Chlorine dioxide (ClO_2)
Reactive bromine species	*Reactive bromine species*
Atomic bromine, $Br^{\bullet}$	Hypobromous acid (HOBr)
	Bromine gas (Br_2)
	Bromine chloride (BrCl)[a]
Reactive nitrogen species	*Reactive nitrogen species*
Nitric oxide, $NO^{\bullet}$	Nitrous acid, HNO_2
Nitrogen dioxide, $NO_2^{\bullet}$[c]	Nitrosyl cation, NO^+
Nitrate radical, $NO_3^{\bullet}$[c,f]	Nitroxyl anion, NO^-
	Peroxynitrite, $ONOO^-$[d]
	Dinitrogen tetroxide, N_2O_4
	Dinitrogen trioxide, N_2O_3
	Peroxynitrate, O_2NOO^-[d]
	Alkyl peroxynitrites, ROONO
	Peroxynitrous acid, ONOOH[d]
	Nitronium cation, NO_2^+
	Alkyl peroxynitrates, RO_2ONO
	Nitryl chloride, NO_2Cl
	Peroxyacetyl nitrate, $CH_3C(O)OONO_2$[c]

ROS is a collective term that includes both oxygen radicals and certain nonradicals that are oxidizing agents and/or are easily converted into radicals (HOCl, HOBr, O_3, $ONOO^-$, $^1O_2$, and H_2O_2). All oxygen radicals are ROS, but not all ROS are oxygen radicals. Reactive nitrogen species is a similar collective term that includes $NO^{\bullet}$ and $NO_2^{\bullet}$ as well as nonradicals such as HNO_2 and N_2O_4. Reactive is not always an appropriate term: H_2O_2, $NO^{\bullet}$, and $O_2^{\bullet -}$ react fast with few molecules, whereas $OH^{\bullet}$ reacts fast with almost everything. Species such as $RO_2^{\bullet}$, $NO_3^{\bullet}$, $RO^{\bullet}$, HOCl, HOBr, $CO_3^{\bullet -}$, $CO_2^{\bullet -}$, $NO_2^{\bullet}$, $ONOO^-$, NO_2^+, and O_3 have intermediate reactivities (Adapted from Halliwell 2006)

[a]HOBr and BrCl could also be regarded as active bromine species

[b]HOCl and HOBr are often included as ROS

[c]Oxidizing species formed in polluted air are toxic to plants and animals

[d]$ONOO^-$, ONOOH, and O_2NOO^- are often included as ROS

[e]NO_2Cl can also be regarded as a reactive nitrogen species

[f]This species may cause formation of allergenic nitrated proteins in pollens (After Halliwell 2006)

occurs at complex I (NADH/ubiquinone oxidoreductase) and complex III (Kohen and Nyska 2002). Superoxide radical ($O_2^{\bullet -}$) is then often converted into H_2O_2 by mitochondrial superoxide dismutase. The hydrogen peroxide thus formed can cross mitochondrial membranes to reach the cytoplasm (Cadenas and Davies 2000). Another enzyme involved in radical generation is NADPH oxidase (NOX) which is found in professional phagocytic cells and nonphagocytic cells. It is known to play a crucial role in different diseases (Babior 1999; Vignais 2002; Lambeth 2007), such as chronic liver diseases (CLDs) (De Minicis and Brenner 2007). Upon stimulation of phagocytic cells, NOX is transported to the plasma membrane where it interacts with Cyt b558. This increases the biological activity of NOX and subsequent generation of ROS. In comparison with the other members of the NOX family, NOX from nonphagocytic cells is similar in structure and function; however the difference lies in redox signaling. The nonphagocytic NOX is constitutive and generates a very low level of ROS, while in response to a number of factors, both its activity and ROS generation increase. 5-Lipoxygenase (5-LOX) is yet another mixed function oxidase that is otherwise involved in the synthesis of leukotrienes from arachidonic acid. LOX can also be stimulated with similar stimuli that stimulate NOX, particularly growth factors and cytokines (Novo and Parola 2008). Alternative mechanisms that explain the generation of reactive oxygen species include ruffling of membrane through the intervention of the small GTPase Rac1 and a SOD isoform, respectively, after a stimulus from growth factors and cytokines (Soberman 2003). In many subcellular compartments, ROS can also be generated through several oxidases, peroxidases, oxygenases, and cytochrome P450 isoforms. Additionally, xanthine oxidase (Pritsos 2000), nitric oxide synthase (Vasquez-Vivar and Kalyanaraman 2000), and peroxisomal oxidases (Rojkind et al. 2002) have also been suggested to be involved in ROS generation. Moreover, an enzyme lysyl oxidase that catalyzes the formation of the aldehyde precursors of cross-links in connective tissue proteins collagen and elastin was also found to generate H_2O_2.

3.2.2 Defense Mechanism Against Oxidative Stress

Reactive oxygen species have recently been found to be involved in various signaling processes, yet the control over generation of ROS is extremely important for the prevention of oxidative stress. One of the primary means that works against the generation of ROS is antioxidant system. Antioxidant defense has emerged throughout a long evolutionary process in response to the changing concentration of oxygen. This system is unique in its abilities primarily, due to its direct interaction with different types of ROS and its capacity to protect a wide variety of biological targets including several biomolecules and structural components. The system contains two major classes: enzymatic antioxidants and nonenzymatic antioxidants.

3.2.2.1 Enzymatic Antioxidants

Among enzymatic antioxidants, primary defense is provided by superoxide dismutase that converts superoxides into less reactive peroxides. SOD is compartmentalized into mitochondria, cytosol, and extracellular matrix in three isoforms, Mn-SOD (SOD I), Cu-Zn-SOD (SOD II), and EC-SOD (SOD III), respectively. Further, this H_2O_2 is detoxified with the help of catalase and peroxidases into water. Catalase is unique due to its high Km value and therefore remains active during high concentrations of H_2O_2. In contrast to catalase, peroxidase is known to possess high affinity (lower Km) and therefore remains active even at low H_2O_2 concentrations. Peroxidases may further be broadly classified into two groups, one dependent on glutathione (GSH) called glutathione peroxidase and other dependent on thioredoxin (Trx) called peroxiredoxin (Prx); Prx are most important antioxidants in erythrocytes as indicated by their highest abundance next to hemoglobin (Rhee et al. 2005), while in the other cellular systems, glutathione peroxidases are most prevalent and have an additional capacity to neutralize organic peroxides apart from hydrogen peroxide. Some of the commonly occurring cellular antioxidant enzymes, their activities, and associated enzyme commission numbers are enlisted in Table 3.2.

3.2.2.2 Nonenzymatic Antioxidants

Among the major nonenzymatic antioxidants, the most abundant antioxidants are glutathione and thioredoxin. Glutathione is a cysteine-containing dipeptide that augments the activities of glutathione peroxidase and also maintains cellular proteins in reduced state. The recycling of oxidized glutathione (GSSG) to reduced glutathione (GSH) requires NADPH in the presence of enzyme glutathione reductase (GR), and therefore, this conversion is expensive for the cells.

Table 3.2 Summary of chemical reactions of various cellular antioxidant enzymes

Enzyme	Reaction catalyzed	EC number
Superoxide dismutase	$O_2^{\bullet-} + O_2^{\bullet-} + 2H^+ \leftrightarrow 2H_2O_2 + O_2$	1.15.1.1
Catalase	$2H_2O_2 \leftrightarrow O_2 + 2H_2O$	1.11.1.6
Glutathione peroxidase	2GSH + PUFA-OOH ↔ GSSG + PUFA +$2H_2O$	1.11.1.12
Glutathione S-transferases	RX + GSH ↔ HX + R-S-GSH[a]	2.5.1.18
Phospholipid-hydroperoxide glutathione peroxidase	2GSH + PUFA-OOH (H_2O_2) ↔ GSSG +$2H_2O$[b]	1.11.1.9
Ascorbate peroxidase	AA + H_2O_2 ↔ DHA + $2H_2O$	1.11.1.11
Guaiacol-type peroxidase	Donor + H_2O_2 ↔ oxidized donor +$2H_2O$[c]	1.11.1.7
Monodehydroascorbate reductase	NADH +2MDHA ↔ NAD^+ +2AA	1.6.5.4
Dehydroascorbate reductase	2GSH + DHA ↔ GSSG +AA	1.8.5.1
Glutathione reductase	NADPH + GSSG ↔ $NADP^+$ + 2GSH	1.6.4.2

[a]R may be an aliphatic, aromatic, or heterocyclic group; X may be a sulfate, nitrite, or halide group
[b]Reaction with H_2O_2 is slow
[c]AA acts as an electron donor (After Mehlhorn et al. 1996)

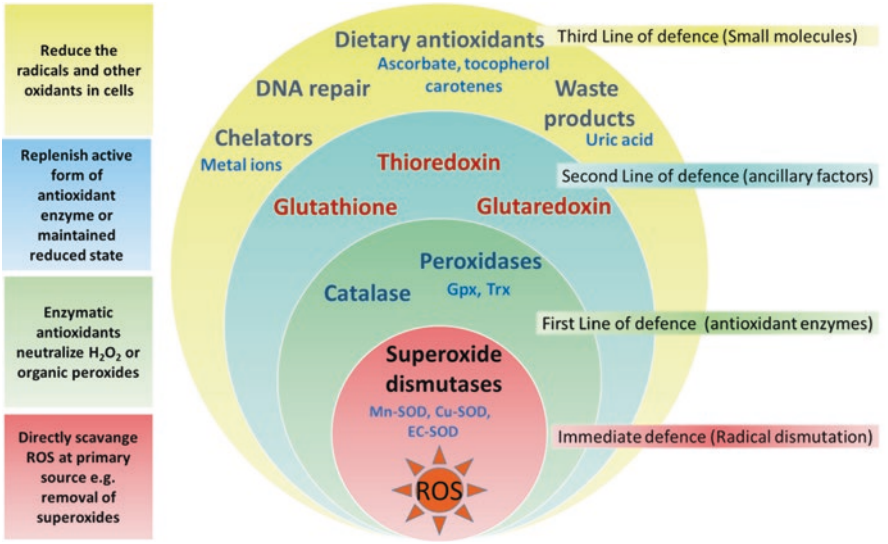

Fig. 3.1 Defense against oxidative stress: at primary level *en source* generation of radicals is dismutated by SOD, which further produces hydrogen peroxide and organic peroxides. These are neutralized by catalase and GSH- or TRX-dependent peroxidases. Glutathione, glutaredoxin, and thioredoxin feed the maintenance of active form of enzyme and reduced state of proteins. Finally, many small metabolites like uric acid, metal chelators, or even dietary antioxidants such as ascorbate, carotenes, and tocopherol scavenge radicals

Thioredoxin is another protein that maintains protein thiolation and augments the activity of peroxiredoxin.

Apart from these dedicated nonenzymatic antioxidants, metabolites such as uric acid, and melatonin, many dietary antioxidants including vitamin E (alpha-tocopherol), carotenes, lycopene, vitamin C (ascorbate), and several polyphenols are known to scavenge the radical and nonradical oxidant species (Catoni et al. 2008). Based on the above discussion on antioxidant defense, the entire antioxidant defense may be stratified into four strata: (a) immediate defense that directly scavenges ROS at source, (b) first line of defense dependent on antioxidant enzymes, (c) second line of defense that includes ancillary factors augmenting conventional antioxidant defense, and (d) third line of antioxidant defense that includes small metabolites and dietary antioxidants including metal chelators and vitamins (Fig. 3.1).

3.3 Antioxidant Supplementation for the Oxidative Stress

Although most of the animal and plant cells are all equipped with a dynamic repertoire of antioxidant enzymes and nonenzymatic antioxidants that respond and adapt quickly to radical burst, the exogenous/dietary supplementation is propounded as one of the key interventions to ameliorate oxidative stress (Halliwell 2006). Recent clinical studies have shown that an inverse correlation exists between the plasma

levels of antioxidants such as vitamins E and C/phytonutrients and cardiovascular disease and cancer (Cameron and Pauling 1976; Willett and MacMahon 1984; Radimer et al. 2004). Therefore, in order to maintain optimal body function, supplementation of antioxidant has become an increasingly popular. Attempts are being made to discover or develop novel antioxidants either of natural or synthetic origin with better efficiency and low-dose requirements.

3.4 Challenges in Antioxidant Research

There have been a growing number of evidences about the challenges posed in the antioxidant research. A large-scale research on phytoextracts and other classical antioxidant types has created a type of antioxidant therapy, yet the success story of antioxidant research remains pacified by a number of questions. One of the leading articles entitled "The myth of antioxidants" in the Scientific American by Melinda Wenner Moyer states that

> for decades researchers assumed that highly reactive molecules called free radicals caused aging by damaging cells and thus undermining the functioning of tissues and organs. Recent experiments, however, show that increases in certain free radicals in mice and worms correlate with longer life span. Indeed, in some circumstances, free radicals seem to signal cellular repair networks. If these results are confirmed, they may suggest that taking antioxidants in the form of vitamins or other supplements can do more harm than good in otherwise healthy individuals (article).

Furthermore, in the aforesaid study, it was demonstrated that one possibility exists that a specific concentration of free radicals stimulates the internal repair mechanisms of an organism. In their experiment on roundworms, it was found that genetically engineered worms which produced large amount of certain free radicals lived longer than the normal worms, which surprised the researchers. Furthermore, when the mutant worms were fed with antioxidants, the longevity was affected in contrast to animals fed with antioxidants.

In another classical review on the comparative analysis of benefits and ill effects of the antioxidants, Jaouad Bouayed comments that the antioxidants are double-edged swords (Bouayed and Bohn 2010). The beneficial or harmful effects of natural compounds as antioxidants may occur independently due to their antioxidative properties as well as due to the activation of specific cellular pathways such as inflammatory processes and nitrogen and dicarbonyl metabolisms. Due to dual active roles of organic compounds such as oxidative properties and antioxidant properties, they are known to act as double-edged molecules. For an illustrative purpose, a study showed that β-carotene at low doses exhibited antioxidant (Palozza et al. 2002) as well as anti-inflammatory (Yeh et al. 2009) behavior in HL-60 cell line of human origin, whereas at high concentrations, it showed the prooxidant activity (Palozza et al. 2002) and pro-inflammatory effects (Yeh et al. 2009). Additionally, an increase in the production of pro-inflammatory mediators such as tumor necrosis factor-α (TNFα) and interleukin-8 (IL-8) has been established in

various studies. Several reasons have been reported for the ill effects of the antioxidants, the primary reason being higher doses and reductive stress. In addition to the effects of antioxidant concentration, the metal ions are also known to be involved in the oxidative stress phenomenon. Galati et al. have shown that in the presence of transition metals, EGCG causes oxidative damage to DNA (Galati and O'Brien 2004). Also, dietary antioxidants especially phenolic compounds can show prooxidant activities when metal ions are present in their vicinity, perhaps due to their reducing capacity and ability to form chelates (Decker 1997; Azam et al. 2004; Galati and O'Brien 2004). Another reason in the perturbation of the cellular processes by the excessive use of antioxidants is known to be caused by modulation of cellular pathways, e.g., by modulation of intracellular signaling, such as the nuclear factor kappa B (NFκB) or by binding to the ATP-binding sites of a large number of proteins and the mitogen-activated protein kinase (MAPK). The aforesaid events have been recently reviewed by Williams et al. (Williams et al. 2004).

There are several such studies that suggest the dark side of the antioxidant, and this may be one of the reasons for very slow induction of antioxidants such as drugs and their inclusion in various therapeutic regimes. Similarly, despite of thousands of studies per year that describe benefits of the antioxidants, only a very few could make it to clinics.

3.5 Nanoceria as Promising Next-Generation Antioxidant

Considering the major anomalies of the antioxidants, the primary ill effect or drawback was the requirement of high doses/repeated doses (high or repeated doses are usually proven beneficial in many in vivo and in vivo studies). The secondary ill effects were results of downstream cellular signaling or generation of new molecule on reduction. However, nanomaterials get rid of some of these issues and therefore provide a new domain for the antioxidant research. A nonmaterial is a matter that has a defined size of 1 nm−100 nm at least in one dimension. Nanomaterials are of particular interest due to significant change in their physicochemical properties on reducing a material to nanoscale. A classical example of this phenomenon is gold. Interaction of gold nanoparticles with light is affected by environment surrounding them, the size of particles, and overall dimensions of particles. Interaction of light photos with the free electrons on the surface of nanoparticles results in the formation of a concerted wave of oscillating electrons known as surface plasmons. The difference in the type of surface plasmons is the underlying cause of difference in the color of gold nanoparticles of different sizes. In small nanoparticles of size closer to 30 nm, absorption of light occurs in the blue-green portion of the spectrum (~450 nm) while red light (~700 nm) is reflected, giving the solution a rich red color. In particle of size greater than 30 nm, the surface plasmon shows absorption of red light, and blue light is reflected, giving the solutions with a pale blue appearance (Aldrich.com), so is true with various other materials, when they are reduced

to nanoscale. A landmark discovery was made in the year 2006 when a study demonstrated the use of cerium oxide nanoparticles in protecting retinal damage caused due to oxidative stress (Chen et al. 2006). The study created an interest in the scientific community and resulted in the surge of nanoscale antioxidants. The primary benefits of using nanoceria over conventional antioxidants were its low-dosage requirement, and it is self-recycling. We will now follow an elaborate discussion on cerium oxide nanoparticles.

The element cerium is the second member of the lanthanide series with molecular weight 58 u and most abundant element of the rare earth family. The concentration of Ce in earth crust is close levels of the major industrial metals Ni, Cu, and Zn (http://www.molycorp.com/). Ground-state cerium possesses electronic configuration Xe-$4f^1 5d^1 6s^2$, which is largely responsible for its effective cycling between its two ionic states, the ceric ion, Ce^{4+}, and cerous ion, Ce^{3+}. This shift in the reduction/oxidation state/behavior is mainly responsible for its behavior. Fundamental principles of chemistry suggest that higher oxidized state has smaller ionic radii; in this case it is 97 pm, while the lower oxidation state has the ionic radius of 114 pm. However, beyond the fundamental observations of chemistry, the lighter oxygen di-anion in the crystal lattice of ceria is much larger having ionic radius of 135 pm, which is shorter than even the Ce^{3+} cerium ion. In the crystalline state, it has been observed that one cerium atom is coordinated by eight oxygen atoms, whereas each oxygen atom is coordinated to four atoms. The complete unit cell Ce_4O_8 measures 0.51 nm on an edge, and the crystal lattice type is a face-centered cubic (fcc) fluorite lattice structure.

Most of the catalytic activities in biological systems are explained on the basis of oxygen vacancies in crystalline nanoceria. An oxygen vacancy is defined as a missing oxygen atom in either of the eight coordinate positions in a ceria unit cell (Campbell and Peden 2005). Although it is not easy to visualize the oxygen vacancies in the crystal lattice, computational models have been used to demonstrate the presence of such areas in the ceria crystals. Esch and coworkers have shown that using scanning tunneling microscopy coupled with density functional calculations revealed the surface oxygen vacancies generated at extremely high temperatures (Esch et al. 2005). Although it is still unclear that what is the absolute charge on cerium atom with vacancies and the calculation of total number of vacancies is yet not completely determined, one consensus exists among the researchers that with decreasing particle size, a number of cerium atoms in the reduced state often increase. Deshpande, Seal, and colleagues showed in a computational calculation that on increasing Ce^{3+} concentration from 17 to 44%, a decrease in the particle size is observed from 30 to 3 nm. This is one of the very strong reasons for scaling down the cerium oxide to nanosize for the benefits in biological models (Deshpande et al. 2005). Another important feature of the cerium oxide particles at nanosize scale is that the lattice expands in size as the particles are reduced to smaller size, leading to a change in oxygen release and reabsorption process (Reed et al. 2014). Let us now discuss the scientific evidences which support the benefits of cerium oxide nanoparticles in cell culture systems and animal models of oxidative stress.

3.5.1 In Vitro Evidences

Well before the biological activity of cerium oxide was known, it begun to be used as an anti-abrasive in polishing, as a catalyst for power generation in in fuel cells and in fuel-borne additives. Studies demonstrating successful therapeutic or prophylactic effects of nanoceria have exponentially grown over the past decade. As mentioned previously, switching between Ce^{3+} and Ce^{4+}oxidation states has been identified as the primary event responsible for free radical scavenging in autocatalytic manner, making nanoceria superlative to existing antioxidants. Oxygen defects in crystal lattice enable nanoceria to scavenge various radical species such as superoxide, hydrogen peroxide, and hydroxyl and nitric oxide radicals and therefore depend upon surface oxidation states; nanoceria mimics the activities of cellular antioxidant enzymes, superoxide dismutase (SOD) (Heckert et al. 2008), catalase (Pirmohamed et al. 2010), and oxidase (Asati et al. 2009) and is a catalytic amplifier for alkaline phosphatase activities (Hayat et al. 2014) and peroxynitrite-scavenging activities (Dowding et al. 2012).

A number of studies performed using in vitro models support the antioxidant and cytoprotective effects of CNPs in cardiac cells (Niu et al. 2007), monocytes (Hussain et al. 2012), pancreatic cells (Wason et al. 2013), and endothelial cells (Chen et al. 2013). Studies suggested that CNPs reduced apoptosis by modulating apoptotic pathways and by direct scavenging of radicals. Several in vitro studies indicating the possible benefits of the nanoceria in cell culture are summarized in Table 3.3.

Table 3.3 Summary of key studies on cell culture systems indicating the benefits of nanoceria

S. No.	Study model	Size (nm)	Concentration	References
In vitro studies on antioxidant properties of nanoceria				
1	Hemoglobin	3–8	50–100 μM	Dowding et al. (2012)
2	MCF-7 breast cancer	3–5	10 nM	Tarnuzzer et al. (2005) and Colon et al. (2009)
3	CRL-1541 colon cell line	3–5	1–100 nM	
4	HT-1080 cell line	16	1–10 nM	Clark et al. (2010)
5	Cardiac progenitor cells	5–8	59–290 μM	Pagliari et al. (2012)
6	J774A.1 macrophage	3–5	10 μM	Hirst et al. (2009)
7	A549 (carcinoma)	30, 50, 300	29.4–235 μM	
8	HT22 hippocampal cells	6, 12	20–40 μM	Schubert et al. (2006)
9	SH-Sy5Y neuroblastoma	6–16	600 μM	D'Angelo B et al. (2009)
10	Rat spinal cord neurons	3–5	10 nM	Das et al. (2007)
11	Mixed culture of rat neurons	7,10, 50	10 nM	
12	Mouse hippocampal slices	NA	0.6–5 μM	

Table 3.4 Various in vivo evidences showing benefits of cerium oxide nanoparticles

S. No.	Study model	Size (nm)	Concentration	References
In vivo antioxidant properties of nanoceria				
1	Rats	3–5	1–20 nmol	Chen et al. (2006)
2	Knockout mice that develop intraretinal and subretinal neovascular lesions	3–5	172 nmol	
3	Mouse model of retinal degeneration	Not reported	20 nmol	
4	Rats	25	0.0001 nmol/kg	
5	Athymic nude mice	3–5	0.06 nmol/kg	Colon et al. (2009)
6	Transgenic mouse model of cardiomyopathy	7	~300 nmol/kg	Niu et al. (2007)

3.5.2 In Vivo Evidences

Various in vivo studies have also confirmed the biological benefits of nanoceria in retinal protection (Chen et al. 2013) from light-induced damage, anti-inflammatory activity in the liver (Suzanne and Steller 2009), cardioprotection (Niu et al. 2007), and neuroprotection (Estevez and Erlichman 2014). Furthermore nanoceria has also been proved to be beneficial in the protection of cancer (Figueroa) and as a regenerative medicine (Das et al. 2014). Biological studies with beneficial effects of nanoceria are summarized in Table 3.4.

Recently, we demonstrated some of the leading biological effects of the nanoceria including its benefits in moving the mitochondrial membrane potential (Arya et al. 2014), amelioration of lung inflammation during hypobaric hypoxia (Arya et al. 2013), reduction of oxidative stress in primary neuronal culture, and hippocampal neurogenesis (Arya et al. 2016) by using size-restricted and well-characterized cerium oxide nanoparticles.

Toxicological assessment of nanoceria is essential along with its classical pharmacokinetic studies to upgrade the nanoceria status from basic research to clinical trials. Several studies were recently reviewed by Yokel et al. and have provided evidences for both ecotoxicity of and biodistribution of nanoceria (Yokel et al. 2012; Hirst et al. 2013; Molina et al. 2014). The oral, dermal, pulmonary, and intravenous routes of exposure have been explored for ecotoxicology. The intravenous route is particularly important as it provides a suitable reference for the pharmacokinetics and fate of nanoceria in different tissues and organs.

After reaching the bloodstream, nanoceria is primarily distributed to mononuclear phagocytic systems. Scientific evidences suggest that the distribution of nanoceria is not greatly affected by the shape of the particles, dosing schedule, and amount of dose. However, the route of administration is an important factor that affects the distribution pattern. Distribution of nanoceria from the lung to the rest of the body is less than 1% of the given dose. This amount is further reduced if dose is

given from the gastrointestinal tract route. A very slow clearance rate burdens the organ as it may persist there for months. Although the acute toxicity of nanoceria is very low, accumulation of large amount of doses produces granuloma in the lung and liver and also fibrosis in the lung (Yokel et al. 2013).

The biodistribution of a smaller particle generally in the range of 3–5 nm and 15–20 nm agglomerate has been determined using fluorescent-tagged crystalline ceria in CD-1 mice 7 days after 2 or 5 weekly IV 0.5 mg kg^{-1} injections (Karakoti et al. 2008) (Hirst et al. 2013). The highest cerium concentration was observed in the spleen which was followed closely by the liver. Most of the doses were accumulated in the liver and spleen than in the lung (0.15%) or kidney (0.008%). No cerium oxide was detectible in the brain using conventional techniques. Also, clearance of cerium into urine was not found. Studies have also shown that nanoceria does not saturate the organs, but a very little nanoceria was cleared during 35 days after the exposure.

After 10 min of an intravenous (IV) injection, the percentage of the nanoceria left in circulation was less than 2% of the total infused dose for the particles ranging 10–50 nm in size. However, in contrast to this, particles of less than 5 nm showed more than 35% in circulation. Probably, these particles were too small to be readily recognized by macrophages and were therefore avoided opsonin adsorption or blood cell attachment (Hirst et al. 2011). This nanoceria was cleared with initial half-life of 15 min. Similarly, another modified form of citrate-EDTA-coated nanoceria with 2.9 nm in diameter was cleared from blood with a half-life of 3.7 h. Sometimes, an increase of ceria in blood was also observed 2–4 h post-infusion in the case of larger nanoceria particles (Heckman et al. 2013).

Pharmacokinetics of non-IV route was studied by Yokel et al., and they showed that IP injection of nanoceria particles of size 3–5 nm tagged with fluorescent labels at the dose of 0.5 mg kg^{-1} BW was accumulated at the concentration of ~4.2, 1.25, 0.02, and 0.01% of the total dose in the liver, spleen, lung, and kidney, respectively (Yokel et al. 2014).

Nanoceria is not always beneficial and antioxidant; it may cause toxicity through oxidative stress. The adverse effects of nanoceria are often observed at the locations away from the site of exposure and may be attributed to translocation of nanoceria or released biomolecules. Additionally the change in the crystal defects and also the interactions of biomolecules with the nanoceria may also play an important role in toxicity of nanoceria. We also witnessed elevated ROS and hyperpolarization of mitochondrial membrane during our studies, but the evidences were scanty and random. Increased toxicity has also been related to greater surface of Ce^{3+} oxidation state, which becomes more prominent with decreasing particle size and increasing ratio of surface area to volume. The toxic levels of nanoceria via different routes and concentrations are enlisted in Table 3.5.

It is now understood that there exists the risk of long-term exposure to nanoceria based on previous biopersistence toxicity evaluation, which may be a cause of adverse health effects. However, current understating of nanoceria is based on the in vitro and in vivo evidences that suggested that a dose of 50 mg/Kg BW is much below than any toxic doses and therefore can be effectively used to assess the advantageous effects of nanoceria in biological systems. Owing to extensive scientific knowledge gained in the

Table 3.5 Toxicological studies on nanoceria

S. No.	Model system	Particle size (μm)	Concentration (μmol/kg)	References
In vitro studies on toxic effects of nanoceria				
1	3 T3 rodent fibroblast and MSTO-211H human mesothelioma cells	19	20,000–90,000	
2	BEAS-2 human bronchial epithelial cells	15–45	6000–230,000	
In vivo studies on toxic effects of nanoceria				
1	Mice	~130	290–2325 μmol/kg	
2	Rats	15–30	641 mg/m^3	
3	Rats	30	290–4350 μmol/kg	Yokel et al. (2012)
4	Rats	5	495 μmol/kg	
5	Rats	5	495 μmol/kg	Tseng et al. (2014)
6	Rats	30	495 μmol/kg	Yokel et al. (2012)

past one decade, nanoceria can be foreseen as a potential therapeutic and prophylactic of several diseases directly associated with oxidative stress and needs to be carried forward to the subclinical and clinical levels for further investigation.

3.6 Nanoceria as Promising Tool in Diagnosis of Oxidative Stress

Recently, a study at author's lab had demonstrated the use of cerium oxide as potential molecule to develop biosensors for the detection of hydrogen peroxide in the animal/human samples. Hydrogen peroxide (H_2O_2) is one of the secondary reactive oxygen species formed by enzymatic neutralization of superoxides. It is also known to link with ailments such as inflammation, rheumatoid arthritis, diabetes, and cancer. Moreover, in comparison with other reactive species, it is relatively more stable reactive molecular species present in living systems. Due to its widespread roles and relatively more stability, H_2O_2 is a preferred choice for developing biosensors, involved in disease diagnosis. Nanoceria that has been described as one of the potent antioxidants and known to undergo electronic transition is preferred for ROS sensing. These properties were exploited by us, recently for the development of an H_2O_2 biosensor, to sense and quantify peroxide levels. For the development of biosensor, nanoceria was synthesized using different capping agents: hexamethylenetetramine (HMTA) and fructose. HMTA capping gave better and clearer crystalline structure. Amperometric responses were measured by increasing H_2O_2 concentration. The authors tested the CeO_2-HMTA and CeO2-fructose sensitivity and found that it was 21.13 and 9.6 l Acm^{-2} mM^{-1}, respectively. The response time was observed as 4.8 s and 6.5 s for CeO_2-HMTA and CeO_2-fructose, respectively. The limit of detection is as low as 0.6 M and 2.0 M for CeO_2-HMTA and CeO_2-fructose, respectively. Ceria-HMTA was further tested for its antioxidant activity in an animal cell line in vitro,

and the results confirmed its activity. For more detailed description, readers are advised to complete article by Ujjain et al. (Ujjain et al. 2014). This study further strengthens the notion that this novel class of antioxidant is more powerful in terms of its commercial applicability as cerium oxide nanoparticles are not only potential drug candidates ready for their clinical trials but also represent diagnostic opportunities due to their unique electrical properties.

3.7 Future Guideline

As discussed in the beginning of the chapter, the complexity of ROS generation and thereby resulting oxidative stress and its coexistence with antioxidants is much intricate and needs to be unbound step-by-step. An intensified global system biology approach may be needed to answer some of the most difficult questions of the antioxidant biology: How do oxidative stress pathways cross talk? What is the combinatorial effect of antioxidants? How do antioxidants work in different individuals? Is there any threshold for antioxidant supplementation or it is always context specific? Apart from these basic questions on antioxidants, we must also explore the novel classes of antioxidants especially the nanomaterials mimicking as antioxidant enzymes or scavengers due to their enormous benefits over conventional antioxidants. Cerium oxide nanomaterials which have emerged as one of the efficient antioxidants circumvent the common disadvantages of the conventional antioxidants such as high- and repeated-dose requirement. Additionally, the synthetic and inorganic antioxidants also have diagnostic abilities and can also be effectively used for the development of oxidative stress biosensors in the future. However, a word of caution must always be kept while dealing with nanomaterials, as their behavior changes swiftly on changing the shape and size. Hence, with changing dimensions, the benefits may change into harms without even a trail of hint.

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Gold Nanoparticle-Based Methods for Detection of Oxidative Stress Biomarkers

4

Sanjay Singh

4.1 Introduction

Recent developments in the material science to the nanoscale have led to engineer the nanomaterials that can revolutionize the biosciences. Most importantly, the ability to manipulate materials at the nanoscale to use them for targeted delivery of drugs and genes, detection of biomolecules, enhanced industrial catalytic processes and harness the light energy more efficiently than ever (Singh et al. 2012; Singh 2013; Savaliya et al. 2016; Lu et al. 2014; Schauermann et al. 2013; Pelley 2005). In biological sciences research, the discovery of new biomarkers and other biochemical processes has made the possibility of prevention of diseases such as cancer, neurodegenerative, diabetes, and cardiovascular diseases (Xu et al. 2015; Chen-Plotkin 2014; Maiese et al. 2011; Gerszten et al. 2011). In this context, nanomaterials have been successfully incorporated to improve the sensitivity and specificity of detection of biomarkers (Cretich et al. 2015). Additionally, with the use of nanoparticles, the biomolecules or chemical species present in extremely low concentrations in biological systems can also be easily detected from the complex composition. Among several types of nanoparticles such as iron oxide, quantum dots, carbon nanotubes, etc., gold nanoparticles (AuNPs) are mostly used for biomarker detection (Azzazy et al. 2011; Abdelhamid and Wu 2016; Karakoti et al. 2015).

Oxidative stress is linked to the compromised capability of a biological system to neutralize the effect of an overproduction of reactive oxygen species (ROS) and other related radicals (Fig. 4.1). Oxidative stress is known to cause cytotoxicity, DNA damage, genetic aberration, cancer, and several other related environmental pollution-mediated diseases (Tucker et al. 2015; El Assar et al. 2013). Oxidative stress has been associated with the cause of autism in children, diabetes, and cancer (Smaga

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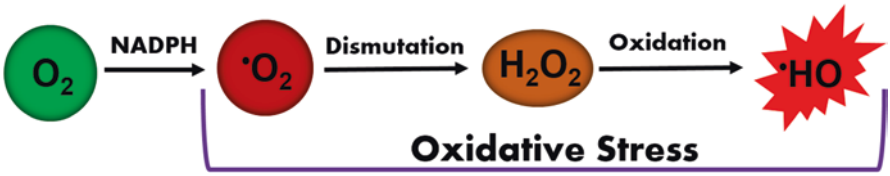

Fig. 4.1 Schematic representation showing most common reactive oxygen species generated in biological system

et al. 2015). In order to estimate the amount of oxidative stress, several biomarkers have been identified, including glutathione (GSH), cysteine (Cys), 3-nitrotyrosine (3-NT), and homocysteine (Hcys). GSH is a tripeptide (glycine, glutamate, and cysteine), present as an important antioxidant in the cytoplasm of plants, animals, fungi, and some bacteria. It is the primary biomolecule, which prevents the cells from the damage caused by oxidative stress. GSH is also responsible for maintaining the reductive environment in the cytoplasm through the redox balance between GSH/GSSH. Mechanistically, GSH reduces the disulfide bonds formed in cellular proteins to cysteines by donating an electron. During this reaction, GSH is converted into an oxidized form, glutathione disulfide (GSSH). In the presence of oxidative stress, nitric oxide is converted into 3-NT, which has been found associated with diabetic patients (Rabbani and Thornalley 2008). Similarly, Hcys has known biomarker for cardiovascular deterioration (Carlsson 2006). Hyperhomocysteinemia occurs due to the endothelial cell injury by enhanced oxidative stress and reduced bioavailability of nitric oxide and other factors, which lead to the increased deposition of LDL on the arterial wall, thereby activation of the coagulation cascade. While these biomarkers, in conjunction with available advanced instrumental assays, can give accurate information about these diseases, wide screening methods are still needed to precisely identify the oxidative stress and related diseases. Further, the development of small, portable, inexpensive, rapid and low-cost operation platforms for biomarker analysis would add value to the screening methods. Therefore, this chapter has been designed to comprehensively cover the methods of oxidative stress detection biomarkers using AuNPs as model nanoparticle system.

4.2 Oxidative Stress Biomarkers

Owing to the extremely high reactivity and short life span of ROS/RNS (reactive nitrogen species), it is expected that suitable biomarker would be the damaged/stressed species isolated from tissues and biological fluids. Although ROS/RNS can be directly detected by ESR (electron spin resonance) with or without spin-trapping reagents or by chemiluminescence, these methods have limited success in clinical studies due to the need of expensive instruments and instability of short-lived reactive oxygen species. Additionally, ROS/RNS themselves cannot be designated as a biomarker for oxidative stress due to the lack of specificity and or sensitivity. Therefore, biomarkers are defined as a characteristic molecule/species which are

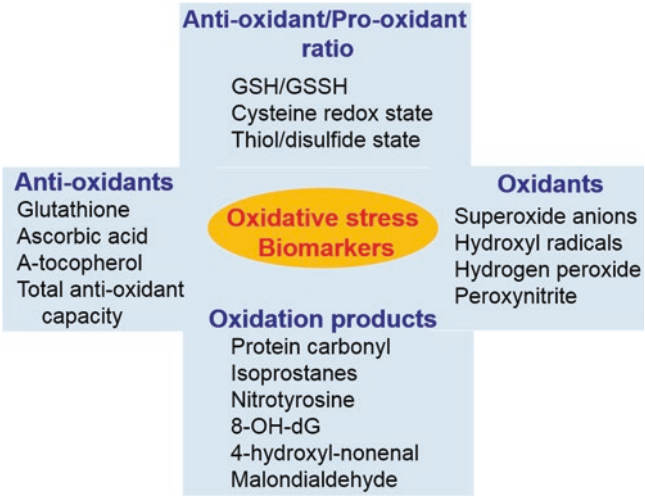

Fig. 4.2 List of biomarkers used for estimation of oxidative stress

specific and can be quantitatively measured and evaluated as an indicator of normal biological processes, disease condition, and treatment response to a therapeutic interference. Generally, biomarkers can be proteins, lipids, nucleotides, and whole cells, which have been shown to exhibit the pathological state of the cell/tissues (Nery et al. 2009; Savaliya et al. 2015). A list of biomarkers used for estimation of oxidative stress is provided in Fig. 4.2.

4.3 AuNPs-Based Colorimetric Assays for Biomarkers of Oxidative Stress

AuNPs exhibit size- and shape-dependent optical properties, which can be easily used for the detection of biomarkers with the help of a spectrophotometer. The biomolecule induces a shift in surface plasmon resonance (SPR) absorbance band of AuNPs, which can be followed to give insight about the biomarker. It is well known that the plasmon oscillation frequency is extremely sensitive and dependent on the dielectric surrounding of nanoparticles, suspension media, and the distance between the nanoparticles (Olson et al. 2015). The SPR of AuNPs-based biomolecule detection has been extensively studied by several research groups from all over the world (Guo et al. 2011; Kim et al. 2009; Pelossof et al. 2011; Li et al. 2016).

4.3.1 Lipid-Based Biomarkers Detection

Oxidized low-density lipoproteins (OxLDL), such as oxidized phosphatidylcholines (OxPCs), are shown to be upregulated in cardiovascular diseases, therefore

described as a potential biomarker (Trpkovic et al. 2015). Hinterwirth et al. have reported the use of AuNPs, conjugated with anti-OxLDL antibodies, for extraction and concentration of OxPCs (Hinterwirth et al. 2013). In this method, selective trapping of OxLDLs from plasma was obtained, which was further detected by liquid chromatography/tandem mass spectrometry (LC-MS/MS). Antibodies were bioconjugated with AuNPs via a bifunctional polyethylene glycol (PEG) spacer and protein G linkage. The controlled conjugation chemistry allowed authors to determine the dissociation constant (Kd) of the OxLDL binding to the AuNPs-antibody conjugate (Fig. 4.3). Further, apparent Kd was also determined for individual PC and their oxidation products, which lead to the assessment of potential antigenicity of (Ox) PCs bound to OxLDLs. This method was one of the best methods to offer new possibilities for targeting lipids in lipoproteins as well as screening lipid-based oxidative stress biomarker. Similarly, other OxLDLs, such as malondialdehyde-modified LDL, play a major role as a useful marker for atherosclerosis and are also reported to be successfully captured and characterized by AuNPs synthesized and functionalized by distinct chemistries. In an attempt by Haller et al., AuNPs were conjugated with anti-MDA-LDL antibodies, which rendered them the selective recognition and capture of MDA-LDL from complex biological sample (Haller et al. 2015). The study explored the optimization of binding affinities and saturation capacities of MDA-LDL-antibody-conjugated AuNPs by following several

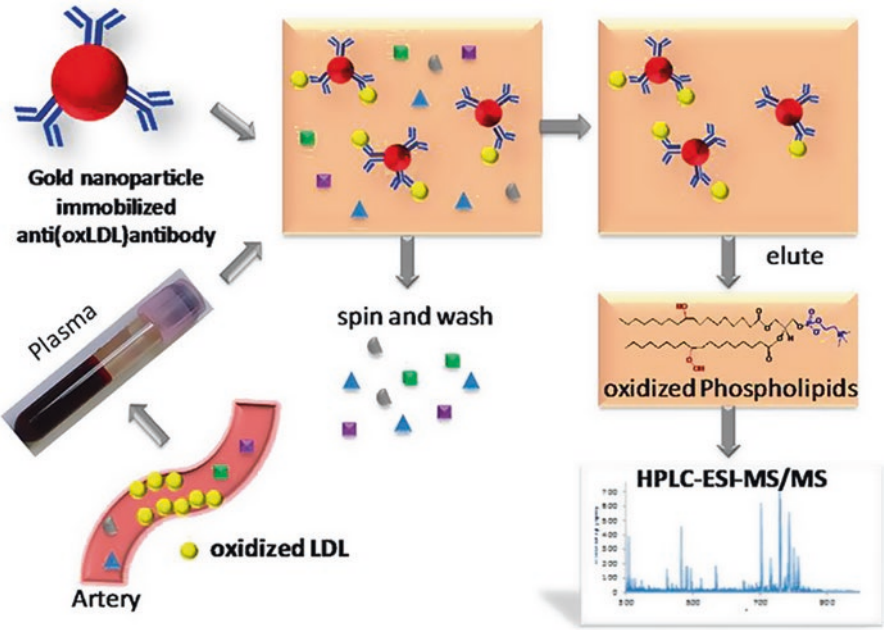

Fig. 4.3 Schematic diagram showing steps of detection of oxidized low-density lipoproteins using AuNPs (Reprinted with permission from reference no. 28, copyright 2013 American Chemical Society)

immobilization approaches, such as (i) direct adsorption of antibodies on AuNPs surface, (ii) amide bond formation between amino groups of antibody and carboxylic group-terminated PEGylated AuNPs, (iii) oxidized carbohydrate moiety of antibody attachment with hydrazine-derivatized AuNPs, and (iv) cysteine-tagged protein A-modified AuNPs. Best conjugation method resulted in three antibodies attached with single AuNPs. The maximum binding capacity was obtained with AuNPs modified with cysteine-tagged protein A, which supported a saturation capacity of 2.24 ± 0.04 μgmL^{-1} AuNPs for MDA-LDL with an affinity Kd of $5.25 \pm 0.11 \times 10^{-10}$ M. It also revealed high specificity for MDA-LDL over copper (II)-oxidized LDL and also for native human LDL. Therefore, this method could be used for specific extraction of MDA-LDL from plasma samples as oxidative stress biomarker, which could further be analyzed by LC-MS/MS, thus allowing sensitive and selective detection of MDA-LDL from complex biological samples. Apolipoprotein E (ApoE) has been assigned as one of the best biomarkers for Alzheimer's and other cardiovascular diseases. It is generally found in the chylomicron and intermediate-density lipoprotein (IDLs) and a major cholesterol carrier in the brain. AuNPs-based nanosensor, which constitutes porous magnetic microspheres as efficient capturing/pre-concentrating platform, has been constructed for efficient detection of ApoE-based Alzheimer's disease (AD) biomarker. The carboxyl functional groups present on iron oxide nanoparticles immobilize the antibodies, which allows the enhanced efficiency in the sensing AD biomarkers from human serum samples. This method was used to detect ApoE concentration at clinically relevant concentrations from cerebrospinal fluid, serum, and plasma samples of patients suffering from AD (de la Escosura-Muniz et al. 2015).

4.3.2 Hydrogen Peroxide Detection

Hydrogen peroxide (H_2O_2) is present in high concentration in the biological system as it performs certain essential functions such as signaling. It is also shown to accumulate at the site of tissue damage, which acts as a signal to attract white blood cells (WBCs) to initiate the healing process. Additionally, H_2O_2 are decomposed in hydroxyl ($HO^{\bullet}$) radicals, which are highly reactive and known to react with neighboring biomolecules and hinder their normal functioning. AuNPs and its composites are shown to sense the presence of H_2O_2, which depicts the pathological condition. A list of AuNPs-based methods for H_2O_2 detection is summarized in Table 4.1. Maji et al. have synthesized a hybrid material consisting of immobilized AuNPs in mesoporous silica covered reduced graphene oxide for H_2O_2 sensing (Maji et al. 2014). Using this method, H_2O_2 can be detected non-enzymatically from 0.5 μM to 50 mM range with a detection limit of 60 nM and good electrochemical sensitivity (39.2 $\mu A\ mM^{-1}\ cm^{-2}$). The advantage of this method is that it does not interfere with the common interfering reagents. Additionally, the hybrid nanomaterial developed was nontoxic and the sensor could sensitively detect H_2O_2 from urine sample as well as nanomolar peroxide produced by living tumor cells (Fig. 4.4).

Table 4.1 List of AuNPs-based methods for H_2O_2 detection

Method of detection	LOD	Results	References
Polymer stabilized AuNPs for detection of H_2O_2	0.7 μM	Enzyme-free biosensor for the electroanalytical detection of H_2O_2 using AuNPs. This novel hydrogen peroxide sensor displayed good reproducibility and long-term stability	Sophia and Muralidharan (2015)
Positively charged AuNPs exhibits peroxidase mimetic activity and their application in H_2O_2 and glucose sensing	5×10^{-7} M	AuNPs (+) possess peroxidase-like activity which provides colorimetric detection of H_2O_2, and it also provides colorimetric detection of glucose. AuNPs (+) have advantage over natural enzymes like easy synthesis, robustness, and stability under extreme environmental conditions	Jv et al. (2010)
AuNPs and cytochrome-c hybrid on ITO (indium tin oxide) interface for amperometric detection of H_2O_2	0.5 μM	Fabrication of ITO/AuNPs/cytochrome-c used as electrode which shows good electrolytic reduction of H_2O_2. Hybrid system with AuNPs archives direct electron transfer of cytochrome-c which allows the development of efficient biosensor	Yagati et al. (2012)
Naked-eye detection of H_2O_2 by oxidation stimulated aggregation of AuNPs	10 μM	AuNPs-based system dependent upon quinone method rearrangement and hydrophilicity change of colloidal AuNPs for naked-eye detection of H_2O_2. It provides quantitative detection and shows linear relation of aggregation of AuNPs	Wu et al. (2016a)
Deposition of AuNPs onto a three-dimensional porous carbonized chicken eggshell membrane for selective and sensitive detection of H_2O_2	3 μM	This HRP-AuNPs-CESM-GCE electrode was used as HRP biosensor for detection of H_2O_2. The electrocatalytic response of the immobilized HRP electrode toward the reduction of H_2O_2 was evaluated from the amperometric current-time response. This sensor has advantages of ease of construction, rapid response, low cost, and convenient use. It has high sensitivity with the linear range of 0.01–2.7 mM H_2O_2	Zhang et al. (2015)

(continued)

Table 4.1 (continued)

Method of detection	LOD	Results	References
With the assist of dual molecules, 2-(N-morpholino) ethane sulfonic acid and sodium citrate, shape-controlled development of AuNPs for H_2O_2, and oxidase biosensing	1 μM	$AuCl_4^-$/MES/Na_3Cit/ H_2O_2 reaction system can be employed for detection of H_2O_2 by the "naked eye" and has been applied to probe substrate or enzyme in oxidase-based reactions	Peng et al. (2014)
An unmodified AuNPs-based method for H_2O_2 detection is developed using horseradish peroxidase and o-phenylenediamine as the catalyst and substrate	1.3×10^{-6} M	A rapid, label-free, colorimetric AuNPs-based detection method of H_2O_2. It has sensitivity with a linear range of 1.3×10^{-6} and 4.1×10^{-5} M H_2O_2	Wu et al. (2007)
Electrode-modified ultrathin AuCu nanowires for detection of H_2O_2 in nanomolar concentration	2 nM	Detection of trace amount of H_2O_2 released from raw 264.7 cells provides real-time quantification of H_2O_2 in biological environment	Wang et al. (2015)
Colorimetric detection of H_2O_2 on the basis of Fenton reaction with AuNPs	40 nM L^{-1}	A visual assay utilizes Fenton reagent, an arbitrary ssDNA and AuNPs in the presence of salt for the sensing of H_2O_2 based on DNA cleavage, and stimulates the aggregation of AuNPs. The concentration of H_2O_2 in the linear range of 2.0×10^{-7}–8.0×10^{-6} mol L^{-1}	Sang et al. (2010)

Similarly, a core-shell-like arrangement of platinum-coated gold nanoparticles was used by Li et al.; they showed an electrochemical reduction of hydrogen peroxide under the physiological condition (Li et al. 2013). The ultrathin layer of Pt was found to enhance the sensing capability. They found that at an applied potential of 0.08 V (vs. Ag/AgCl), the current reduction of H_2O_2, was directly proportional to its concentration in the range of 1–450 μM, with the detection limit of 0.18 μM. Koposova et al. have also reported the use of ultrathin Au nanowires and NPs assembled with horseradish peroxidase enzyme for bio-electrochemical detection of H_2O_2 (Koposova et al. 2014). Currently, more efforts are being devoted to visual and single-step detection of analytes; therefore, strategies have been developed to detect H_2O_2 as well. In one attempt by Sang et al., a novel concept was used wherein they used ssDNA (single-stranded DNA)-coated AuNPs for H_2O_2 detection. DNA-stabilized AuNPs are known to be unaffected by salt-induced aggregation due to

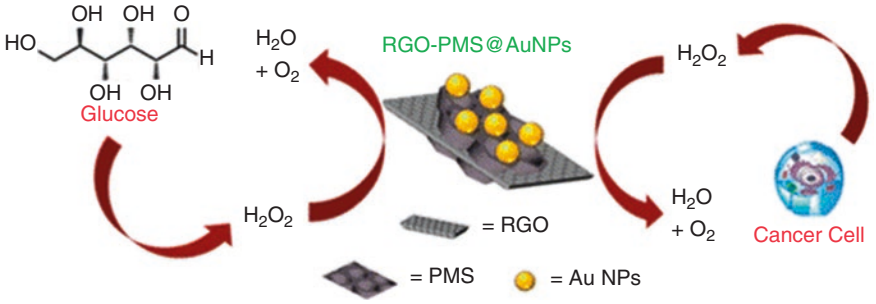

Fig. 4.4 Two-dimensional (2-D) hybrid material (RGO-PMS@AuNPs), fabricated by the immobilization of ultra-small AuNPs, ~3 nm, onto sandwich-like periodic mesoporous silica-coated reduced graphene oxide, was employed for both electrocatalytic application and cancer cell detection (Reprinted with permission from reference no. 31, copyright 2014 American Chemical Society)

strong electrostatic repulsion; however, in the presence of hydroxyl radicals (HO*), AuNPs undergo aggregation because of the cleavage of stabilizing ssDNA (Sang et al. 2010). As expected, the aggregated AuNPs show a change in color from ruby red to blue, which could be seen by the naked eyes, and the changes in surface plasmon resonance (SPR) light scattering could be measured with a common spectrofluorometer. The measured values of Abs (650)/Abs (520) of the shifted SPR wavelength were found directly proportional to the H_2O_2 concentration in the range of 2.0×10^{-7}–8.0×10^{-6} mol L^{-1} with the limit of determination being 40 nmol L^{-1}. Further, this technique was successfully used for detection of the content of H_2O_2 in rat encephalon extract with 98–103% recovery. Additionally, hybrids of AuNPs with proteins and enzymes have also been reported to be one of the excellent H_2O_2 sensors. One of such method was to hybridize AuNPs with cytochrome-c on indium tin oxide (ITO) electrodes using immobilization method (Yagati et al. 2012). AuNPs were conjugated with ITO electrodes by 3-mercaptopropyltrimethoxysilane (3-MPTMS), and the electrode was further modified with 11-mercaptoundecanoic acid (11-MUA), which leads to the attachment of cytochrome-c. During differential pulse voltammetry (DPV) and amperometric I-T measurements on the modified electrode, the system showed a linear response after H_2O_2 addition. The electron transfer rate constant was determined to be 0.69 s^{-1} with a limit of detection of 0.5 μM. A similar attempt was made by Nandini et al., wherein they immobilized the catalase derived from *Pichia pastoris* on a tubular AuNPs surface, and this nanocomposite was used for H_2O_2 detection (Nandini et al. 2014). Authors used cyclic voltammetry, differential pulse voltammetry, and chronoamperometry for analysis and studied various parameters such as working potential, pH, and thermal stability of the modified electrode. It was observed that the composite exhibits a linear response range from 50 μM to 18.5 mM of H_2O_2, a quick response time of 7 s, an excellent sensitivity of 26.2 mA mM^{-1} cm^{-2}, and a detection limit of 0.12 μM. This method showed good reproducibility and high stability and operates at a biocompatible microenvironment.

4.3.3 Superoxide Anion Detection

In biology, superoxides are considered to be extremely toxic, which are managed by the immune system to destroy foreign microorganisms. Superoxides are produced in large quantity by phagocytes with the use of NADPH oxidase enzyme, which is known as an oxygen-dependent killing mechanism of foreign pathogens. In the biological system, superoxides are also produced in mitochondrial respiration (as a by-product) in complex-1 and II and with the help of other notable enzymes such as xanthine oxidase, which upon reaction with hypoxanthine produces a lot of superoxide anions (Grivennikova et al. 2016; Muller et al. 2008). Owing to the importance of superoxides in biology, several attempts have been successfully shown the sensitive detection using AuNPs. Well-known surface properties of AuNPs have made the surface modification extremely easy; therefore, several proteins and enzymes have been successfully immobilized on the surface of AuNPs. Interestingly, these enzymes still retain their catalytic activity after surface immobilization. Similarly, superoxide dismutase (SOD) enzyme is immobilized on AuNPs surface for scavenging of superoxide anions (Santhosh et al. 2011). In this method, direct electron transfer was achieved between the enzyme and electrode with an electron transfer rate constant of 8.93 s^{-1}. With the applied potential of +300 mV, AuNPs-SOD composite showed highly sensitive detection of $O_2^{\cdot -}$. Additionally, a novel amperometric biosensor for superoxide anion based on SOD immobilized on AuNPs-chitosan-ionic liquid biocomposite film has been constructed by Wang et al. (Wang et al. 2013). In this method, cyclic voltammetry and chronoamperometry were used to evaluate the electrochemical performance of constructed biosensor. Authors observed a pair of quasi-reversible redox peaks of SOD with a formal potential of 0.257 V at the constructed SOD-AuNPs composite at physiologically relevant condition (Fig. 4.5). Based on the several electrochemical parameter evaluation, it was shown that the synthesized biosensor showed specific reactivity of SOD toward superoxides and exhibited a fast amperometric response (<5 s), wide linear range (5.6–2.7 × 10^3 nM), low detection limit (1.7 nM), and excellent selectivity for the real-time measurement of superoxide anions. Liu et al. have reported a novel AuNPs-based for direct electron transfer measurement from copper, zinc superoxide dismutase, and used it for an efficient superoxide anion biosensor (Liu et al. 2008). The advantage of this method is that it does not involve the use of any mediators or promoters for electron transfer. This method used pyramidal, rodlike, and spherical Au nanostructures, and the detailed study revealed that the thermodynamic and kinetic parameters of the electron transfer is dependent on the morphology of the electrodeposited gold nanostructures. The SOD was found strongly confined on all three types of AuNPs surface; thus, the direct electron transfer between AuNPs and SOD was further eased out. This method offered a dual electrochemical approach to detect superoxides, in which it could be detected at both on anode and cathode. The biosensor displayed excellent analytical performance, for example, wide linear range, low LOD, quick response time, and excellent reproducibility at both the electrodes. The common interfering factors such as uric acid, ascorbic acid, and hydrogen peroxide were not found affecting the results.

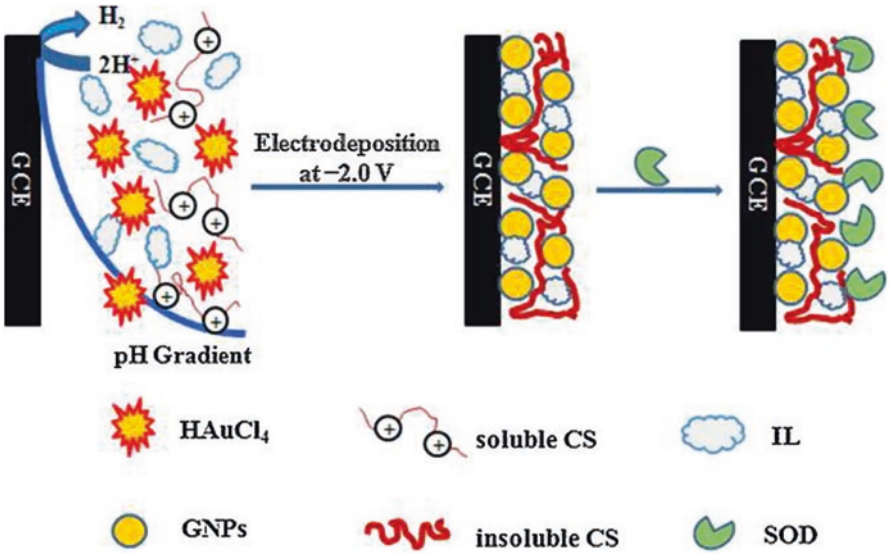

Fig. 4.5 Schematic representation of the assembly process of SOD/GNPs-CS-IL/GCE and detection of superoxide anions (Reprinted with permission from reference no. 47, copyright 2013 Elsevier)

The discovery of new sensitive techniques has provided an extra advantage to the detection techniques, and therefore the limit of detection has gone up to the molecular levels. Surface-enhanced Raman spectroscopy or surface-enhanced Raman scattering (SERS) is one of such techniques discovered in mid-70. It is a surface-sensitive technique, based on the enhancement of the Raman scattering by molecules adsorbed on rough metal surfaces or by nanomaterials (Wilson and Willets 2013). The enhancement of scattering may be as high as ten (Gerszten et al. 2011), which suggest that this technique may sense the information from single molecules. Qu et al. have used this technique with AuNPs and cytochrome-c composite to construct superoxide nanosensors (Qu et al. 2013). This method relies on the alterations in the SERS spectra of the oxidized and reduced form of cytochrome-c; therefore, this nanosensor was able to investigate superoxide concentration by quantifying the SERS spectra of the reduced cytochrome-c. Authors reported the LOD of 1.0×10^{-8} M. Most importantly, this sensor was unaffected with the other reactive oxygen species and also additional biologically relevant species. Therefore, this nanosensor was further used to check the concentration of superoxides in living HeLa and normal human liver cells in real time and in a noninvasive manner. Further Chen et al. have developed a new multi-modified core-shell Au@Ag nanoprobe for real-time monitoring of the entire autophagy process at a single-cell level (Chen et al. 2015). Understanding the mechanism of autophagy is essential as it controls the systematic degradation and recycling of cellular components. The role of autophagy is vital to understand the mechanism of human diseases, new drug development, and approaches to cell survival by conserving the cellular energy levels.

Several approaches have been revealed to understand the mechanism of action of autophagy; however, its real-time monitoring is still infancy. In this context, Chen et al. proposed a solution, wherein a real-time, in situ detection of superoxide radicals may impart essential insight about real-time monitoring of autophagy, as superoxides are one of the major regulators of autophagy (Chen et al. 2015). In this study, the developed nanoprobe was etched by superoxides, which resulted in a notable change in the absorbance wavelength of surface plasmon resonance spectra. The alteration in absorbance wavelength was in agreement with the level of superoxide radicals. This observation was also confirmed with the simulated studies. The sensor response enabled its application in real-time in situ quantification of superoxides during the process of autophagy. Upon applying to the cells the "relay probe," operation revealed two types of superoxide regulating autophagy processes, which were successfully traced from the beginning to the end, and the possible mechanism was also suggested.

4.3.4 Hydroxyl Radical Detection

In the biological system, hydroxyl radicals are produced during immune action as a by-product but more frequently by macrophages and microglia cells when exposed to pathogens. Since hydroxyl radicals are extremely reactive, they can virtually damage all types of biomolecules of cells/tissues. Therefore, these radicals can be very dangerous to the normal functioning of carbohydrates, nucleic acids, lipids, and amino acids. Additionally, there is no enzyme in the cell which can specifically eliminate the hydroxyl radicals from the system. The endogenous antioxidants such as melatonin and GSH and dietary antioxidants such as mannitol, ascorbic acid, and vitamin E can be used for the scavenging of the hydroxyl radicals from the biological systems. Therefore, the easy detection and sensitive quantification of hydroxyl radicals are needed to avoid its deleterious effects on the cells. Tang et al. reported the use of AuNPs as quencher module in fluorescent probes for DNA damage caused by intracellular hydroxyl radicals (Tang et al. 2008). In this method, AuNPs (15 nm) were surface modified with DNA oligomers having thiol functional group at the 3′ positions while 5′ end modified with a fluorophore. Upon exposure to hydroxyl radicals, the FRET would switch off due to the radical-induced DNA strand break into ssDNA. However, the broken ssDNA would retain the fluorescence, which can be estimated by spectrophotometry. Hydroxyl radicals were generated artificially by Fenton chemistry, showing linear response range from 8.0 nM to 1.0 μM with a detection limit of 2.4 nM. Authors used confocal microscopy to image the hydroxyl radicals in macrophages and HepG2 cells and observed a good sensing response. This method could be further used for the investigation of the mechanism behind hydroxyl radical-mediated injury and diseases. In another approach, Wu et al. have shown the use of AuNPs-based biobarcode nanoparticles for construction of a DNA-based biosensor for detection of hydroxyl radicals electrochemically (Wu et al. 2012). The biosensor platform was constructed using a planar Au-electrode immobilized with thiolated DNA1 (SH-DNA1). The hydroxyl radicals were

artificially generated by Fenton chemistry, which could induce damage to the immobilized DNA layer on AuNPs. Further, an intercalating probe, methylene blue, was used to monitor the extent of DNA damage. Additionally, to enhance the sensitivity of biosensor, DNA2-modified AuNPs were used as response signal amplifier. This DNA-based biosensor could quantitatively sense hydroxyl radicals up to 10 mM with a detection limit of 3 μM.

AuNPs have recently shown applications in microwave-assisted cancer hyperthermia, where interaction with an electromagnetic radiation leads to the generation of hydroxyl radicals, which mediates the enhanced cancer cell killing, mostly further than the pure heating effects. In a method reported by Paudel et al., 500 mM of 5,5-dimethyl-1-pyrroline N-oxide (DMPO) in 20 ppm AuNPs was scanned with an electron paramagnetic resonance (EPR) spectrometer to generate and detect hydroxyl radicals(Paudel et al. 2016). It was found that due to the exposure of AuNPs-DMPO to microwave, hydroxyl radical signals were obtained under EPR. Authors further report that AuNPs can generate hydroxyl radicals in aqueous media when exposed to a microwave field. Therefore, if these radicals are generated in the close vicinity of DNA of cells by accurate localization of particles, the resulting hyperthermia can exhibit enhanced cell killing utilizing both the in situ elevated temperature and localized hydroxyl radical generation.

4.3.5 Malondialdehyde Detection

Malondialdehyde (MDA) is one of the most common biomarkers of oxidative stress and is used for the diagnosis of many diseases. MDA is one of the low-molecular-weight end products, which is synthesized by the decomposition of some primary and secondary lipid peroxidation products. Although the most common method to detect the MDA formation, due to lipid peroxidation, is the use of 2-thiobarbituric acid (TBA), the use of AuNPs has been limited. Mechanistically, at acidic pH and high temperature, MDA readily participates in a nucleophilic addition reaction with TBA, thus producing a red- to pink-colored (Fig. 4.6) aqueous soluble adduct (Singh et al. 2009). The advantage with this adduct is that it can be measured quantitatively by absorbance and fluorescence. This method is also famous as "TBA test" to routinely use for qualitative and quantitative detection of lipid peroxidation in a wide range of sample types. It should also be mentioned here that not all kinds of lipid

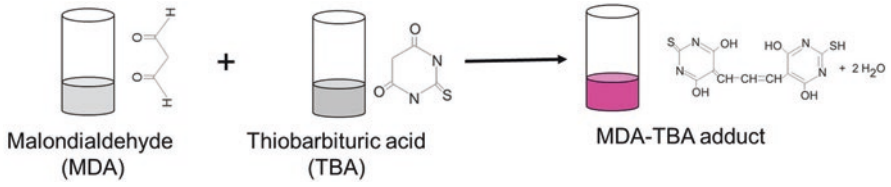

Fig. 4.6 Schematic representation of the formation of MDA-TBA adduct for colorimetric detection of lipid peroxidation

peroxidation produces MDA and that too in very low quantity. Additionally, MDA is not the only end product of lipid peroxidation; however, other factors or stimulus for lipid peroxidation may also induce the MDA formation from lipids of cells/tissues. On the other hand, TBA is not selective only to MDA, rather it can form an adduct with several other nonlipid-related products as well as fatty acid-based peroxide-derived decomposition products, which are not MDA. It has also been observed in many cases that MDA-TBA adduct formation is an indicator of lipid peroxidation; however, in other cases, no qualitative or quantitative information about MDA content, TBA reactivity, and lipid peroxidation can be obtained from the test samples(Janero 1990). Therefore, the use of "TBA test" for the interpretation of MDA content in the sample with respect to lipid peroxidation requires careful discretion and correlation of data with other methods of analysis of MDA formation and decomposition of lipid peroxides. In a report by Zamani-Kalajahi et al., a poly-taurine film was deposited on Au surface to develop an electrochemical biosensor for detection of MDA (Zamani-Kalajahi et al. 2014). In this study, repetitive cyclic voltammetry (CV) was used to deposit poly-taurine on Au surface in PBS. The poly-taurine/Au-electrode was used to follow the electroanalytical behavior of MDA by differential pulse voltammetry. This method was used to detect MDA from human serum and exhaled breath condensate and revealed that this electrochemical sensor can quantitate the MDA levels from healthy individuals, as the limit of detection of this method ranges from 0·78 to 3·10 mM, which is the normal concentration range. Further, it was also reported that this nanosensor can detect MDA up to the level of 34 and 0·995 nM in human serum and exhaled breath condensate samples, respectively. Thus, this study can provide a base for the investigation of other AuNPs-based methods to sense MDA and other related lipid peroxidation products efficiently. Further, the invention of a noninvasive method for MDA detection from humans could offer it to be used routinely in a pathological laboratory.

4.3.6 C-Reactive Protein (CRP) Detection

CRP is a pentameric protein, known to be found in blood plasma in high concentration during inflammation and oxidative stress (Huang et al. 2012; Tracy et al. 2014). It is synthesized by the liver; however, the production increases in response to interleukin-6 secretion by macrophages and T cells. The main function of CRP is to bind with the phosphocholine, which is expressed on the surface of necrotic and apoptotic cells and bacterial cells followed by the activation of complement system thus promote phagocytosis by macrophages (Kushner and Antonelli 2015). The level of CRP increases in blood in response to a wide variety of conditions such as inflammation (due to viral, bacterial, or fungal infections) and diseases mediated by oxidative stress (cancer, cardiovascular, fibrosis and inflammation, coronary heart disease, rheumatoid arthritis, and tissue damage)(Li et al. 2015; Allin and Nordestgaard 2011; Zhang et al. 2010; Kojima et al. 2013). Since its level in the blood rises very quickly (up to 50 k fold within 2 h), it is considered as one of best markers of inflammation and thus oxidative stress. The blood level of CRP has been divided

into three categories, <1.0 mg/L (low risk), between 1.0 and 3.0 mg/L (average), and >3.0 mg/L (high risk). Owing to the importance of CRP levels in the blood, several methods of detection have been developed to effectively sense the concentration. These methods are based on ELISA, immune turbidimetry, nephelometry, rapid immunodiffusion, and visual agglutination. These methods face limited sensitivity; therefore, AuNPs-based methods are developed by several research groups worldwide, with a sensitivity of femtomolar levels (Table 4.2).

Further, it is also imperative to develop easy-to-use CRP sensor, which is quick in detection but is cost-effective as well. These properties are vital for the success of various diagnostic applications. Incorporating AuNPs, lateral flow assay (LFA)-based strip sensor has been developed, which possess several advantages such as rapid, one-step, and high-throughput analysis (Oh et al. 2014). Oh et al. have developed a new strategy to overcome the "hook effect" of CRP, which is a major hurdle of the LFA kind sensor (Oh et al. 2014). They constructed a three-line LFA strip sensor by adding an extra antigen line along with the traditional two-line LFA sensor. This allowed detecting CRP within a broad concentration range in the human serum (Fig. 4.7). This LFA strip sensor was able to linearly detect CRP concentration from 1 ng/mL to 500 μg/mL within 10 min (detection range of 0.69 ng/mL–1.02 mg/mL). With the use of this three-line LFA sensor, authors could measure 50 clinical samples with a detection range of 0.4–84.7 μg/mL. The use of aptamers with AuNPs has also shown successful detection of CRP up to pM concentrations. In one such effort by Wu et al. DNA aptamers against CRP were used in a microfluidic chip platform (Wu et al. 2016b). These aptamers were coated on AuNPs surface and used for the construction of an AuNPs-based enhanced surface plasmon resonance biosensor. Upon interaction between CRP with aptamer-conjugated AuNPs, the alteration in surface plasmon resonance pattern was monitored, which could detect CRP from concentrations ranging from 10 pM to 100 nM in diluted human serum. Although aptamers are excellent recognition molecules used for specific detection of biomolecules, their complex synthesis method is one of the major hurdles in the production of biosensors in large quantity. Therefore, other specific ligands have been used which act as artificial protein recognition agent. Recently, Kitayama et al. have reported the synthesis of AuNPs coated with poly(2-methacryloyloxyethyl phosphorylcholine), which shows specific recognition to CRP (Kitayama and Takeuchi 2014). The SPR pattern of AuNPs is altered upon CRP binding, which acts as the recognition signal. This nanosensing method could detect CRP in human serum solution with a limit of detection of ~50 ng/mL. Authors also reported that the nonspecific absorption of other proteins from serum was negligible. This method offers several advantages such as low cost, not needing expensive instruments, easy experimental procedure, zero manpower training, and high stability. AuNPs are also known to exhibit excellent catalytic activity in generating chemiluminescence by luminol and H_2O_2. This property of AuNPs has also been exploited to synthesize a quantitative analytical method to measure CRP in human serum. In this strategy, the chemiluminescence intensity of luminol in the presence of CRP and its ligand, O-phosphorylethanolamine, was greatly enhanced due to the aggregation of AuNPs after the addition of 0.5 M NaCl. Since CRP stabilizes AuNPs, in the absence of O-phosphorylethanolamine,

Table 4.2 List of AuNPs-based methods for C-reactive protein detection

Method of detection	LOD	Results	References
Synthesis of biomimetic block copolymer poly(2-methacryloyloxyethyl phosphorylcholine)-b-poly(N-methacryloyl-(L)-tyrosine methylester)-protected AuNPs	20–40 nM	The system may be used for direct colorimetric screening of inflammation and infection by the naked eye	Iwasaki et al. (2014)
AuNPs amplified surface plasmon resonance imaging aptasensor	7×10^{-21}	Potential applications in detection of proteinaceous and non-proteinaceous molecules found in blood, urine, and other specimens due to attomolar sensitivity	Vance and Sandros (2014)
AuNPs enhanced surface plasmon resonance based on aptamer-antibody sandwich assay	10 pM–100 nM	The aptamer-based biosensor showed high selectivity along with specificity for CRP detection and also had an advantage of regeneration	Wu et al. (2016b)
Localized surface plasmon resonance using PMPC-g-AuNPs by surface-initiated atom transfer radical polymerization	~50 ng/ml	PMPC-g-AuNPs avoid nonspecific binding and works as highly specific nanosensors for CRP based on LSPR properties	Kitayama and Takeuchi (2014)
Electrochemical impendence immunosensing method	10 ng–10 μg	The technique used to immobilize the antibody on the electrode surface and the methodology employed to analyze the bioelectrode response are sufficiently general, reliable, and sensitive for the early detection of acute myocardial infarction	Mishra et al. (2014)
Quantitative method of detection based on the catalytic activity of AuNPs and luminol H_2O_2 chemiluminescence	1.88 fM	The approach offers label-free quantification of samples at very low concentrations without using antibody, with 164 times greater LOD than ELISA	Islam and Kang (2011)

(continued)

Table 4.2 (continued)

Method of detection	LOD	Results	References
Quartz crystal microbalance (QCM) immunosensor based on 20 nm AuNPs conjugated anti-CRP antibodies.	20 ng/ml	The results show the use of AuNPs in immunosensor format which can be regenerated using 3 mol urea solutions. By integrating microfluidic system with a shorter regeneration cycle, the amount usage of reagents and samples can be reduced	Ding et al. (2013)
Development of Au-based working electrode immunosensor	2.2 ng/ml	The developed CRP immunosensor was able to detect a diagnostically relevant range of the biomarker in serum without the need for signal amplification using nanoparticles	Fakanya and Tothill (2014)
Colorimetric rolling circle immunoassay	30 fg/ml	The assay is cost-effective and maintains the temperature conditions and label-free quantification of protein	Wang et al. (2014)
Homogenous assay method based on AuNPs aggregation induced colorimetric response	10–5 μg/ml	The determination of CRP levels in serum improved from 100 ng/ml to 10 ng/ml by eliminating the hook effect and inducing aggregation of AuNPs	Byun et al. (2013)

thus the chemiluminescence intensity of luminol is ordinary, and the particles are well dispersed. This method displayed linear enhancement in chemiluminescence intensity in proportion to CRP concentration with the range of 1.88 fM–1.925 pM and limit of detection 1.88 fM. Authors claim that the sensitivity of detection of this method was 164 times better than that of the conventional, ELISA method (Islam and Kang 2011).

4.3.7 Glutathione (GSH) Detection

GSH is an important endogenous antioxidant biomolecule present in eukaryotes (plants and animals) and prokaryotes (bacteria and fungi). It is composed of cysteine, glycine, and glutamic acid, conjugated with gamma peptide linkages. Being present in high concentration (~5–7 mM) in the cytoplasm, GSH is capable of scavenging the free radicals and thus thwarting the damage caused by oxidative stress to important cellular components and organelles (Singh et al. 2016). Mechanistically,

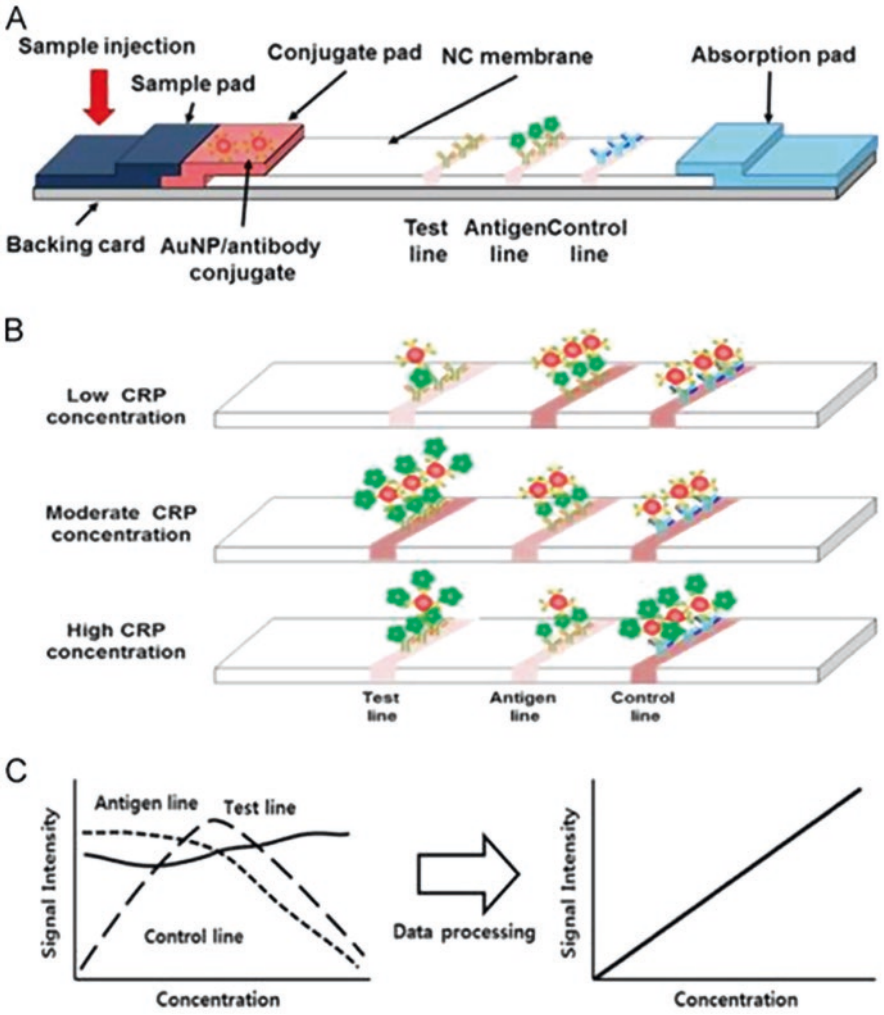

Fig. 4.7 Schematic diagram of the antigen-introduced three-line LFA strip sensor. Structure (**a**) detection principle (**b**) and data processing (**c**) (Reprinted with permission from reference no. 75, copyright 2014 Elsevier)

the thiol group in GSH acts as a reducing agent and thus forms disulfide bonds which lead to the formation of cysteines within the proteins of the cell cytoplasm. In doing so, GSH overall acts as an electron donor and itself gets converted into oxidized glutathione (GSSH). Additionally, the redox-based interconversion of GSH to GSSH and from GSSH to GSH with the use of NADPH (an electron donor) makes GSH an exceptional antioxidant molecule, which can keep on scavenging the free radicals. The ratio of GSH/GSSH in cell cytoplasm is frequently used as a qualitative and quantitative measure of oxidative stress. Although, there are several

methods reported for the quantitative measure of GSH in cell cytoplasm as an indicator of oxidative stress, the spectrophotometric/microplate reader assay methods are more famous and are readily in use. Among them, 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB)-based colorimetric determination of GSH is very common. In this method, oxidation of GSH by DTNB is performed which gives a yellow-colored product known as 5′-thio-2-nitrobenzoic acid (TNB), which can be quantitated by spectrophotometer by measuring the absorbance at 412 nm. In fact, this method has two parts: first part involves the steps for the preparation of cell cytoplasm for assay and the second part comprises the detection of total glutathione (GSH and GSSG) in the testing material. This method has several advantages such as simple, convenient, quick (~ 15 min), sensitive, and accurate and can be performed in a 96-well plate with the detection limit of 0.103 nM. The assay can be applied to a variety of samples such as whole blood, blood plasma, serum, lung lavage fluid, cerebrospinal fluid, urine, tissues, and cell extracts (Rahman et al. 2006). Depletion of GSH has been correlated with the pathogenesis of several diseases such as Parkinson's disease. Mytilineou et al. have reported that due to oxidative stress, GSH level is compromised, which leads to the development of Parkinson's disease (Mytilineou et al. 2002). Drop in GSH level is one of the early symptoms of this disease. To elaborate this study, authors treated mesencephalic cell culture model with a known GSH synthesis inhibitor, L-buthionine sulfoximine (BSO). It was also reported that drop in GSH level leads to phospholipase A2-dependent release of arachidonic acid, which cause damage to cells through lipoxygenase metabolism. Ultimately, superoxide radicals are generated, which could be one of the reasons for the toxicity cascades followed by GSH depletion.

Despite above discussed method, specific assays to GSH are still infancy, which could be applied to detect GSH in human serum with satisfactory results. Additionally, the linkage of the recent discovery of disease biomarkers with GSH, amino acid determination in fields such as food processing, biochemistry, pharmaceutical, clinical analysis, etc. are some areas which would require a great demand for methods of sensitive detection of GSH. In this context, incorporation of nanomaterials has provided an opportunity to enhance the GSH detection limit up to fM levels. Among them, AuNPs have been investigated in great extent, and several methods are reported (Table 4.3). Chen et al. have developed a sensitive method, which is based on resonance light scattering technique for GSH detection using AuNPs (Chen et al. 2012). It is well known that AuNPs exhibit nanoparticle distance-dependent optical properties. GSH-decorated AuNPs were synthesized, which undergo aggregation in the presence of Cu^{2+} ions. Here, Cu^{2+} induces the formation of glutathione-Cu^{2+} complex, which results in bringing AuNPs close to each other. Due to aggregation of AuNPs, the resonance light scattering intensity is greatly altered with the color of AuNPs change from red to blue and a shift in absorbance wavelength. This method can determine the concentration of GSH by the naked eye, absorbance or fluorescence spectrometer in 20 mins with a limit of detection of 10 nM. Photoluminescence (PL)-based detection of GSH using AuNPs has also been investigated, which could be applied for the imaging and labeling of intracellular thiols in cells. Su et al. showed that GSH-stabilized AuNPs show

Table 4.3 List of AuNPs-based methods for GSH detection

Method	LOD	Results	References
Resonance light scattering assay for detection of GSH by AuNPs as a colorimetric probes	10 nM	GSH can be measured by observing color change with the naked eye. RLS-based assay has a concentration range for GSH detection lies between 40 to 280 nM. Dynamic range of a sensor can be tunable by tuning AuNPs size and concentration	Chen et al. (2012)
Colorimetric probe for detection of GSH based on anti-aggregation property of AuNPs via pH modulation	12 nM	Discriminative determination of GSH over cysteine and homocysteine in PBS buffer solution based on regulation of pH and anti-aggregation of AuNPs. Cysteine and homocysteine cannot show any interference but demonstrate its high sensitivity and selectivity toward the detection of GSH. This method is rapid, convenient, and cost-effective	Li et al. (2017)
Quasi-stable AuNPs assembly for colorimetric detection of GSH	0.5 μM	Selective and sensitive method for detection of GSH. The concentration range of detection of GSH 0.5–1.25 μM	Hu et al. (2013)
Development of dispersion-dominated approach for visual detection of GSH using AuNPs	11×10^{-9} M	Qualitative assessment of cellular GSH level in cancer cells A549 and HeLa and normal cells HASMC and HAF. GSH prevents the aggregation of arginine-modified AuNPs via mercury-thiol interaction, which helps in GSH sensing by the naked eye	Xianyu et al. (2015)
Conjugation of AuNPs to SiNPs via disulfide bonds for sensing of GSH	0.5 μM	OFF-ON probe for GSH detection. GSH is a disulfide-reducing agent; it cleaves the disulfide bridge between two nanohybrids and releasing the AuNPs from nanohybrid; thus fluorescently dark SiNPs–S–S–AuNPs resumes strong fluorescence emission (ON state). Fluorescence of SiNPs was quenched by AuNPs bound on SiNPs surface via disulfide linkage, and nanohybrid becomes fluorescently dark (OFF state)	Shi et al. (2013)
Developing AuNPs-based colorimetric sensor for GSH detection via anti-aggregation of AuNPs	8 nM	Selective and sensitive detection method for GSH depends upon anti-aggregation of AuNPs in the piperazinebisdithiocarbamate system	Li et al. (2011)
Polyethylene amine-coated AuNPs for glutathione sensing and nucleotide delivery	20 nM	PEI- AuNPs have a good catalytic activity of peroxidase mimics and GSH sensing based on competitive inhibition	Pandey et al. (2016)

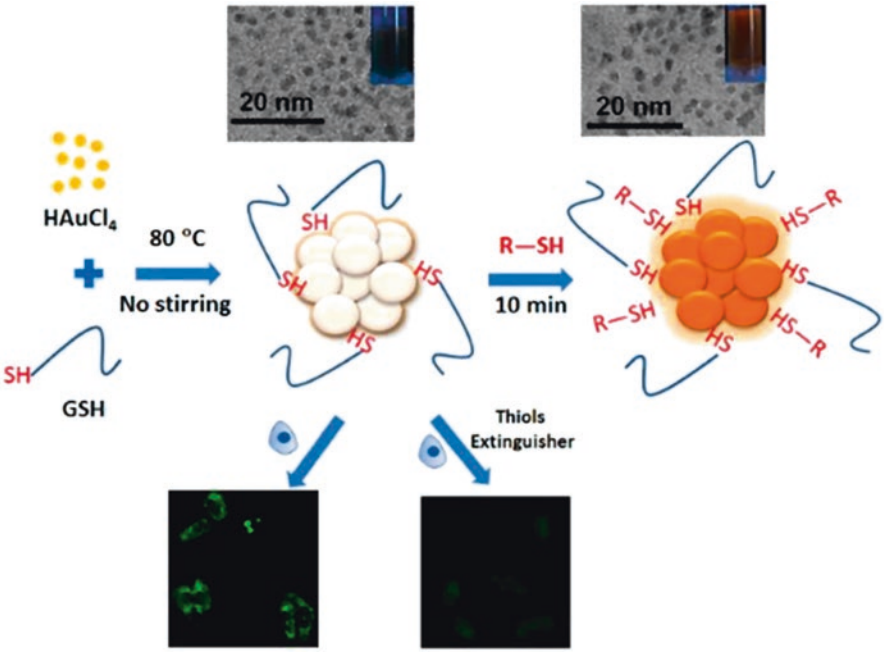

Fig. 4.8 Schematic representation of detection of GSH using thiol-coated AuNCs (~ 2 nm). The fluorescence of AuNCs gets quenched after exposure with thiol extinguisher (Reprinted with permission from reference no. 86, copyright 2015 American Chemical Society)

enhancement of PL intensity when exposed to thiols (Su et al. 2015). In this method of detection of GSH using thiol-coated AuNCs (~ 2 nm), the fluorescence of AuNCs gets quenched after exposure with thiols extinguisher (Fig. 4.8). AuNPs-based immune sensor has also been reported by several groups. The combination of ellipsometry and Kretschmann surface plasmon resonance was used for low-concentration detection of GSH (Garcia-Marin et al. 2014). A thin film of AuNPs coated with anti-GSH antibody was synthesized, which was used for the measure of surface plasmon polariton (SPP) excitation. It was found that at low GSH concentration, changes in refractive index was very small, probably due to the small molecular weight of GSH, thus an only negligible shift in plasmon resonant energy was seen. Interestingly, when changes induced by AuNPs adsorption were monitored by an ellipsometer, using Ψ and Δ ellipsometric functions, the resonant energy linearly shifts as the relative concentration of free GSH increases. Here, concentrations of free GSH were varied, but functionalized AuNPs concentration was fixed to a constant amount. Additionally, Güçlü et al. have developed a sensitive and selective method to detect thiols from biological samples using AuNPs coated with Ellman's reagent (Guclu et al. 2013). We have earlier in this section discussed that Ellman's reagent-based GSH measurement is one of the very commonly used methods. The developed sensor gave linear response over a wide concentration range of standard GSH and was comparable to the conventional DTNB

assay. Further, the common biologically interfering components like amino acids, flavonoids, vitamins, and plasma antioxidants did not obstruct the proposed sensing method.

4.3.8 Cysteine Detection

The cysteine residues in cytoplasmic proteins are one of the most vulnerable biomolecules to be affected by the oxidative stress, therefore considered as one of the best oxidative stress biomarkers (Ho et al. 2013). Depending on the magnitude of the oxidative stress, the ROS and RNS can alter the normal metabolic processes, which could eventually lead to toxicity and cell death. Cysteines are mostly vulnerable to the posttranslational modification of proteins due to oxidative stress. Additionally, they also function as redox switches that dictate the cellular response to oxidative stress. Therefore, the sensitive detection of cysteines is imperative. Recently, the methods involved in identifying the proteins containing oxidized cysteine residues are mass spectrometry and chemoselective functionalization, which are labor intensive and time-consuming, and the data analysis requires trained manpower. Further, in order to understand how oxidative stress and cell responses are related to aging and disease progression, it is expected that methods with the capability of sensitive detection real-time monitoring of cysteine residues are needed.

Several methods for detection of cysteine have been put forward, which utilize the properties of AuNPs. One such effort was made by Bagci et al., using AuNPs synthesized at room temperature and reduced by apple juice as reducing agent (Bagci et al. 2015). Due to the strong affinity of AuNPs with thiols, an addition of cysteine residues to AuNPs suspension resulted in the aggregation of AuNPs and color change of the suspension. The localized SPR absorbance peak was red shifted, measured by a spectrophotometer. Authors also studied the effect of potential interfering agents such as glucose, ascorbic acid, K^+, Na^+, Ca^{2+}, Zn^{2+}, Ag^+, Ni^{2+}, Cu^{2+}, Co^{2+}, and Hg^{2+}, which did not show any drop in the sensing capability of the assay. A linear sensing pattern of cysteine was obtained from 2 to 100 μM with a limit of detection of 50 nM. Further, Deng et al. developed a nanosensor for detection of cysteine from homocysteine and GSH with multiple techniques such as colorimetric, PL and, upconversion photoluminescence (UCP) (Deng et al. 2015). The nanosensor was composed of nitrogen-doped carbon dots (NC) and AuNPs in a core-shell manner (Au core-NC shell), which showed a different response to cysteine, homocysteine, and GSH with colorimetric, PL, and UCP signals. However, the discrimination effect for cysteine was proposed to be originated from conformations and interaction difference (with AuNPs) of the thiols groups among the three thiols tested. It was also suggested that among the three thiols, cysteine could quickly penetrate the NC shell and access the AuNPs, thus induce the dispersion of the aggregated NC-AuNPs. Further, the dispersion of NC-AuNPs would change from purple (aggregate) to red (dispersed) and the recovery of PL and UCP of NC. This method gave a detection limit of 4 nM during the detection of cysteine in human serum. Potentiodynamic method has also been reported for the detection of cysteine

using AuNPs. Kannan et al. have reported a sensitive method for stable determination of L-cysteine at physiological pH using a core-shell of AuNPs aminomercaptothiadiazole-modified glassy carbon (GC) electrode (Kannan and John 2011). During measurement, the bare GC electrode did not show any response for cysteine at physiological pH, whereas electrode modified with core-shell AuNPs exhibited a well-defined oxidation signal at 0.51 V. The amperometric current was increased linearly from 10 nM to 140 nM concentration of cysteine with the limit of detection of 3 pM, when cysteine residues are spiked into human blood serum and urine samples. Chen et al. demonstrated a simple and colorimetric cysteine detection method using ssDNA-decorated AuNPs (Chen et al. 2009). Cysteine is well known to quickly establish Au-S bond with AuNPs; therefore, when nanoparticles stabilized with ssDNA, it displays stable colloidal suspension of wine-red color even in the presence of salt solution. However, when ssDNA-stabilized AuNPs are exposed to cysteines, they undergo aggregation, resulting in a characteristic color change from wine-red to blue upon addition of salt. This color change was also followed by the change in absorbance intensity from wine-red (525 nm) to blue (640 nm). Therefore, a ratio of absorbance at 640/525 nm was calculated, which was linearly dependent on the cysteine concentration (0.1–5 μM). Further, the specificity of cysteine to this method was also checked by using other amino acids, which did not show any change in color suggested that this method is specific to cysteine.

4.3.9 8-Hydroxy-2′-Deoxyguanosine Detection

There is growing evidence that oxidative stress leads to permanent damage to membrane lipids, proteins, and nucleic acids. 8-Hydroxy-2′-deoxyguanosine (8-OHdG) is one of the well-known biomarkers for free radical-induced oxidative lesions in nuclear and mitochondrial DNA (Yahia et al. 2016). Methods are developed for the detection of 8-OHdG from urine, which serves as a good biomarker for risk assessment of various ailments such as cancer and neurodegenerative diseases. This biomarker plays a pivotal role in quantitating the endogenous DNA damage due to oxidative stress in cancer initiation, promotion, and metastasis. It is also used to estimate the oxidative stress-mediated DNA damage in humans due to exposure to tobacco smoke, asbestos fibers, heavy metals, and polycyclic aromatic hydrocarbons. The common methods of quantitative detection of 8-OHdG include HPLC equipped with electrochemical detection, gas chromatography-mass spectrometry (GC-MS), and HPLC-tandem mass spectrometry (Valavanidis et al. 2009). Although these techniques provide qualitative and quantitative results, they face many drawbacks such as that they require trained manpower and are time-consuming and costly. In this quest, several quick methods have also been investigated. Jia et al. have reported a new biosensor for 8-OHdG detection by combining the single-stranded ssDNA and graphene nanosheets (GNs) (Jia et al. 2015). The excellent conductivity of GNs enabled the electrochemical detection of 8-OHdG (Fig. 4.9). The fabricated biosensor showed high electrocatalytic activity during the oxidation of 8-OHdG with the sensitivity of 13.23 (±0.03) $\mu A \mu M^{-1}$, 5.827 (±0.008) $\mu A \mu M^{-1}$,

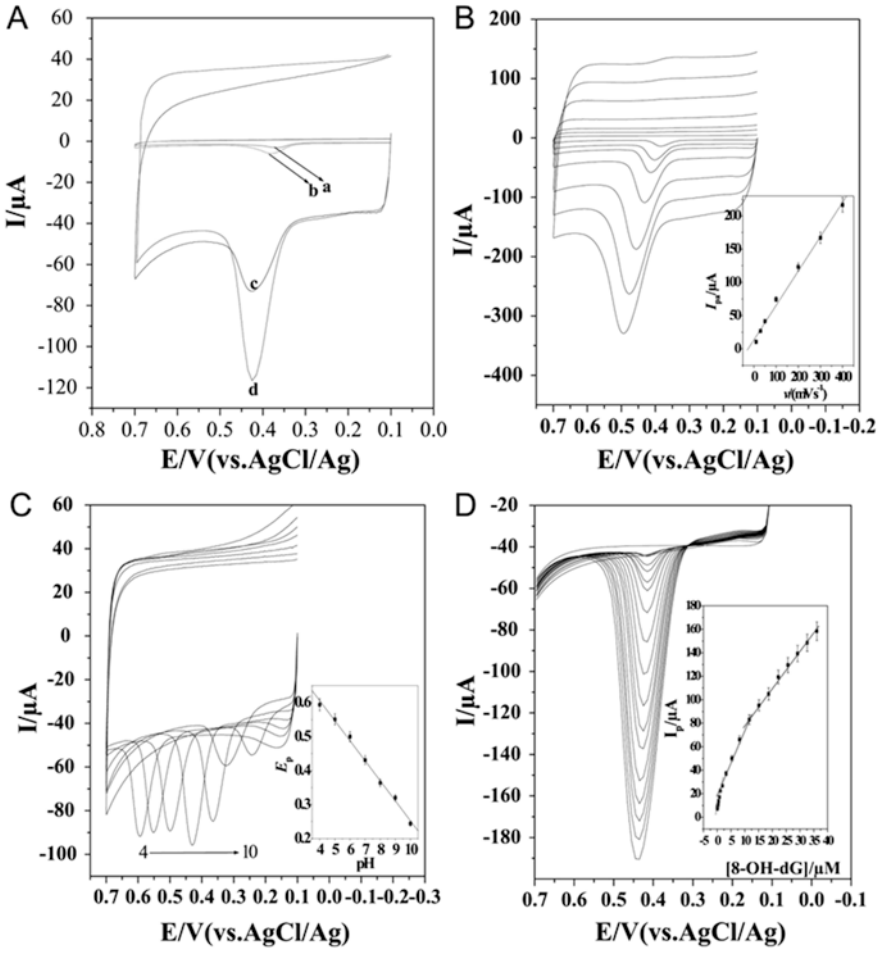

Fig. 4.9 (**a**) CVs of (a) GCE, (b) ssDNA/GCE, (c) GNs/GCE, and (d) ssDNA/GNs/GCE in 0.1 M PBS (pH 7.0) containing 14 μM 8-OHdG at scan rate of 100 mV s^{-1}. (**b**) CVs of ssDNA/GNs/GCE at different scan rates (10–400 mV s^{-1}) in 0.1 M PBS (pH 7.0) containing 7 μM 8-OHdG. Inset is the plot of I_p (μA) vs. v(mV s^{-1}). (**c**) CVs of 7 μM 8-OHdG on ssDNA/GNs/GCE in different pHs: 4, 5, 6, 7, 8, 9, and 10. Inset is the plot of E_p (V) vs. pH. (**d**) CVs of ssDNA/GNs/GCE upon successive addition of 8-OHdG into 0.1 M PBS (pH 7.0). Inset is calibration curve of I_p (μA) vs. [8-OHdG] (μM). All error bars represent the standard deviation for three independent measurements (Reprinted with permission from reference no. 96, copyright 2015 Elsevier)

and 3.086 (±0.005) μAμM^{-1} in the concentration ranges of 0.0056–1.155 μM, 1.155–11.655 μM, and 11.655–36.155 μM, respectively. The detection limit of this method was 0.875 nM, which did not show interference with uric acid. Further, a fluorescence aptasensor was also developed for detection of 8-OHdG using 8-OHdG aptamer. This method uses 8-OHdG aptamer as a recognition probe with N-methyl mesoporphyrin IX (NMM) as a reporter. The conformational alteration of a

K(+)-stabilized G-quadruplex to an 8-OHdG-stabilized one was followed by addition of 8-OHdG in the solution of aptamer K(+). The resulting sharp change in fluorescence intensity offers a linear response in proportion to the concentration of 8-OHdG, in the range of 3.96 nM–211 nM with a limit of detection of 1.19 nM. Since the constructed aptasensor consists of only aptamer and a dye NMM, it is cheaper, easy to design, and more applicable (Liu et al. 2016). AuNPs have also been used for the sensitive detection of 8-OHdG by electrochemical and colorimetric methods. The chiral property of AuNPs has also been extensively studied in context with circular dichroism (CD) spectroscopy. Owing to a simple and sensitive analytical method, CD with AuNPs has been used for the quantitative detection of 8-OHdG. The DNA-induced chiro plasmonic assemblies of AuNPs show high CD signals. However, in presence of 8-OHdG, the recognition and affinity constants of aptamer and 8-OHdG destroyed the hybrid of aptamer and its complementary sequence; thus the destroyed AuNPs dimers show low CD signal. The CD signal intensity was linearly proportional to the concentration of 8-OHdG ranging from 0.05 to 2 nM, with a correlation coefficient of 0.9951 with a detection limit of 33 pM. This method has successfully detected 8-OHdG from human serum sample and, therefore, holds promise for clinical examinations. Gao et al. have also reported a simple, quick, and low-cost colorimetric method to quantitate 8-OHdG using DNA-decorated AuNPs, which measures about four orders of magnitude greater than typical organic dyes (Gao et al. 2016). It is an optical property-dependent method, which uses sharp melting transition and unique distance-dependent plasmonic properties of AuNPs. Based on these unique plasmonic properties, the AuNPs-linked triplex receptor was made which could selectively detect 8-OHdG in the presence of guanine. The receptors were conjugated to AuNPs using 3′-thiol through two pyrimidine-rich non-complementary strands. Three purine-rich spacer strand were also used to amplify the stabilization effect induced by 8-OHdG binding to AuNPs. However, in the absence of 8-OHdG, these linkers cause aggregation of AuNPs.

4.4 Future Directions

Several pharmacological interventions in oxidative stress-related diseases fail because the initiation of treatment started too late to stop the damage caused to tissues. Very frequently, these impairments are extensive and irreversible. One of the reasons for such a failure is the shortage of adequate assay/method to detect the oxidative stress early enough to arrest the pathology and thus minimize the deleterious consequences. Nanoparticle-enabled nanosensors are being used to enhance the potential of disease diagnostics, but limited success is observed when applied for early stage detection. Although nanotechnology-based sensors have the potential to be incorporated into point-of-care devices, they are still at an early stage of development. Unlike conventional fluorophores, fluorescence nanomaterial does not bleach; therefore, several simple spectrophotometer readable assays have been developed. Among nanomaterials, AuNPs offer exceptional optical properties which could be exploited to develop an easy method of free radical detection. Owing to the

biocompatibility of AuNPs, in vivo real-time detection and imaging of free radicals could also be possible. Additionally, other morphology AuNPs such as Au-nanorods could be used, which have optical resonance in the near-infrared spectral window. This would allow deeper penetration within the tissues, and since near-infrared light is transparent to biological tissues, photochemical damage could be avoided. Further, with regard to the tunability of surface plasmon absorption peak, by varying the aspect ratio of Au-nanorods, the sensitivity to free radicals can be optimized. This could further be extended to the sensing of one type of free radical with a fixed aspect ratio Au-nanorods. Additionally, spherical and nanostructures of gold could be explored for the two-photon luminescence (TPL)-based detection of markers of oxidative stress. TPL-based imaging offers several advantages such as excellent dark-field imaging and high signal-to-noise ratio. It is an attractive option for intracellular and deep-tissue imaging as a strong background signal from tissues impedes the detection of low-concentration signals. Although TPL-based imaging has been successfully employed in bio-imaging, strategies should also be made to consider them for detection of changes in free radical, in response to oxidative stress, level in the cytoplasm under physiological, and pathological situations (Yellen and Mongeon 2015; Mongeon et al. 2016). Quantum dots (QDs) are known to exhibit high quantum yields than organic fluorophores, but nanocrystals of gold (AuNCs) are also reported to show comparative fluorescence (Maysinger et al. 2015). In this context, strategies could be made to use the exceptional fluorescence or luminescence of AuNCs or AuNPs for sensitive detection of free radical species from cells/tissues. Additionally, owing to the plasmonic resonance in AuNPs, a "plasmonic resonance energy transfer (PRET)" can also be considered for qualitative and quantitative measurement of markers of oxidative stress in biological cells/tissues. PRET has been successfully used in determination of disrupted redox homeostasis, neuroinflammation, and protein shedding (Altmeppen et al. 2013; Saftig and Bovolenta 2015).

"Lab-on-a-chip" is another direction where current detection and treatment are moving rapidly. Therefore, it is expected that in a few years, many of these AuNPs-based oxidative stress biomarker detection modalities would be merged into such a chip-based portable device, which can run the tests rapidly and noninvasively. Such chips could be integrated with the cell phones, enabling the tests to be run from homes, offices, or even from remote locations. The results could be stored in the cell phone and shared with the doctor. This would allow the doctor to access the medical or treatment history of the patient; therefore, it may be possible for a doctor to prescribe the needed medicines to the patient. Additionally, the trend of "personalized medicine" would be more appreciated in the near future, and it could be befitting with the use of nanotechnology. It may be possible to decide which drug will perform best for a particular patient, suffering from a disease originated from oxidative stress, and the correct drug dose to be prescribed. This will improve the drug efficacy and safety and minimize the nonspecific side effects.

4.5 Conclusion

AuNPs-based detection of oxidative stress biomarkers presented in this chapter is mostly established in laboratory conditions or biomarkers spiked within the human serum; therefore, their use under true disease conditions remains to be explored. Additionally, currently available biosensors are not sensitive enough to detect the biomarkers present in an extremely low concentration, therefore require significant improvements in design and sensitivity to provide a reliable and reproducible measurement of biomarkers. Further, the precise location of oxidative stress biomarkers in cells is also another topic of interest, which needs to be explored in great detail. The uptake and co-localization of AuNPs-based oxidative stress biomarker sensors in cells are another challenge and required to be addressed by combining more techniques. Further advances in oxidative stress biology, biomarker development, and nanotechnology in the near future could be combined with the advanced techniques, which may provide a way of more successful detection and treatment modality of oxidative stress and related disease. This could be achieved with the combined efforts of oxidative stress biologists, nanotechnologist, electronic engineers, and chemists.

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Conflict of Interest None.

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Hydroxamic Acids as Potent Antioxidants and Their Methods of Evaluation

5

Samir Mehndiratta, Kunal Nepali, and Mantosh Kumar Satapathy

Hydroxamic acids are a potent class of drugs that act epigenetically to control various pharmacological functions and are currently used for the treatment of various cancers. To better understand their function and role, one must first understand the difference between genetics and epigenetics.

5.1 Genetics vs. Epigenetics

Both genetic and epigenetic aberrations control the initiation and progression of cancer. Epigenetic modifications are possibly reversible and allow the cancerous cells to revert to a more normal state unlike genetic alterations, which are hardly possibly to reverse. With the growing understanding of these modulations, numerous drugs capable of targeting specific enzymes involved in the epigenetic regulation of gene expression are emerging as an effective approach to chemoprevention and chemotherapy. The term "epigenetic" describes the mitotically and meiotically heritable states of gene expression which are not related to changes in DNA sequence (Bird 2002). Epigenetic events play a crucial role in biology, and numerous researches over the past decade have established their role in carcinogenesis and tumor progression. DNA methylation and modifications of histone tail are two of the most studied epigenetic events. The smallest structural unit of chromatin comprises 147 bp of DNA wrapped around a core of eight histones (an octamer consisting of H3/H4 tetramer and two H2A/H2B dimers) known as nucleosome. The

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amino-terminal tails of highly conserved histone proteins extrude out of the nucleosome and are potential sites of posttranslational modifications such as phosphorylation, methylation, ubiquitylation, sumoylation, ADP-ribosylation, glycosylation, biotinylation, and carbonylation including acetylation by histone acetyltransferases (HATs) and histone deacetylation by histone deacetylases (HDACs). These posttranslational changes act as a regulator of gene expression by influencing the compactness of chromatin in various tissue types (Jenuwein and Allis 2001). Acetyl groups, for example, tend to neutralize the positive charges on the basic amino histone tails thus weakening electrostatic interactions between the negatively charged phosphate backbone of DNA and histones (Margueron et al. 2005). In a similar manner, acetylation of lysine residues on histones H3 and H4 is related to activation or opening of chromatin, allowing accesses of various transcription factors to the promoters of target genes. On contrary, deacetylation of lysine residues by histone deacetylases (HDACs) results in chromatin compaction and inactivation of genes (Margueron et al. 2005; Namdar et al. 2010).

HDAC inhibitors (HDIs) cause reactivation of tumor suppressor genes and/or other genes that are crucial for the normal functioning of cells by targeting aberrantly heterochromatic regions of histones. It has been observed that reactivation of tumor suppressor genes and thus restoration of DNA repair pathways by epigenetic drugs result in more chemosensitive cells, which can then be targeted by another type of therapy (Namdar et al. 2010). Thus it could be anticipated that HDAC inhibitors could be used therapeutically as single agents or as part of a combination with other therapeutic modalities, such as chemotherapy, immunotherapy, or radiotherapy in the future. Epigenetic therapy could be applied for chemopreventive approaches for patients who have been diagnosed with aberrant epigenetic alterations but have not yet acquired neoplastic lesions. Epimutations (epigenetic mutations) might be used as an indicator of the likelihood of developing cancer in individuals with no history of malignancy but have been diagnosed with epimutations. If these epimutations are dealt on time with DNA methylation and HDAC inhibitors, it is expected that it could delay/completely prevent tumorigenesis in these individuals. Development of a detailed tissue-type-based map of specific epigenetic patterns in their normal and in cancerous states would make it possible to detect premalignant epimutations (Laird 2003). Furthermore, comprehensive knowledge of the epigenome would open up new pathways for the development of various target-specific drugs of the genome in which an epimutation has occurred (Yoo and Jones 2006).

5.2 HDAC, HDIs, and Cancer (Wagner et al. 2010)

With the advancement in knowledge of epigenetics, it has become evident that HDACs are potential therapeutic targets with the ability to reverse aberrant epigenetic states associated with cancer (Reddy et al. 2004). Various studies in cancer cell lines and tumor tissue revealed changes in the acetylation levels and the expression of the HDAC enzymes (Bolden et al. 2006). Research has suggested that aberrant recruitment of HDACs to promoters plays a crucial role in hematologic malignancies

(Pandolfi 2001). Common chromosomal translocations or overexpression of repressive transcription factors in these diseases creates oncogenic DNA-binding fusion proteins that physically interact with HDACs. Minucci and Pelicci demonstrated on a molecular level the involvement of HDACs in cancer onset in the first model disease of acute promyelocytic leukemia in 2006. In acute promyelocytic leukemia, 100% of the patients show formation of fusion proteins of the retinoic acid receptor-α with the promyelocytic leukemia, the promyelocytic zinc finger, or other proteins. These fusion proteins further recruit HDAC-containing repressor complexes which further suppress the expression of specific target proteins (Pandolfi 2001). In some cancers, transcriptional repressor recruits complexes containing HDAC enzymes such as in B-cell lymphoma (Ropero and Esteller 2007). These complexes cause activation of BCL-6 resulting in transcriptional silencing. BCL-6 is overexpressed in 40% of diffuse large B-cell lymphomas (Pasqualucci et al. 2003).

However, the expression of the HDAC enzymes is not always consistent and can be up- or downregulated in various types of cancer, and considerable variation in the expression levels of HDAC enzymes has been observed in tumors of the same entity. Expression of class I HDACs is found to be higher in most of tumor samples compared to the corresponding normal tissue, but class II HDACs seemed to be downregulated and high expression correlated with a better prognosis (Weichert 2009). Increased HDAC activity accelerates hypoacetylation of histones in the promoter area of tumor repressor genes, thus resulting in transcriptional suppression (Santos-Rosa and Caldas 2005). It is very rare to find mutations in genes encoding for HDACs. So far, only one truncating mutation of HDAC2 in colorectal and endometrial tumors has been found. Somatic HDAC4 mutations were found in breast and colorectal cancer, and some reports suggest germline polymorphisms in various HDACs. The functional significance for these sequence alterations is yet to be illustrated (Ganesan et al. 2009). Inhibition of HDAC by HDAC inhibitors (HDIs) causes changes in the acetylation status of compact chromatin and other nonhistone proteins, resulting in changes in gene expression, induction of apoptosis, inhibition of angiogenesis, metastasis, and cell cycle arrest (Ma et al. 2009). The overall number of genes regulated by HDACs is relatively small (Van Lint et al. 1996), and the genes induced by HDIs play a role in cell growth, differentiation, and survival. Growing evidence shows that HDIs have immunomodulatory effects. This can result in an increased reorganization of malignant cells by the immune system due to an increased presence of surface antigens (Magner et al. 2000a). Additionally, HDIs can enhance immune cell activity by altering cytokine secretion. But the HDAC inhibitor suberoylanilide hydroxamic acid (SAHA, Vorinostat) has also been demonstrated to suppress the production of pro-inflammatory cytokines that play a role in the pathogenesis of acute graft-versus-host disease (GVHD) (Reddy et al. 2004). These immunomodulatory effects may contribute to the antitumor activity of HDIs. Table 5.1 illustrates the various findings about HDIs and their roles in cancer.

HDACs are classified into four different classes depending on sequence homology to the yeast original enzymes and domain organization (Table 5.2)

Table 5.1 Various findings about HDIs and their roles in cancer

Who?	When?	What?
Leder et al. (1975)	1975	DMSO ability to induce cellular differentiation
Richon et al. (1998)	1998	The concentrations necessary to cause growth inhibition are the same as needed to induce hyper-acetylation of histones
Richon et al. (2000)	2000	G1 cell cycle arrest is a result of the induction of the CDKN1A gene, which encodes the cyclin-dependent kinase inhibitor WAF1
Magner et al. (2000b)	2000	HDIs can upregulate the expression of major histocompatibility complex class I and II proteins
Deroanne et al. (2002), Rossig et al. (2002), and Michaelis et al. (2004)	2002, 2002, and 2004	HDIs have antiangiogenic and anti-metastatic effects. Antiangiogenic properties result from a decrease in expression of proangiogenic genes like vascular endothelial growth factor and endothelial nitric oxide synthase
Liu et al. (2003), Joseph et al. (2004), and Mazieres et al. (2007)	2003, 2004, and 2007	Upregulation of gene expression of metastatic suppressors and downregulation of genes that promote metastasis are responsible for the anti-metastatic effects of HDIs
Bolden et al. (2006)	2006	HDIs cause cell cycle arrest in G1 and/or G2 phase, thus leading to inhibition of cell growth
Bolden et al. (2006)	2006	Various members of the TNF receptor superfamily and ligands become transcriptionally activated upon HDI treatment
Ma et al. (2009)	2009	HDIs can also lead to the induction of apoptosis. The inhibitors can initiate extrinsic (death receptor) and intrinsic (mitochondrial) pathways

5.3 Chemical Classification of HDAC Inhibitors and Their Development (Bertrand 2010; Dokmanovic et al. 2007)

The development of the HDAC inhibitors (HDIs) dates back in 1971 when it was first observed that DMSO induces erythroid differentiation in murine erythroleukemia cells (MELC) (Marks and Breslow 2007). In 1998 SAHA was found to inhibit HDACs after the identification of its ability to inhibit growth of transformed cell and inducer of cell death in 1996. Since then, it took almost 8 years for SAHA to be get approved as first HDAC inhibitor for the third-line treatment of cutaneous T-cell lymphoma in the USA. With the approval of SAHA in 2006, it opened a new era of HDAC inhibitor development, and till date four HDAC inhibitors have been approved by FDA: SAHA (Zolinza, Vorinostat, **1**) in October 2006 for cutaneous T-cell lymphoma, romidepsin (FK228, Istodax, **11**) in November 2009 for cutaneous T-cell lymphoma, PXD101 (Belinostat, **2**) has been granted orphan drug and fast track designation by the FDA and was approved in the USA for the use against peripheral T-cell lymphoma in July 2014, and panobinostat (Farydak, **3**) in February 2015 for the treatment of multiple myeloma.Various HDAC inhibitors have been developed (Table 5.3) and are currently at different stages of clinical trials; thus,

Table 5.2 Classification of HDACs (West and Johnstone 2014; Witt et al. 2009; Wikipedia)

Class	Members (size, AA)	Catalytic sites	Subcellular localization	Tissue distribution	Substrates	Expression in cancer	KD function in cancer
I	HDAC1 (483)	1	Nucleus	Ubiquitous	Androgen receptor, SHP, p53, MyoD, E2F1, STAT3	Elevated in gastric, breast, colorectal, HL, lung, liver	Inhibition of proliferation and induction of autophagy; increase in p21 and p27
	HDAC2 (488)	1	Nucleus	Ubiquitous	Glucocorticoid receptor, YY1, BCL6, STAT3	Elevated in gastric, prostate, colorectal, HL, CTCL	Differentiation, apoptosis, and p53-independent p21 expression
	HDAC3 (428)	1	Nucleus	Ubiquitous	SHP, YY1, GATA1, RELA, STAT3, MEF2D	Elevated in, breast, ALL, colorectal, HL	Differentiation genes, disrupts cell cycle
	HDAC8 (377)	1	Nucleus/cytoplasm	Ubiquitous?	–	Elevated in neuroblastoma	Induces differentiation and cell cycle arrest and inhibits clonogenic growth
IIA	HDAC4 (1084)	1	Nucleus/cytoplasm	Heart, skeletal muscle, brain	GCMA, GATA1, HP1	Breast cancer	Inhibits expression and functional activity of HIF-1a
	HDAC5 (1122)	1	Nucleus/cytoplasm	Heart, skeletal muscle, brain	GCMA, SMAD7, HP1	Elevated in medulloblastoma, decreased in lung	On differentiation interacts with GATA-1
	HDAC7 (855)	1	Nucleus/ cytoplasm/mitochondria	Heart, skeletal muscle, pancreas, placenta	PLAG1, PLAG2	Elevated in ALL, decreased in lung	Silencing alters morphology, migration, and tube-forming capacity
	HDAC9 (1011)	1	Nucleus/cytoplasm	Brain, skeletal muscle	–	Elevated in ALL, medulloblastoma	Cardiac defect

(continued)

Table 5.2 (continued)

Class	Members (size, AA)	Catalytic sites	Subcellular localization	Tissue distribution	Substrates	Expression in cancer	KD function in cancer
IIB	HDAC6 (1215)	2	Mostly cytoplasm	Heart, liver, kidney, placenta	α-Tubulin, HSP90, SHP, SMAD7	Elevated in breast and CTCL, decreased in lung	On inhibition acetylates HSP90 and disruption of its chaperone function; KD leads to downregulation of HIF-1a, VEGFR1/2
	HDAC10 (669)	1	Mostly cytoplasm	Liver, spleen, kidney	–	–	Downregulates VEGFR
III	Sirtuins in mammals (SIRT1–7)	–	–	–	–	–	–
	Sir2 in the yeast *S. cerevisiae*	–	–	–	–	–	–
IV	HDAC11 (347)	2	Nucleus/cytoplasm	Brain, heart, skeletal muscle, kidney	–	Elevated in breast, renal, liver	–

AA amino acids, *KD* knockdown, *HL* Hodgkin lymphoma, *ALL* acute lymphoblastic leukemia, *CTCL* cutaneous T-cell lymphoma

more potent and selective HDAC inhibitors for the treatment of various cancers could be expected in the near future.

5.4 Modifications of FDA-Approved HDIs

Many researchers have worked on these four FDA-approved HDAC inhibitors and have done many structural modifications to develop potent HDAC inhibitors of SAHA (Suzuki et al. 2004; Zhang et al. 2013), FK228 (Saijo et al. 2012), PXD101 (Wang et al. 2013), and LBH589 (Mehndiratta et al. 2014, 2016; Huang et al. 2015). Figure 5.1 illustrates some of these examples.

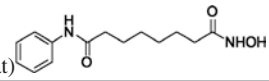
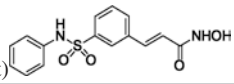
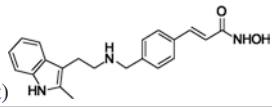
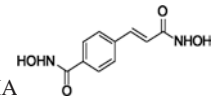
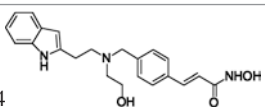
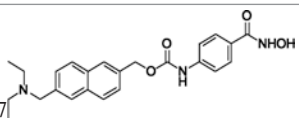
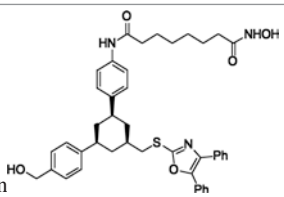
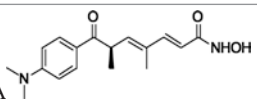
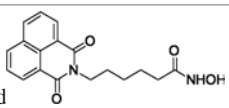
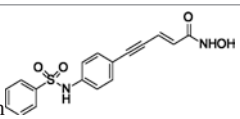
5.5 Role of HDIs in Diseases Other than Cancer

HDACs counteract the action of HAT by reversing the hyper-acetylation and suppressing the genes or gene silencing (Dokmanovic et al. 2007; Adcock et al. 2005). Although HDAC inhibitors (Table 5.3) inhibit the action of HDACs and thus increase the hyper-acetylation, reports have suggested that almost equal numbers of genes are induced and are inhibited. The effects of HDAC inhibitors vary according to cell type and stimulus (Dokmanovic et al. 2007; Glauben et al. 2009), and this supports the idea that they have other potential therapeutic applications such as anti-inflammation, immune modulation (Wang et al. 2009), antirheumatic (Lin et al. 2007), and anti-HIV properties (Archin et al. 2009; Routy 2005). Numerous studies involving mainly SAHA (**1**) (Leoni et al. 2002; Dinarello 2010), TSA (**8**) (Choi et al. 2005; Nasu et al. 2008), MS-275 (**15**) (Choo et al. 2010; Zhang et al. 2010), and ITF2357 (**6**) (Leoni et al. 2005; Joosten et al. 2011) have shown promising evidence of their anti-inflammatory properties. Song et al. have reported that panobinostat (LBH589, **3**) suppresses various pro-inflammatory cytokines, indicating its immunomodulatory and anti-inflammatory potential (Song et al. 2011). HDACs have found to play an important role in aging, and recent studies have reported promising antioxidant and antiaging effects of HDIs (Vaiserman and Pasyukova 2012; Baltan 2012; Vaiserman et al. 2013). Thus, this could be anticipated that in the coming few years, HDIs could be a well-established class of drugs with uses other than cancer such as antiaging/antioxidant molecules.

5.6 Methods to Measure Antioxidation Potential (Alam et al. 2013; Antolovich et al. 2002; Shahidi and Zhong 2015)

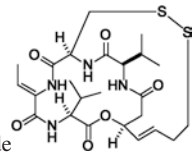
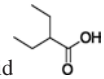
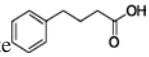
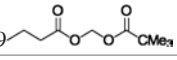
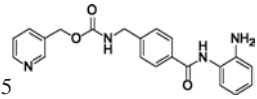
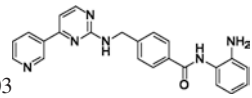
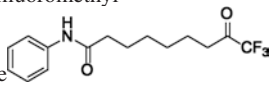
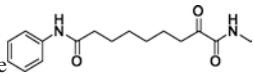
There are more than 400 methods reported for evaluating the antioxidant potential of various molecules. However, they are derived from 29 basic methods. Out of these 29 basic methods, only few methods are the most commonly used for in vitro and in vivo evaluation of antioxidant activity of the chemical entities, and

Table 5.3 Chemical structures of various HDI and their respective HDAC targets

Class	Compound/structure	HDAC target
Hydroxamates	**1** SAHA (Vorinostat) 	Class I and II
	2 PXD101 (Belinostat) 	Class I and II
	3 LBH-589 (panobinostat) 	Class I and II
	4 CBHA 	Not available
	5 LAQ-824 	Class I and II
	6 ITF2357 	Class I and II
	7 Tubacin 	Class IIb
	8 TSA 	Class I and II
	9 Scriptaid 	Not available
	10 Oxamflatin 	Not available

(continued)

Table 5.3 (continued)

Class	Compound/structure	HDAC target
Cyclic peptide	 **11** FK-228/depsipeptide	Class I
Aliphatic acids (short-chain fatty acids)	 **12** Valproic acid	Class I and IIa
	 **13** Phenyl butyrate	Class I and IIa
	 **14** AN-9	Not available
Benzamides	 **15** MS-275	HDAC1, HDAC2, HDAC3
	 **16** MGCD0103	Class I
Keto derivatives	**17** Trifluoromethyl  ketone	Not available
	 **18** Alpha-ketoamide	Not available

1,1-diphenyl-2-picrylhydrazyl (α,α-diphenyl-β-picrylhydrazyl) (DPPH) assay and lipid peroxidation (LPO) assay are the most commonly used assays for this purpose. Apart from these two, below is the list of some assays that are used most frequently for determining antioxidant potential of various pharmacologically active compounds including HDIs:

I. In vitro *assays*:
   (a) DPPH scavenging activity assay
   (b) Hydroxyl radical scavenging activity assay
   (c) Superoxide radical scavenging activity assay
   (d) Hydrogen peroxide scavenging (H_2O_2) assay
   (e) Nitric oxide scavenging activity assay
   (f) Ferric reducing antioxidant power (FRAP) assay

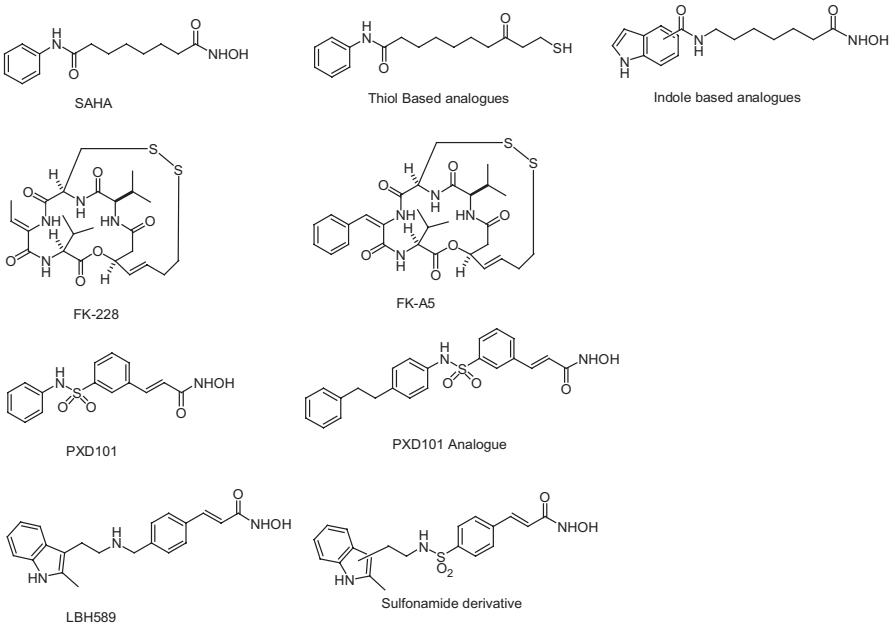
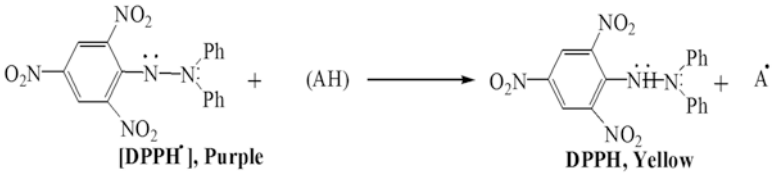


Fig. 5.1 Chemical modifications of FDA-approved HDAC inhibitors

(g) Oxygen radical absorbance capacity (ORAC) method
(h) β-Carotene linoleic acid method/conjugated diene assay
(i) Metal chelating activity

These are the few most commonly used assays for the evaluation of antioxidant potential of the compounds. Out of these assays, the first three are the most widely used, and their method/procedures are described in detail below.

(a) DPPH Scavenging Activity 1,1-Diphenyl-2-picrylhydrazyl radical (DPPH) is a nitrogen-centered free radical that shows strong absorbance at 517 nm. DPPH assay is based on the measurement of the scavenging ability of antioxidants (AH) toward the stable DPPH radical. The free stable radical is reduced to the corresponding hydrazine when it reacts with hydrogen donors; this ability was evaluated by more frequently used discoloration assay, which evaluates the absorbance decrease at 517 nm produced by the addition of the antioxidant to a DPPH solution in ethanol or methanol. This method is widely used to check the free radical scavenging antioxidants. To evaluate the antioxidant activity of specific compounds or extracts, the antioxidant was allowed to react with a stable radical $DPPH^{\bullet}$ in a methanol solution. The extent of DPPH radical scavenged was determined by the decrease in intensity of violet color in the form of IC_{50} values.



$$[DPPH^{\bullet}] + A^{\bullet} \longrightarrow DPPH\text{--}A$$

$$A^{\bullet} + A^{\bullet} \longrightarrow A\text{--}A$$

In this Rxn, DPPH reacts with the free radical and shows a color change from purple to yellow by making a complex with free radical. In its radical form, DPPH• absorbs at 515 nm, but upon reduction the absorption disappears or decreases. In the DPPH• free radical method, antioxidant efficiency is measured at ambient temperature and thus eliminates the risk of thermal degradation of the molecule tested. The use of DPPH• provides the easy and rapid way to evaluate the antiradical activities of antioxidants. It measures the hydrogen-donating ability of antioxidants in a relatively short time as compared to other methods, and spectrophotometric characterization is possible. The scavenging reaction between DPPH• and antioxidant can be expressed as shown above. In the above reaction, A• is the free radical of antioxidant that combines with another A• to form A–A•. The change in optical density of DPPH radicals is the prime observation in order to evaluate the antioxidant potential through free radical scavenging by the test samples. The sample extract (0.2 mL) is diluted with methanol, and 2 mL of DPPH solution (0.5 mM) is added, and the absorbance is measured at 517 nm after 30 min. The percentage of the DPPH radical scavenging is calculated using the equation as given below:

$$\%\text{Radical scavenging power} = \frac{\text{Absorbance}\left(\text{control} - \text{sample}\right)}{\text{Absorbance of control}} \times 100$$

(b) Hydroxyl Radical Scavenging Activity In the biological system, hydroxyl radical is one of most reactive oxygen species that reacts with phospholipids (polyunsaturated fatty acid moieties) of the cell membrane leading to cell damage. Kunchandy and Rao in 1990 had developed an effective method to measure the scavenging ability of hydroxyl radicals. The method includes a reaction mixture (1.0 mL) comprising of 100 µL of 2-deoxy-D-ribose (28 mM in 20 mM KH_2PO_4-KOH buffer, pH 7.4), 500 µL of the extract, 200 µL EDTA (1.04 mM) and 200 µM $FeCl_3$ (1:1 v/v), 100 µL of H_2O_2 (1.0 mM), and 100 µL ascorbic acid (1.0 mM) which is incubated at 37 °C for 1 h. After incubation, 1 mL of thiobarbituric acid (1%) and 1.0 mL of trichloroacetic acid (2.8%) are added and again incubated at 100 °C for 20 min. After cooling, absorbance is measured at 532 nm, against a blank sample.

(c) Superoxide Radical Scavenging Activity (SOD) Despite superoxide anion being a weak oxidant, its end products are hydroxyl radicals as well as singlet oxygen which led to oxidative stress (Meyer and Isaksen, 1995). Robak and Gryglewski in 1988 developed a method to measure superoxide anion scavenging activity. The superoxide anion radicals are generated by 3.0 mL of Tris-HCl buffer (16 mM, pH 8.0), having 0.5 mL of nitroblue tetrazolium (NBT) (0.3 mM), 0.5 mL NADH (0.936 mM) solution, 1.0 mL extract, and 0.5 mL Tris-HCl buffer (16 mM, pH 8.0). A solution of 0.5 mL phenazine methosulfate (PMS) (0.12 mM) was added in to the mixture to initiate reaction and incubated at 25 °C for 5 min, and then the absorbance is measured at 560 nm against a blank sample.

II. In vivo *assays*:
   (a) Lipid peroxidation (LPO) assay
   (b) Superoxide dismutase (SOD) method
   (c) Catalase (CAT)
   (d) Glutathione reductase (GR) assay
   (e) Reduced glutathione (GSH) estimation
   (f) Ferric reducing ability of the plasma
   (g) Glutathione peroxidase (GSHPx) estimation
   (h) Glutathione-S-transferase (GSt)
   (i) LDL assay

Out of these in vivo assays, the first three are the most widely used and accepted assays for the evaluation of antioxidant potential of the compounds in in vivo and are therefore discussed in detail below.

(a) Lipid Peroxidation (LPO) Assay LPO, an autocatalytic process, is a common consequence of cell death due to peroxidative tissue damage in diseases like inflammation, cancer and toxicity of xenobiotics, and aging. Malondialdehyde (MDA) is an indicator of lipid peroxidation and is one of the end products generated during lipid peroxidation. During oxidative degeneration, malondialdehyde (MDA) is formed as a product of free oxygen radicals. Okhawa in 1979 described the LPO assay. Using a Teflon glass homogenizer, tissues are homogenized in 0.1 M buffer pH 7.4, and LPO is determined by measuring the amounts of malondialdehyde (MDA) produced primarily. Tissue homogenate (0.2 mL) + 0.2 mL of 8.1% sodium dodecyl sulfate (SDS) + 1.5 mL of 20% acetic acid and 1.5 mL of 8% TBA are mixed, and volume is adjusted to 4 mL using distilled water in different tubes. The mixture is heated at 95 °C on a water bath for 60 min using glass balls as condenser. After heating tubes, final volume was made to 5 mL in each tube. Five mL of butanol/pyridine (15:1) mixture is added, and the contents are vortexed thoroughly for 2 min. Centrifugation was done at 3000 rpm for 10 min, and the upper organic layer is taken to measure optical density at 532 nm against an appropriate blank without the sample. The levels of lipid peroxides can be expressed as n moles of thiobarbituric acid reactive substances (TBARS)/mg protein using an extinction coefficient of 1.56×10^5 ML cm^{-1}.

(b) Superoxide Dismutase (SOD) Method Mccord and Fridovich in 1969 described this method for determination of antioxidant activity of a sample. Erythrocyte lysate prepared from the 5% RBC suspension is used to estimate antioxidant activity. To 50 µL of the lysate, 75 mM of Tris-HCl buffer (pH 8.2), 30 mM EDTA, and 2 mM of pyrogallol are added, and increased absorbance is recorded at 420 nm for 3 min by spectrophotometer. Fifty percent inhibition of the rate of autoxidation of pyrogallol is equal to one unit of enzyme activity and is determined by change in absorbance/min at 420 nm. The activity of SOD is expressed as units/mg protein.

(c) Catalase (CAT) Aebi in 1984 described this method to determine catalase in erythrocyte lysate. Fifty microliter of the lysate is added to a cuvette containing 2 mL of phosphate buffer (pH 7.0) and 1 mL of 30 mM H_2O_2. The activity of catalase is measured for 1 min at 240 nm using spectrophotometer. The molar extinction coefficient of H_2O_2 (43.6 M cm^{-1}) is used to determine the catalase activity. One unit of activity is equal to 1 mmol of H_2O_2 degraded per minute and is expressed as units per milligram of protein.

These are the few methods that are used to evaluate antioxidant activity. These methods are in use for many years to evaluate potent antioxidants which are in the market in the form of drugs. Results obtained from these assays are highly reliable and reproducible. It will be interesting to see that in the future, potent HDIs evaluated by these methods will come up in the market as antioxidants for the treatment of oxidative stress leading to aging.

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Oxidative Stress-Related MicroRNAs as Diagnostic Markers: A Newer Insight in Diagnostics

6

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6.1 MicroRNA Biogenesis and Mechanism of Action

MicroRNAs (miRNAs) are evolutionarily conserved small noncoding RNAs (18–25 nucleotides). miRNAs are transcripts of individual genes having their own promoter or produced from protein coding genes by transcribing spliced portions intragenically. RNA polymerase II produces pri-miRNA, a primary transcript (Czech and Hannon 2011). It has basic structural features of mRNA transcripts such as 7-methylguanosine cap and poly-(A) tail. Introns are also present sometimes. Drosha ribonuclease along with DGCR8 (double-stranded RNA-binding protein) recognizes pri-miRNAs for further processing. In humans, the DGCR8 microprocessor complex subunit is encoded by *DGCR8* gene. It is localized in cell nucleus and binds to Drosha to form the microprocessor complex which cleaves the characteristic stem-loop structure, i.e., pri-miRNA, which is processed further to miRNA fragments by enzyme Dicer (Bertoli et al. 2015). pri-miRNAs produces precursor miRNAs (approx. 70 nucleotides). It is also known as pre-miRNAs. pre-miRNAs is also produced from some intronic miRNAs (also known as mirtrons) by using splicing machinery because they bypass Drosha processing (Czech and Hannon 2011). The pre-miRNAs are then transported to cytoplasm from the nucleus by exportin 5 (XPO5). In cytoplasm, RNase III enzyme Dicer 1 along with AGO2 (DICER complex) and transactivation-responsive RNA-binding protein 2 (TARBP2) cleaves pre-miRNAs resulting in a double-stranded miRNA-miRNA* duplex formation (Bertoli et al. 2015). Two strands then separate. The mature miRNA, also known as guide strand, gets integrated into the RNA-induced silencing complex (RISC). Two routes

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are followed by the passage miRNA* strand, i.e., either it is loaded in RISC or it is degraded. To repress the expression of target, AGO protein of the RISC is guided by mature miRNA to the complementary mRNA sequence (Czech and Hannon 2011).

miRNA has a 6–8-nucleotide seed sequence at the 5′ end which determines specificity for its binding to target mRNA (Czech and Hannon 2011). The complementarity between seed sequence and the loaded miRNA causes a measurable decline in the expression of target mRNA. Complementarity matching may take place in any segment of mRNA. However, it is likely to occur in 3′ untranslated region of a mRNA (3′ UTR) (Bertoli et al. 2015). Degree of homology between 3′ UTR of mRNA and miRNA determines whether translation will be repressed or target mRNAs will be degraded. Since each miRNA has got capability to regulate the expression of many genes, it might be inferred that each miRNA is capable of controlling many cellular signaling pathways simultaneously.

There are reports which advocate an entirely different opinion about the mechanism of miRNA action. Some findings suggested that miRNAs could enhance target mRNA translation by inducing AU-rich region through recruitment of protein complexes at this site. Else, they could in some way raise the level of target mRNA by altering repressor proteins which inhibit the translation process. miRNAs could also be implicated in increased ribosome biogenesis, thus affecting protein biosynthesis, or bypassing cell cycle arrest, which ultimately activates repression of target gene (Bertoli et al. 2015). The role of miRNAs as biomarkers has been found to be a tempting area in health and diseases. They will revolutionize the diagnostic and patient care processes including screening and diagnosis of diseases, evaluation of disease progression, and identification of accurate treatment. Since they control the target-specific gene expression, their dysregulation is concerned with modulation of biochemical processes at molecular level in cells and tissues ultimately progressing toward diseases. Diagnostic miRNAs can be found at both extracellular (whole blood, sera, plasma, and urine) and intracellular levels. Studies also revealed that miRNAs found in seminal fluid or cerebrospinal fluid may also be used to study the expression profile of miRNAs as prognostic marker for particular disease (Bertoli et al. 2016). Isolation and characterization of miRNAs are mostly done on samples derived from body fluids and tissues. Their extraordinary stability in blood and urine makes them attractive target for noninvasive tests.

6.2 Oxidative Stress- and Disease-Related miRNAs in Cardiovascular Diseases

Oxidative stress-linked modulation of various miRNAs is implicated in endothelial and vascular dysfunction. It has been observed that characteristic features are shown by miR-200 family in oxidative stress-induced vascular cell response. miR-141 and miR-200c are members of miR-200 family. They have been found as the most upregulated miRNAs in response to oxidative stress in endothelial cells (ECs) (Magenta et al. 2011). Magenta et al. (2013) reported that glutathione reductase inhibitor 1,3-bis(2 chloroethyl)-1-nitrosourea (BCNU), which inhibits the

formation of reduced glutathione from oxidized glutathione, has ability to increase expression of miR-200c. They described the significance of miR-200 family upregulation, and especially of miR-200c in ECs reaction to oxidative stress, indicating the important role of ZEB1 (zinc finger E-box-binding homeobox 1) downmodulation in ROS-induced apoptosis and senescence. Several studies have established that oxidative stress is involved in upregulation of miR-200 family. Experiments on oxidative stress induction in a cell model with tert-butyl hydroperoxide (t-BHP) proved upregulation of miR-200c and miR-141. miRNA profiling studies on H_2O_2-induced oxidative stress in primary hippocampal neurons of mouse further supported upregulation of miR-200c. Nitric oxide (NO), a free radical known to play important role in endothelium metabolism, also stimulates overexpression of miR-200 family. These events and ZEB2 knockdown bring forth the expression of CXCR4 and Flk1, which are early cardiovascular and mesendoderm precursor markers in mouse embryonic stem cells (Magenta et al. 2011). A relationship between the expression of miR-200 family and oxidative stress has also been portrayed by workers in other systems (Mateescu et al. 2011). The same seed sequences are present in miR-141 and miR-200a. These sequences are used to target mitogen-activated protein kinase (MAPK) p38α, a signal molecule involved in regulation of cell proliferation, stress management, and cell survival. This illustration highlights the potential mechanism of action used in many cell types by miR family (Magenta et al. 2011).

Upregulation of an NAD^+-dependent class III histone deacetylase, sirtuin 1 or SIRT1, is reported in many organisms during caloric restriction, aging, and extension of the life span processes. SIRT1 plays a major role in metabolic regulation at cell, tissue, and organ levels by actively deacetylating countless enzymes, stress-responsive transcription factors, and co-regulators. It also has intense anti-inflammatory and antioxidant activity, suggesting a beneficial role for SIRT1 upregulation in endothelial cell biology (Magenta et al. 2011). However, decreased expression of SIRT1 is found during aging and overproduction of ROS (reactive oxygen species) which is related to EC dysfunction. Activation of SIRT1 in ECs mitigates oxidative stress, increases bioavailability of NO through eNOS induction, promotes biogenesis of mitochondria, and averts endothelial senescence. Interaction between oxidative stress-miRNA-SIRT1 pathways plays crucial role in vascular disease development processes. Its role has been highlighted in abdominal aortic aneurysm and atherosclerosis. miR-217 increases with age progression and affects endothelial senescence. miR-217 negatively modulates expression of SIRT1, resulting in loss of functional interaction with eNOS and Forkhead box protein O1 (FoxO1) which are main endothelial targets of SIRT1 (Menghini et al. 2001). Inverse relationships between FoxO1 acetylation and miR-217 level as well as between expression of SIRT1 in atherosclerotic plaques and miR-217 level are explicit. HIF1A destabilization is directly related to SIRT1 expression which is downregulated by miR-199a. Low SIRT1 consecutively causes inactivation of prolyl hydroxylase domain-containing protein 2 (PHD2) which is essential for HIF1A destabilization. Similarly miR-34a also targets SIRT1.

In cultured bone marrow cells (BMCs), overexpression of miR-34a stimulates cell death and SIRT1 downregulation, while its inhibition stops H_2O_2-induced cell death. It has been observed that BMCs, with blocked miR-34a expression ex vivo, when injected in mice after acute myocardial infarction boost cardiac function (Magenta et al. 2011). Targeting of SIRT1 by oxidative stress-induced miR-200a further confirmed the major role played by miR-200 family in EC dysfunction resulting from oxidative stress. It has been reported that stress-induced increased expression of miR-21 safeguards ECs by promoting eNOS and NO levels and lowering apoptosis. Overexpression of miR-21 in atherosclerotic plaques is also associated with decline in mitochondrial antioxidant protein SOD-2 and SPRY-2. This, further, causes activation of ERK/MAP kinase with consequential increase in ROS production and migratory defects in angiogenic progenitor cell (APC). During heart failure, mitochondrial structural modification coupled with size reduction and increased numbers has been observed (Magenta et al. 2011). In addition, mitochondrial damage is related to several manifestations of ventricular dysfunction in congestive heart failure including end-diastolic pressure, ejection volume, and the extent of orthosympathetic stimulation. One study has revealed that mitochondrial integrity is affected by specific miRNAs in cardiomyocytes which include miRNAs of miR-15 family (miR-15b, miR-16, miR-195) and miR-424 having same seed nucleotide sequences. These modulate ATP levels and decrease the expression of ADP-ribosylation factor-like 2 (Arl2) mRNA which is their common protein target. The evidences explained above suggest that miRNAs play key roles in pathophysiological processes, and hence they are good diagnostic biomarker for oxidative stress-mediated vascular ailments (Magenta et al. 2011).

6.3 Oxidative Stress-Induced miRNAs in Liver Injury

Several studies have revealed the role of oxidative stress in the pathophysiology of liver injury. In adult liver, miR-122 constitutes approximately 70% of the total miRNA. These are involved in functional aspects of the liver in health and diseases comprising lipid metabolism, progression of cell cycle, fibrosis, and hepatocellular carcinogenesis. Moreover, miR-122 is a prospective biological marker for diagnosing liver toxicity resulting from alcohol, acetaminophen, and drug-induced liver injury (Zhang et al. 2011). In addition, miR-122 has also been proposed as a biomarker for the early detection and diagnosis of antitubercular drug-induced liver injury (ADLI). Correlation between oxidative stress and miR-122 in ADLI has been proven by measuring alterations in oxidative stress indicators and levels of miR-122 in liver tissue of mice during hepatic damage.

Isoniazid (INH) is used as a first-line drug to treat tuberculosis. However, ADLI is the most frequent offshoot of INH treatment and progresses to liver cirrhosis. ADLI occurs because of excessive oxidative stress and mitochondrial dysfunction attributable to the formation of reactive metabolites during metabolic breakdown of drug. Studies indicate that mitochondrial ribosomal protein S11 (MRPS11) is targeted and regulated by miR-122. It is well known that oxidative stress causes ADLI. It has been

hypothesized that certain types of miR-122, acting as a potential biomarkers for ADLI, regulate the synthesis of mitochondria, thus participating in oxidative stress (Zhang et al. 2011). miR-122, as a dominant liver-specific miRNA, may adjust the targets to affect liver cells. It has been reported that miR-122 expression is more closely correlated with liver damage as compared to levels of GPT and GOT, the serum enzymes indicating that miR-122 is an important biomarker having diagnostic potential. It is concluded that tissue miR-122 is closely involved in INH-induced ADLI and might be implicated in oxidative stress by altering its target levels.

6.4 Oxidative Stress-Induced miRNAs in Neurodegenerative Disease

miRNAs are possibly related to several pathophysiological processes, viz., antioxidant response, cholesterol trafficking, pathological proteins clearance, neuroinflammation, and cell cycle anomalies. All these processes aid in progression of neurodegenerative diseases (NDs). Postmortem of ND brains in humans and animal models (rodent mouse) has reported oxidative stress responses (Coppedè and Migliore 2010). In ND patients, oxidative stress affects calcium homeostasis, peroxidation of membrane lipids, proper protein folding, aggregation, DNA repair, and clearance of damaged proteins. ROS is mainly produced during mitochondrial respiration and inflammatory processes. This indicates a strong linkage among oxidative stress-associated redox processes vis-a-vis mitochondrial dysfunction and neuroinflammatory response. Current scientific literature has highlighted the role of miRNA in pathological and physiological stress-induced responses (Coppedè and Migliore 2010; Prendecki and Dorszewska 2014).

In Parkinson's disease (PD), Alzheimer's disease (AD), and other NDs, undue neuronal apoptosis takes place in different parts of the human brain which adversely affects the central nervous system (CNS). PD and AD are nonhomogeneous group of disorders. The nature of the abnormalities in behavioral and cognitive functions as well as motor responses of the brain are determined by part of brain and types of neurons affected, which are disease specific. However, perfect diagnosis of NDs is possible simply after postmortem and histopathological evaluation of brain tissue because of the considerable heterogeneity of clinical signs in NDs. Today, NDs constitute one of the most important healthcare issues. About 24 million people are affected by AD worldwide. Scientific community across the globe is looking for specific markers essential for the pathogenesis and diagnosis of ND. It appears that miRNA, whose biosynthesis is clearly known, could be one of the potential biomarkers. At present, about 2600 miRNAs are known in humans who are engaged in many pathophysiological processes (Prendecki and Dorszewska 2014). Out of these, specific miRNAs related to pathogenesis and diagnosis of NDs have been identified. Majority of miRNAs are of common occurrence in various NDs. Only a small number of them are exclusive to specific NDs such as miR-132 and miR-212 for frontotemporal dementia; miR-19b, miR-34b/miR-34c, and miR-133b for PD; and let-7f, miR-125b, and miR-193b for AD. Thus ND-specific miRNAs may pave

the way for early and definite diagnosis, and accordingly, clinicians may introduce suitable treatment regimen for particular disease. Association between NDs and oxidative stress-dependent miR-153 has been displayed by workers (Narasimhan et al. 2014). The study reported the effect of paraquat, an environmental pollutant, on PD resulting from increased risk of dopaminergic neurons (DNs) damage. Real-time quantitative PCR analysis demonstrated that paraquat considerably upregulated the expression of brain-enriched miR-153 and downregulated nuclear factor Nrf2 which binds and activates antioxidant response elements (ARE, the transcription initiator) involved in mitigation of oxidative stress. Thus, paraquat induces neurotoxicity by damaging DNs, suggesting a decisive role of ROS (resulting from oxidative stress) interaction in miR-153-Nrf2/ARE pathway (Narasimhan et al. 2014). Intensive efforts are required for the development of miRNA-based methods merging data obtained from the expression studies of various miRNAs of CSF or blood origin. The effort will go ahead in making available, specific, and sensitive assays for early diagnosis of neurodegenerative disorders.

6.5 Oxidative Stress-Induced miRNAs in Sepsis

Infection causing a systemic inflammatory response is known as sepsis which could be responsible for high mortality because of multiple organ dysfunction syndromes (MODS). Studies related to sepsis prevention and treatments during the last 10 years have achieved some success. Nevertheless, sepsis is still the most important cause of ICU mortality (Yao et al. 2015). For diagnosis and appraisal of sepsis conditions before time, biomarkers play significant role. C-reactive protein (CRP), procalcitonin (PCT), N-terminal pro-atrial natriuretic peptide, and interleukins are the early sepsis markers having some limitations. They need to have more precision with respect to their role as a biomarkers. For instance, CRP is not appropriate alone as diagnostic marker because of its high sensitivity and low specificity. However, specificity of PCT is higher than that of CRP. It has been reported that PCT alone is unable to differentiate between bacterial sepsis and nonbacterial systemic inflammatory response syndrome (SIRS). Hence, development of new biomarkers is much needed. Altered level of miRNA expression has been observed in some sepsis patients. The studies on miRNAs in rat model of septic shock revealed that 17 out of 351 miRNAs had higher expression, while only 9 among them notably regulated specific genes (Yao et al. 2015). Sepsis causes upregulation of miR-27a in mice, and its inhibition is correlated with reduction of the inflammatory responses by lowering IL-6 and TNF-α levels (Wang et al. 2014). Hence, miR-27a acts as biomarker for sepsis detection. In addition, it is also a good target for drug action. In PBMCs of sepsis patients, dysregulated miRNAs are present. Downregulation of miR-146a is associated with raised level of IL-6 and monocyte proliferation in sepsis (Zhou et al. 2015). Oxidative stress is involved in sepsis-induced MODS, a major cause of patient death. During sepsis, ROS generation resulting from activation of enzymes (NADPH oxidase and xanthine oxidase) by ischemia reperfusion injury and/or endotoxins produces redox imbalance which eventually causes tissue damage (Von-Dessauer et al. 2011).

miR-25 and oxidative stress levels are inversely related in sepsis patients. Increased stress indicates lower levels of miR-25 whose target is NOX4 (NADPH oxidase 4) gene. Lower levels of miR-25 enhance NOX4 expression which induces ROS production and ultimately triggering the oxidative damage (Yao et al. 2015). Earlier studies have also revealed that linkage of miR-25 with oxidative stress may be the main cause for sepsis-induced MODS. Hence, miR-25 could be used as oxidative stress-mediated prognostic biomarker of sepsis (Varga et al. 2013).

6.6 Oxidative Stress-Induced miRNAs in Diabetes

Diabetes is known to be an important and established threat factor for diverse ailments. Hyperglycemia is a common feature of both type 1 and type 2 diabetes. It enhances ROS production, changes the oxidant status of cells, and quickly modifies membrane function, followed by other malfunctioning in different organs of the body. Endogenous antioxidant defense system gets compromised during oxidative stress which is associated with diabetes-induced decline in different body functions (Kumar and Pandey 2015). Several studies have probed the causes underlying role of oxidative stress in diabetes. Emerging facts specify that miRNAs functioning as translational repressors are key regulators of important biological processes and might be related to the pathophysiology of diabetes. Tissue-specific miRNAs have been recognized in complications associated with diabetes. Dysregulation and differential expression of cardiac-enriched miRNA levels in heart of diabetic animals have been shown to play vital roles in the progression of diabetic cardiomyopathy (Yildirim et al. 2013). In diabetic heart, miR-133 expression level has been shown to change (Feng et al. 2010). Glucose-stimulated apoptosis of cardiomyocytes is mediated by miR-1. However, lowered expression of miR-1 brings about enhancement in the level of a cytoskeleton regulatory protein which induces cardiac hypertrophy (Yu et al. 2008). Hyperglycemia-induced oxidative stress-dependent reduction of four miRNAs (miR-1, miR-133a, miR-133b, and miR-499) has been experimentally shown in diabetic rats. In addition, redox imbalance further influences many intracellular targets of these miRNAs. Therapeutic efficacy of miRNA therapeutic intervention in diabetic complications has been proven, and miR-21 has been identified as a disease target (Yildirim et al. 2013).

Association of particular miRNAs, viz., miR-15a, miR-107, miR-103, and miR-143, with glucose metabolism (insulin induced) and progression of the diabetes have been discussed by several workers. Studies have revealed the linkage between some of the diabetic problems and the modulated expression of miRNA consequently leading to increase in transcription factors, matrix components, and growth factors. Lowered level of miRNA expression causes failure of inhibition at certain targets in diabetic complications. This is the first trend observed in diabetes. Expression of fibronectin (FN) is negatively regulated by miRNA-146a related to retinopathy. Downregulation of miR-146a in diabetic animals finds direct correlation with overexpressed FN (Feng et al. 2013). Further, miR-200b and VEGF expression are inversely related. Therefore, loss of miR-200b in retinopathy results in endothelial

proliferation and permeability due to overexpression of VEGF. Association of miRNA-133a with insulin growth factor 1 receptor also results in cardiomyopathy (Feng et al. 2013). Finally, hyperglycemia-induced oxidative stress led downregulation of miRNA results in augmentation in the level of growth regulators and inflammatory factors. However, some miRNAs are overexpressed in diabetic nephropathy, e.g., miR-377 which is directly related to SOD1/SOD2 levels. The increment in miR-377 level increases the instability of *SOD2* transcripts which ultimately lead to reduced Mn-SOD activity (Wang et al. 2008). This shows that hyperglycemia-induced overexpression of a miRNA compromises with the antioxidant defense. Similarly, hyperglycemia causes overexpression of miR-192 in renal mesangial cells which directly targets zinc finger E-box-binding homeobox 1 (*ZEB1/ZEB2*) resulting in decreased expression of *ZEB1/ZEB2* in the experimental subjects (Kato et al. 2007). Under normal conditions, *ZEB1/ZEB2* is responsible for repressing TGF-β. So decreased *ZEB1/ZEB2* levels in diabetes ultimately causes increase in TGF-β expression and leading to decline in renal function. Above examples demonstrate that inhibition mediated by overexpression of miRNA in the cell can produce varied results on protective signaling in diabetic individuals.

6.7 Oxidative Stress-Induced miRNAs in Cancer

Since hundreds of mRNAs are targeted by a single miRNA, anomalous expression of miRNA may have an effect on a large number of transcripts and strongly affect signaling pathways related to cancer. miRNA was primarily portrayed in 1993, its physiological and pathological significance became known after their characterization in several species in 2001. Microarray expression data obtained from variety of cancers have proved that unusual expression of miRNA is the essential feature rather than the exception. Notably, miRNA overexpression or ablation in mouse models have confirmed the fundamental association between miRNAs and cancer growth, and therefore, miRNAs are making their entry in biomedical fields as potential biomarkers and accepted as targets for therapy. Studies on several mammalian differentiation pathways have revealed that individual miRNAs may serve as switches, for example, smooth muscle cell differentiation is regulated by miR-143 and miR-145, while skin differentiation is regulated by miR-203 (Jansson and Lund 2012). A single miRNA has capability to affect identity of the cell. Early studies on overexpression substantiated that HeLa cells transfected with single miR-124 showed changed pattern of expression exemplifying characteristics of brain expression profile. Brain tissues show higher expression of miR-124 (Lim et al. 2005). miRNAs play critical roles in many vital processes including noise-dampening effect, differentiation, and cellular identity. Hence, functional loss of miRNA may lead to enhanced dedifferentiation, cellular plasticity, and a greater tendency toward oncogenic alterations. miRNAs also play remarkable roles in differentiation of stem cells and pluripotency induction. Recently, human and mouse fibroblasts have shown ability to produce miR-302-induced iPSC (induced pluripotent stem cells) by a single miRNA cluster. At present, about 1400 human miRNAs are known.

Many of them show strong conserved sequences among distantly related animal taxa (Jansson and Lund 2012). Most of the miRNAs put forth their complete functional effects by targeting many mRNAs; some of them might be the part of same cellular pathway. A specific miRNA may be augmented in some cancer types suggesting its oncogenic behavior, while in other cancers it may be downregulated signifying its tumor suppressor function. Therefore, it is imperative to be careful while drawing conclusions regarding usage of miRNA.

6.7.1 Ovarian Cancer

Among gynecological malignancies, epithelial ovarian cancer (EOC) is the major cause of fatality in women worldwide. It accounts for approximately 5% of all cancer cases and about 4.2% of all cancer deaths in women globally. Despite rapid progress in diagnostics and therapeutics, only limited success in the survival rate of ovarian cancer patients ahead of 5 years has been realized after initial diagnosis. Several factors are responsible for the high mortality of ovarian cancer patients. These include the lack of early stages specific symptoms and delayed diagnosis causing problem in designing therapeutic intervention as well as the developing resistance to chemotherapy in cancer cells. This necessitates for better EOC detection and screening approach at early stages coupled with effective treatment regimen for advanced stage patients (Pal et al. 2015).

Redox regulation has been suggested to play important role in cancers. However, its role in tumor prognosis is still elusive. Several studies have shown relationships between oxidative stress and the miRNAs that affect tumorigenesis and chemosensitivity. miR-141 and miR-200a have been shown to alter the oxidative stress response by targeting p38a. Higher concentrations of miR-200a and lower concentrations of p38a are observed in human ovarian adenocarcinomas patients, which act as an oxidative stress marker (Mateescu et al. 2011). Correlation has been found between the level of stress biomarker miR-200a and the improvement in patients' survival under treatment regimen. Therefore, miR-200a could acts as a prognostic marker during stress for evaluation of clinical efficacy in EOC. Besides its tumor-promoting role, oxidative stress also enhances sensitization of cancerous tissue to drug treatment. This could provide explanation to clinical trials using antioxidants with the partial success. Thus, there is an urgent requirement to investigate the biochemical rationale of ovarian cancer for exploration of early diagnostic markers/classifiers which can consistently identify patients for interventional therapy. ROS buildup in cancer cells damages cellular machinery and modifies diverse processes at biochemical and molecular levels including cell proliferation, gene expression, and stability of genome. Among stress-linked modulation of gene expression, the mitogen-activated protein kinase p38α family carries out the redox-sensing function for monitoring oxidative stress status. The sensor function is necessary for controlling tumor progression. The miRNAs of miR-200 family have been shown to alter cellular motility and manage "stemness" and apoptosis. During redox imbalance and ovarian tumorigenesis, the miR-200 performs a new function. Mateescu et al.

(2011) have demonstrated inhibition of p38α by miR-141 and miR-200a (the members of the miR-200 family) which play important role in redox sensing. Upsurge of these miRNAs in mouse imitates deficiency of p38α and supports malignancy.

6.7.2 Breast Cancer

Various reviews have presented the diagnostic, prognostic, and the therapeutic roles of miRNAs in breast cancer (BC). miRNAs can play dual role in BC either by acting as oncogenes or tumor suppressor genes. Outcome of studies has indicated the role of miRNAs as promising biomarkers for diagnostic, prognostic, and therapeutic applications in BC. miR-9, miR-10b, and miR-17-5p are diagnostic makers for BC, while miR-148a and miR-335 are prognostic markers. Some miRNAs, viz., miR-30c, miR-187, and miR-339-5p, are emerging as potential biomarkers for testing therapeutic efficacy of drugs. All these miRs are associated with BC control functions, i.e., proliferation, invasion, metastasis, apoptosis, resting death, and genomic instability (Bertoli et al. 2015). Other new and easily available miRNAs, viz., miR-155 and miR-210, circulating in body fluids have drawn attention as markers for management of BC patients because they are affordable and noninvasive tools. Many circulating miRNA have shown better diagnosis and prognosis results in BC along with better sensitivity. New miRNA-based drugs containing miR-9, miR-21, miR-34a, miR-145, and miR-150 have emerged as potential therapy for BC. Other miRNAs such as miR-21, miR-34a, miR-195, miR-200c, and miR-203 in combination with chemotherapy have also shown a basic response in modulation of other non-miRNA treatments (Bertoli et al. 2015). miRNAs having oncogenic activities are designated as oncomirs. These are constitutively overexpressed and responsible for promoting tumor growth by inhibiting tumor suppressor genes or regulatory genes that affect cell cycle progression and differentiation or apoptosis. miR-21 is an excellent example of oncomirs. Its target tumor suppressor gene is PTEN (phosphatase and tensin homolog), and several studies have shown that miR-21 overexpression, correlating to PTEN downregulation, leads to proliferation and metastasis (Corsini et al. 2012). miRNAs having invasive skills are called metastamiRs. These miRNAs regulate positively or negatively epithelial-to-mesenchymal transition (EMT), loss of cellular adhesion, can play a pro- and anti-metastatic role. Examples include miR-192/miR-215, which targets ZEB1 and ZEB2 (E-cadherin repressors), miR-30, and miR-200 family that regulate the TGF-beta pathway (Corsini et al. 2012).

6.7.3 Prostate Cancer

In males prostate cancer (PC) is the sixth major cause of cancer death and its prevalence increases with age. Like other cancer, oxidative stress also contributes major role in pathophysiology of prostate cancer. Intrinsic and extrinsic factors may cause higher production of ROS in the prostate and thereby affect the function of prostate.

Altered redox status in prostate tissue resulting from imbalance between oxidants and antioxidants plays an important role in the initiation of PC.

Androgens play key role in mitigating ROS imbalance in the prostate. In addition, the transcription factor Nrf2-mediated expression of major antioxidant defense enzymes through the upregulation of ARE is responsible for lowering the ROS levels in prostate cancer. Current reports indicate that in human prostate cancer, Nrf2 and its target genes are considerably downregulated. Thus, cells repeatedly face rising oxidative stress levels that ultimately result in their continuous advancement toward metastatic conditions (Pekarik et al. 2013). One of the approaches to study miRNAs in PC is to analyze the exosomal miRNA profiles of cancerous and non-cancerous prostate samples. It has been shown that miR-711 and miR-4258 have comparatively higher expression in exosomes of PC sample (Liu et al. 2011). Another study has reported that serum level of miR-141 can differentiate between healthy control and PC samples (Huang et al. 2010). It was further substantiated by upregulation of miR-141 in the plasma of PC patients that confirmed its role as diagnostic biomarker (He et al. 2012). The use of miR-141, miR-298, miR-346, and miR-375 as diagnostic markers of PC has been authenticated by using microarray and RT-PCR techniques (Felicetti et al. 2008). Compared with healthy control sera, upregulation of 15 miRNAs (miR-16, miR-92a, miR-103, miR-107, miR-197, miR-34b, miR-328, miR-485-3p, miR-486-5p, miR-92b, miR-574-3p, miR-636, miR-640, miR-766, miR-885-5p) have also been reported in serum of PC patients (Fanjul-Fernandez et al. 2010). In addition, miR-12, miR-34, miR-129-5p, miR-203, miR-302, miR-372, miR-373, and miRNA cluster miR-183-96-182 are known to be involved in oxidative stress-mediated prostate cancer pathophysiology (Bertoli et al. 2016).

6.8 Conclusion

MicroRNAs are specific targets for diagnostic and prognostic applications. They are helpful in evaluation of disease progression and in recognition of the therapeutic target for disease management in patients suffering from many degenerative diseases. Expression of particular target genes is regulated by miRNAs and their dysregulation cause altered biochemical and molecular processes in intracellular milieu. Oxidative stress is concerned in up-/downregulation of numerous miRNAs. Identification of various miRNAs as prospective targets for diagnosis and prognosis of cardiovascular, liver, and neurodegenerative diseases, diabetes, sepsis, and different types of cancers has revolutionized the field of biomarker discovery. Diagnostic miRNAs can be found at extracellular and/or intracellular levels. miRNAs are extremely stable in biological samples making them attractive molecules for noninvasive tests.

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Oxidative Stress Monitoring Using In Vitro Systems: Tools and Findings

7

Aditya Arya and Yasmin Ahmad

7.1 Introduction

Oxidative stress is well-known phenomenon, caused by a shift in the delicate balance between radical generation and scavenging of radical capacity in cells. Reactive oxygen species (ROS) primarily composed of superoxide radicals, hydroxyl radicals, etc. In principle, every molecule including oxygen is known as an oxidant or oxidizing agent if it is capable of accepting electrons (Prior and Cao 1999), and the process of electron loss is known as oxidation. In biology, the process of oxidation is always accompanied by reduction and such reactions are called as redox reactions. Redox reactions are basis for numerous biochemical pathways including biosynthesis and regulation of metabolism. While oxidant and reductant are chemical terms, in biological context these are often known as pro-oxidant and antioxidant, respectively (Kohen and Nyska 2002). Pro-oxidant includes several radical and nonradical species (Halliwell 2006).

Understanding the phenomenon of radical scavenging in real time is challenging primarily due to their transient life and non-availability of highly specific probes for various reactive species. Furthermore, in case of plant-based antioxidant, the separation and purification is costly as well as inefficient due to the complexity of composition. This complexity of studying the effect of plant-based antioxidants is further complicated by synergy of actions that exist between various antioxidants.

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Another challenge in the conventional antioxidant research is the lack reliable and highly specific measurement of antioxidant capacity different biological samples and food products. A large number of scientific evidences and a number of critical reviews have also been published, yet, opinions vary considerably. Moreover, considerable debate about the criteria for the best method is still on. The fact that most of the antioxidant tests are performed in test tubes but not in the live biological milieu further adds to this debate. More than one type of assays for similar parameter and non-availability of indexing the values and their coordinated integration leaves the researches confused about the antioxidant power. Despite the non-consensus on the best and most suitable method for determination of net oxidative stress and gross antioxidant status, the usage and applicability of these assays are massive and provide a huge amount of data which has pushed several drugs into clinical trials, and it is therefore very important to understand the methodology, pros and cons of existing methods and their suitability depending on cost, instrumentation sample type. This chapter outlines the key methods which are currently in use for the qualitative or quantitative estimation of reactive oxygen species, antioxidant defence and oxidative stress in cell culture systems and some of the animal samples such as plasma, which may be analysed in vitro.

7.2 Various Assays for in Vitro Analysis of Oxidative Stress

There are several means of estimating the reactive oxygen species (ROS) in the cellular systems. Usually in vitro systems are the first line of experimentation regimes for any scientific hypothesis, and therefore cell culture and simulated oxidative stress are usually followed by various assays to evaluate the oxidative stress. A number of experimental variants are known for the determination of reactive oxygen species; some of them provide a direct quantitative measure such as biochemical methods. Other methods may be semiquantitative, but either provide a direct visual estimate (such as microscopy) or semiquantitative estimation of ROS in the intracellular compartments (such as flow cytometry). More precise methods such as electron spin resonance methods rely on different principles and therefore can help us to infer transient changes in the ROS especially the rapid ROS burst and its surge. We may broadly categorize the methods for determination of oxidative species primarily on the basis of instrumentation and type of principle. Majority of methods for the ROS estimation are based on spectrometric methods which include UV-Vis spectrophotometry and an extension luminometry and fluorimetry. Spectrometric methods are simple less time-consuming and cost-effective (Fig. 7.1).

7.2.1 Biochemical Assays (Spectrometry Based)

Biochemical assays primarily include those assays in which the reactive oxygen species or their derivatives are estimated using some chemical reaction which result in the formation of coloured complex or noncoloured complex showing high absorbance in ultraviolet radiation or luminescence or fluorescence which may be measured using luminometer or fluorimeter. We will now discuss some of the common methods used

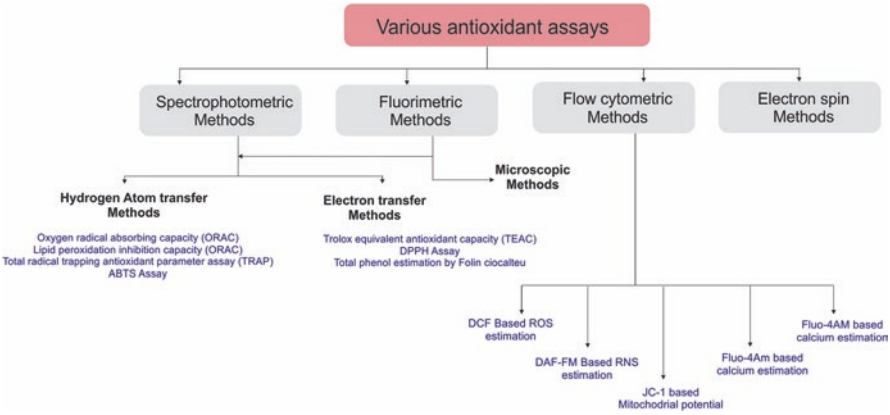

Fig. 7.1 Various methods (assays) for the analysis of antioxidants

for estimation of free radical species and their pros and cons. All the spectrometry-based methods may be broadly grouped into three main categories, hydrogen atom transfer methods (HATMs) and electron transfer methods (ETMs). The third category includes other mechanisms such as peroxidation and other chemical events. The following is the detailed description of commonly used spectrometry-based methods.

7.2.1.1 Hydrogen Atom Transfer Methods

These methods are used for the measurement of the antioxidant capacity or ability to scavenge ROS (e.g. peroxyl radical, ROO•) by the loss of hydrogen ions as shown in equations 1 and 2. ROO• are generally chosen as 64, the reactive species in these assays because of their higher biological relevance and longer half-life (compared to hydroxyl, •OH and superoxide anion radicals, O2 •−). The hydrogen atom transfer (HAT) reaction mechanism, involving the transfer of hydrogen radical/atom (H•) of antioxidants to a peroxyl radical (ROO•) to give more stable free radicals (A• and ArO•), is represented below with the help of chemical equations:

$$\mathrm{ROO}\bullet + \mathrm{AH} \rightarrow \mathrm{ROOH} + \mathrm{A}\bullet \tag{7.1}$$

$$\mathrm{ROO}\bullet + \mathrm{Ar-OH} \rightarrow \mathrm{ROOH} + \mathrm{ArO}\bullet 71 \tag{7.2}$$

$$2\mathrm{ROO}72\bullet \rightarrow \text{Nonradical Products} \tag{7.3}$$

where the radical of the antioxidant (A•) and aryloxyl radical (ArO•) are usually stabilized by resonance. A potent phenolic antioxidant (Ar-OH) need to react faster than the target to be protected with the oxidant (ROS), and A • must be rapidly converted to less reactive species (Apak et al. 2016). Some of the commonly used methods based on HAT are as follows:

A. Oxygen radical absorbing capacity (ORAC) assay

The ORAC assay is one of the commonly used antioxidant assays that involves the inhibition of oxidation of β-phycoerythrin by ROS. Trolox is well-known

reference antioxidant that is used as internal control in the assays. Among several interfering agents, proteins are predominant and reactive groups must be protected during the reaction, due to protein interference especially in plasma. Different radicals have different lag time and therefore different results are expected for the different radicals. Therefore, care must be taken in ORAC assay, when conducted in plasma. As a disadvantage, ORAC cannot be used to determine lipophilic antioxidants but remains limited to the measurement of hydrophilic molecules.

B. Lipid peroxidation inhibition capacity (LPIC) assay

LPIC is an assay that was developed by Zhang et al. in 2006 for assessment of in vitro antioxidant. The lipid peroxidation inhibition capacity (LPIC) method is based on the measurement of both hydrophobic and hydrophilic antioxidants. A lipophilic fluorescent probe 4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-s-indacene-3-undecanoic acid is incorporated in the membrane (Zhang et al. 2006) generating a significance on radical generation which may be further estimated using fluorimetry or flow cytometry.

C. Total radical-trapping antioxidant parameter (TRAP) assay

Total radical-trapping antioxidant parameter or TRAP assay was developed by Wayner et al. It has become the widely used assay to determine total antioxidant activity in biological samples. It is based on the principle of estimating consumption of oxygen under the controlled oxidation of lipids (Wayner et al. 1985). The TRAP results are expressed as the millimolar of peroxyl radicals trapped in plasma. Furthermore, the change in the rate of peroxidation caused by AAPH (2′-azobis (2-amidinopropane) hydrochloride) is monitored indirectly via loss of R-phycoerythrin (R-PE) fluorescence. The lag phase induced by plasma in TRAP assay is same as compared to the lag phase induced by Trolox – a reference antioxidant (Antolovich et al. 2002).

D. ABTS assay

2,2′-Azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) or ABTS assay was developed by Miller et al. They described an alternative technique for the measurement of total antioxidant capacity (TAC) using colorimetry. The ABTS assay is based on the principle of incubation of 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) [ABTS]-(key reagent of the assay) with peroxidase followed by the formation of a relatively stable radical cation, $ABTS^{+}$. Often the ferryl myoglobin is used to facilitate this chemical transition. ABTS+ then forms a relatively stable blue-green colour complex; the absorbance of this coloured solution can be measured at 600 nm using colorimetry or spectrophotometry. Antioxidants present in the fluid samples are known to suppress the formation of coloured complex and therefore remain potential interfering agents (Miller et al. 1993).

7.2.1.2 Electron Transfer Methods (ETMs)

Unlike aforesaid hydrogen atom transfer methods, the electron transfer methods are based on the antioxidant action simulated with a suitable redox potential probe, namely, the antioxidants react with a fluorescent or coloured probe (oxidizing agent) instead of peroxyl radicals. Spectrophotometric ET-based assays measure the capacity of an antioxidant in the reduction of an oxidant, which changes colour when reduced. The degree of colour change (either an increase or decrease of absorbance of the probe at a given wavelength) is correlated to the concentration of antioxidants in the sample. The following are some of the commonly used ET-based antioxidant assays:

A. *TEAC* assay

Trolox equivalent antioxidant capacity (TEAC) assay is used to determine the ability of molecules to scavenge the stabilized free radical of 2,2′-azinobis-(3-ethylbenzothiozoline-6-sulphonic acid). Trolox (a hydrophilic analogue of vitamin E) is used as an internal reference for comparing the antioxidant capacity. This technique is not widely applied due to limited scope for the biological samples that can be used. This limits the comparison of the results across various platforms.

B. FRAP assay

Ferric reducing antioxidant power (*FRAP*) method was developed by Antolovich. The principle of this method is reduction of Fe^{3+} complex of tripyridyltriazine Fe $(TPTZ)^{3+}$, a type of a ferroin analogue, which turns into blue-coloured Fe^{2+} complex $Fe(TPTZ)^{2+}$ by antioxidants in acidic condition (Antolovich et al. 2002). The blue colour thus obtained is measured at 593 nm using spectrophotometry and used for the determination of Fe^{2+} equivalents which corresponds to the antioxidant standard used in the assay. Sanchez et al. have reviewed the method and report it as simple and rapid method for manual as well as automated procedures (Sanchez-Moreno et al. 2000).

C. DPPH assay

An organic compound 2,2-diphenyl-1-picrylhydrazyl or DPPH is the key component of this assay which is capable of trapping the radicals present in the reaction and forming a coloured compound. The coloured complexes thus formed show an absorption at 517 nm which can be evaluated using spectrophotometry. The antioxidant activity is determined by measuring the decrease in the absorbance (Brand-Williams et al. 1995). Studies have shown that quantity of antioxidant necessary to decrease the 50% of the initial DPPH concentration tEC50 was later used to define a new parameter in the antioxidant research known as antiradical efficiency.

D. Total phenols estimation by Folin-Ciocalteu

Phenolic compounds are the commercially important secondary metabolites often extracted from the plants; some of the common examples of phenolic include

phenolic acids, flavonoids, tannins, lignin, etc. Some of these phenolic compounds are known to be antioxidants and confer UV protective abilities. Determination of the phenolic content in the phytoextracts is also one of the primary assays towards the measurement of total antioxidant capacity. One of the most commonly used methods involves the reaction with Folin-Ciocalteu reagent, which can react with the phenolic group and can produce a coloured complex showing maximum absorbance at 650 nm. The method and various studies based on this method have been recently reviewed by Andressa et al. (Blainski et al. 2013). One of the disadvantages of this method is the interference with the proteins containing high-content or aromatic amino acids, which will also react with the aforesaid reagent.

7.2.1.3 Other Related Spectrometric Methods

A. *Dye-based fluorometric assay in cell lysate*

There are a number of spectrophotometric methods which are based on fluorescent probes which are often used to determine the ROS in cell lysate or tissues homogenates. The degree of fluorescence in the sample is directly proportional to the amount of ROS; however, the absolute quantification cannot be done using these methods, and only relative quantification is obtained. Therefore the amount of ROS in samples is usually represented as fold change or arbitrary units. The most commonly used fluorescent probe is DCFH-DA or dichlorofluorescein diacetate, which is cleaved by intracellular esterases, and then cleaved DCF reacts with free radical and forms fluorescent adducts. Recently modified versions of the DCFH-DA are carboxymethyl or CM-DCFH-DA which shows a more persistent fluorescence for prolonged duration. It has been frequently observed in the author's lab and other studies that fluorescence levels are readily affected by the type of sample, time of incubation and the instrument used. Therefore, care must be taken while estimating the ROS levels. There are a number of other fluorescent probes available for determination of calcium ions (Fluo-4 AM), mitochondrial superoxide (MitoSox) and mitochondrial membrane potential (JC-1).

B. *TBARS assay*

Thiobarbituric acid reactive species or TBARS is one of the oldest antioxidant assays and also one of the most widely used methods for the detection of lipid oxidation. In this method a lipid peroxidation product called malondialdehyde (MDA) is estimated after a chemical reaction with thiobarbituric acid. Malondialdehyde is the secondary product of ROS which is formed by the oxidation of unsaturated fatty acids. On reacting with thiobarbituric acid (TBA), malondialdehyde forms a pink pigment which is then measured spectrophotometrically at the absorption maximum of 532–535 nm. Various reference and model molecules are also used to set the standards for this assay and for absolute quantification. Wijewickreme has suggested the use of linoleic acid and its emulsions with detergents such as SDS or Tween (Wijewickreme et al. 1997). The use of ethanol in this assay is a debated issue. Studies by Belguendouz et al. (1997) have revealed that the presence or absence of ethanol did not influence the antioxidant activity of the samples. The method was reviewed by Antolovich et al. (2002).

7.2.2 Flow Cytometry-Based Assays

Flow cytometry is a technique that is based on the separation of cells using hydrodynamic focusing and reading of specific molecule using laser excitation and subsequent detection. The advantage of flow cytometry-based assays is that they represent a semiquantitative assessment and also distinguishes between the cells showing differential ROS content, which can never be distinguished using spectrometric techniques. A significantly large number of fluorescent probes are now commercially available which show a binding with specific type of reactive oxygen species (ROS). A list of commercially available probes used for identification reactive oxygen species in flow cytometry is provided in Table 7.1.

Conventionally, reactive oxygen species is determined using DCFH-DA (as described in Sect. 7.2.1). More advanced type of fluorescent probes which are modified version of DCFH-DA is also being used nowadays. Carboxymethyl derivative (CM-DCFH-DA) is much popular choice due to long shelf life and higher quantum yield. Figures 7.2a and 1.2b illustrate the results of flow cytometry-based assays.

7.2.3 Electron Spin Resonance (ESR)-Based Assays

Electron spin resonance or ESR spectrometry is a state-of-the-art technique which has gained attention recently. This technique is powerful enough to specifically detect the free radicals that are involved in autoxidation and cross-oxidation process. As an advantage, the ESR technique is sensitive to several stable free radicals such as di-*tert*-butyl nitroxide (TBN). However, the drawback is that ESR is not able to detect the reactive, short-lived free radicals involved in autoxidation and oxidation of other biomolecules (lifetime varies from 10^{-29} s for the hydroxyl radical to several seconds for the peroxyl radicals). The anomalies of the ESR have been overcome by using several approaches such as continuous flow systems, pulse radiolysis, UV photolysis and spin trapping methods; among these the spin trap method is the most commonly used. Spin trapping involves the use of an additional compound in the reaction called the spin trap, which is capable of reacting with free radicals to fairly stable adducts. These adducts are later detected with the help of ESR scanner.

Some common examples of spin traps which are commercially available include different types of nitroso compounds such as a-phenyl-*tert*-butylnitrone (PBN), *tert*-nitrosobutane (tNB), 5,5-dimethylpyrroline-*N*-oxide (DMPO), a-(4-pyridyl-1-oxide)-*N*-*tert*-butylnitrone (4-POBN) and 3,5-dibromo-4-nitrosobenzenesulfonic acid (DBNBS) (Antolovich et al. 2002).

7.2.4 Microscopy-Based Assays

Using similar fluorescent probes which we discussed in Sect. 7.2.2., we may also prefer to directly visualize the generation and persistence of the reactive oxygen

Table 7.1 Commonly used fluorescent probes used for ROS/RNS detection that are commercially available

Detection reagents (probes)	Reactive oxygen species
RedoxSensor Red CC-1	Hydrogen peroxide
Carboxy-H_2DCFDA	
CM-H_2DCFDA	
Dihydrocalcein AM	
Dihydrorhodamine	
H_2DCFDA	
Lucigenin	
Luminol	
Proxyl fluorescamine	Hydroxyl radical
TEMPO-9-	
3′-(*p*-Aminophenyl) fluorescein (APF)	
3′-(*p*-Hydroxyphenyl) fluorescein (HPF)	
CM-H_2DCFDA	
Luminol	Hypochlorous acid
Aminophenyl fluorescein (APF)	
Dihydrorhodamine	
Luminol	Nitric oxide
DAF-FM	
DAF-FM diacetate	
2,3-Diaminonaphthalene	
Luminol	Peroxyl radical, includes alkylperoxyl and hydroperoxyl radicals (wherein R = H)
cis-Parinaric acid	
RedoxSensor red CC-1	
BODIPY FL EDA	
BODIPY 665/676	
H_2DCFDA	
Carboxy-H_2DCFDA	
CM-H_2DCFDA	
DPPP	
Luminol	Peroxynitrite anion
Dihydrorhodamine	
3′-(*p*-Aminophenyl) fluorescein (APF)	
3′-(*p*-Hydroxyphenyl) fluorescein (HPF)	
H_2DCFDA	
Carboxy-H_2DCFDA	
CM-H_2DCFDA	
Coelenterazine	
Trans-1-(2′-methoxyvinyl)pyrene	Singlet oxygen
Singlet oxygen sensor green reagent	

(continued)

Table 7.1 (continued)

Detection reagents (probes)	Reactive oxygen species
Lucigenin	Superoxide anion
Luminol	
Coelenterazine	
Dihydroethidium	
Fc OxyBURST Green assay reagent	
OxyBURST Green H_2HFF BSA	
XTT, MTT, NBT	
MCLA	
RedoxSensor Red CC-1	
TEMPO-9-AC	

Adapted and redrawn from www.Lifetechnologies/thermofisher.com

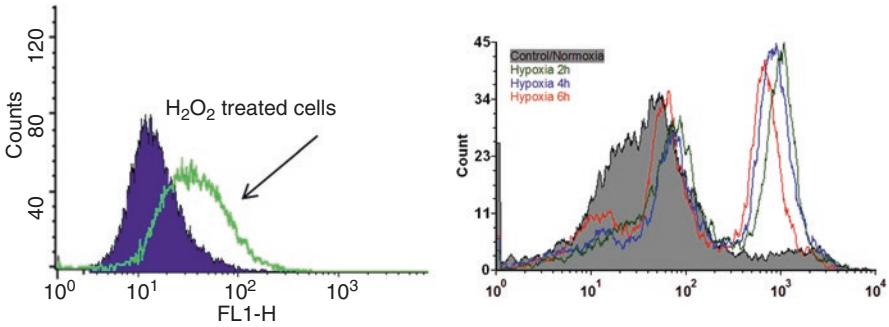

Fig 7.2 Fluorescence histograms of DCFH-DA-stained cells obtained using flow cytometry. (**a**) *Blue-coloured* filled plot represents the fluorescence of control cells, while unfilled *green* histogram represents fluorescence intensity of cells stimulated with hydrogen peroxide (known to induce reactive oxygen species). (**b**) Histogram overlays of various cells stimulated using hydrogen peroxide at different time intervals (*grey-shaded* histogram represents control cells). Note that there are two types of populations in this dataset, some cells which show higher fluorescence (graphs towards extreme *right*), while other populations (towards *left*) show lower fluorescence

species using fluorescent microscopy. This has two advantages; first it can be used to confirm and correlate the results of flow cytometry; second it helps the user to rule out the artefacts.

The most common ROS indicator used is DCFH-DA which shows green fluorescence, and the degree of fluorescence is directly associated with the degree of free radicals generated. Figure 7.2 depicts the difference in the cells under oxidative stress in contrast to normal cells (Fig. 7.3).

Furthermore, a number of dyes (fluorescent probes) are available for the determination of secondary effects of oxidative stress such changes in mitochondrial membrane potential or direct determination of mitochondrial superoxides (MitoSox). The MitoSox-stained cells are shown in Fig 7.4.

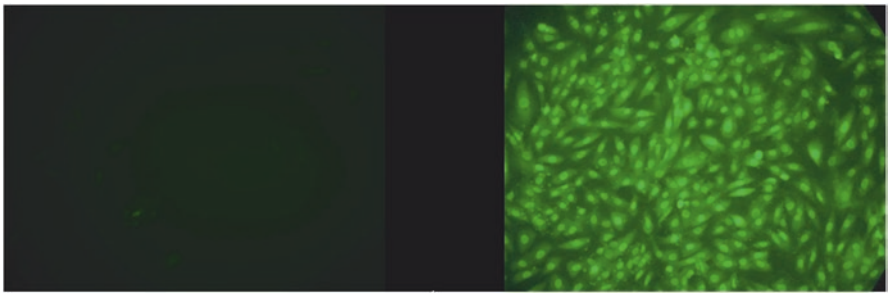

Fig 7.3 Photomicrographs of the cells incubated with DCFH-DA showing relative difference in the fluorescence of cells: (**a**) no oxidative stress or normoxic control and (**b**) cells pre-exposed to hydrogen peroxide

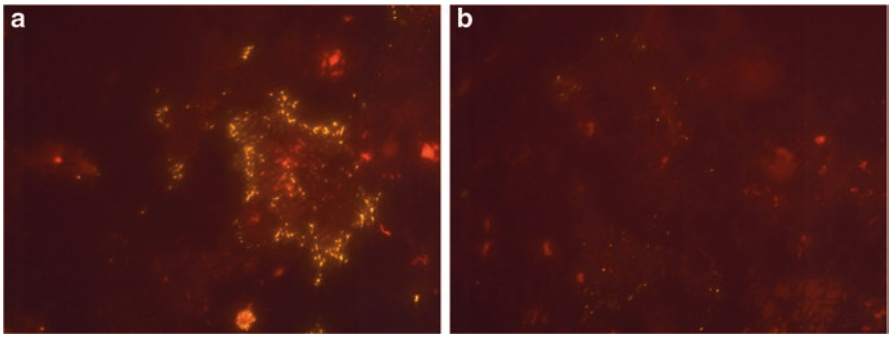

Fig 7.4 Mitochondrial superoxide determination using MitoSox. (**a**) Control cells. (**b**) Cells exposed to simulated oxidative stress

7.3 H9c2 as Preferred Model System

H9c2 (2–1) is basically an incompletely differentiated cell, of cardiac origin, therefore named cardiomyoblast. These were purified and established by B. Kimes and B. Brandt and exhibits many of the properties of the skeletal muscle (Kimes, Brandt, 1976). It is a subclone of the original clonal cell line obtained from embryonic heart tissue of BD1X strain of rat. On terminal differentiation these cells fuse to form multinucleated myotubes and also respond to acetylcholine stimulation showing rhythmic contraction. Fusion occurs faster if the serum concentration in the medium is reduced to 1 %. These cells can be successfully cultured on Dulbecco's Modified Eagle's Medium (DMEM) – a commonly used cell culture media. To make the complete growth medium, DMEM is added with 10% foetal bovine serum, 1% amphotericin and penicillin and streptomycin. CO_2 incubators are used for culturing these cells which maintain of 95% air, 5% carbon dioxide (CO_2) and 37 °C temperature. These cells behave well with the artificial stimulation and different type of oxidative stress simulations. Various studies at the author's lab and many other research labs worldwide have demonstrated the use of H9c2 for oxidative stress-related studies (Fig. 7.5).

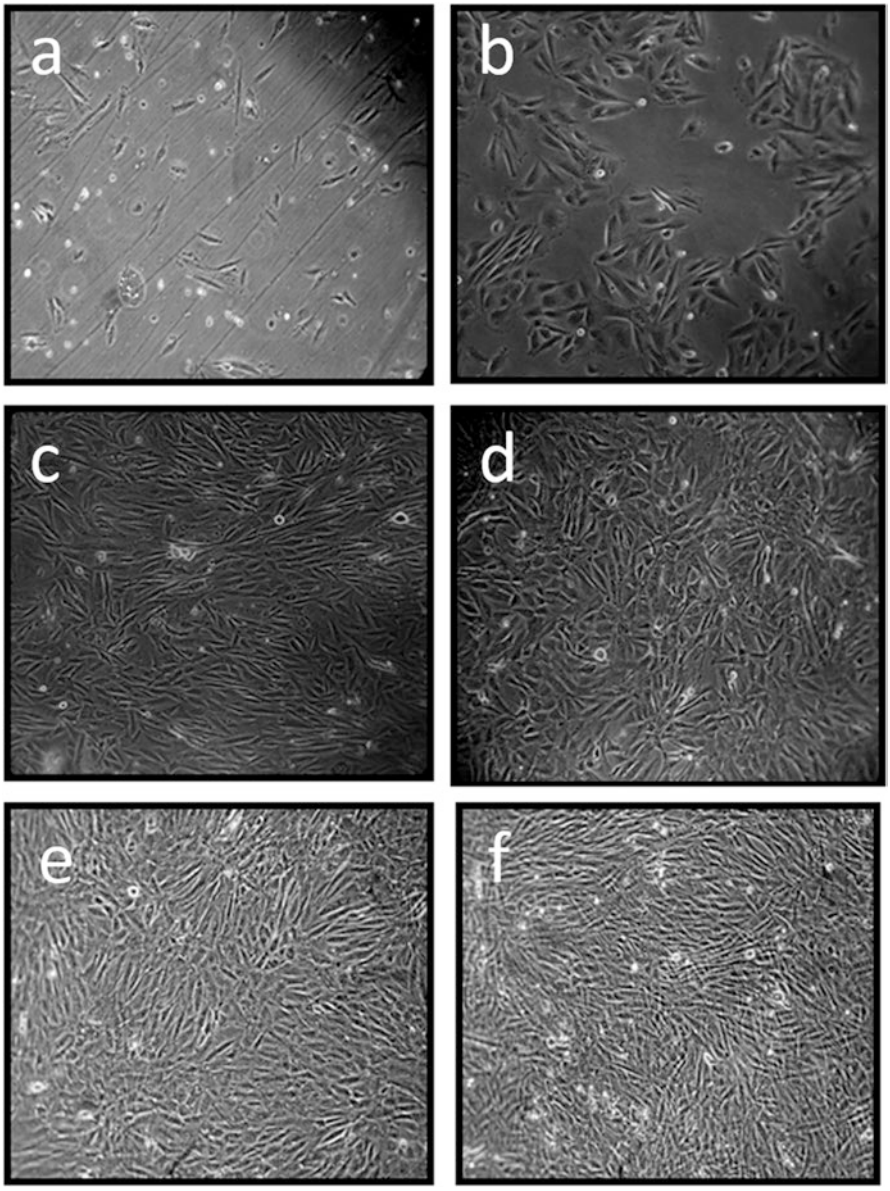

Fig. 7.5 Different stages of growth of H9c2 cardiomyoblasts when grown in DMEM. Phase contrast, 10 X objective. (**a**) Cells after 24 h of seeding start differentiating. (**b**) Rapid division begins after 48 h–72 h. (**c**) Sub-confluence after 72 h. (**d**) Confluence after 4 days. (**e**) Over confluent cells beyond 4 days. (**f**) Cells form overlapping layers if grown beyond 1 week

7.4 Oxidative Stress Biomarkers in Plasma

In most of the cases, for clinical reasons and ethical issues, it is not possible to obtain any type of sample for analysis of oxidative stress and reactive oxygen species (ROS), and therefore some of the non-invasive and semi-invasive techniques are used. Plasma is one of the most preferred samples among biological fluids to determine various pathophysiological conditions. Although aforesaid biochemical methods may be used to analyse the oxidative stress and therefore underlying mechanisms of the oxidative stress induced pathology, but a deeper insight may also be obtained by global proteomics profiling of the plasma samples. Studies at the author's lab have already shown that several proteins that play active role in direct or indirect scavenging of reactive oxygen species are altered during environmental stress. Therefore classical proteomic analysis is also an additional augmentation.

7.5 Future Prospects

Based on the shortcoming of existing methods that they are not highly specific for a particular type of radical and also the life time of the radical is a small fraction of second, it is important to look forward to develop newer methods which could be robust, less time-consuming and cost-effective. Nevertheless, classical methods which predominate the oxidative stress research need to be well understood for their merits and disadvantages before making a choice for experiments. A number of standard methods for detecting the antioxidant potential of the cells that include immunoblotting of the protein involved in antioxidant defence (such as catalase, glutathione peroxidase, thioredoxin reductase, etc.) are often used to determine the status of antioxidant proteins in the cells. Additionally, biological activity using spectrometry-based kinetic methods for antioxidant enzymes or non-enzyme antioxidants such as glutathione is gaining interest. However, there is not any integrated index for overall representation of total oxidative stress level or antioxidant defence of a biological sample. A few labs and commercial manufactures are working on development of such index. Such index may have score for each of the key parameters of oxidative stress and antioxidant defence based on their contribution, ultimately a net value of this index would be a measure to represent the antioxidant potential of the cell or a biological sample. The use of standard antioxidant systems for reference such as Trolox or ascorbate also needs to be revised, and more efficient and sensitive antioxidants must be included in the common antioxidant practice. Cerium oxide nanoparticles have recently emerged as important antioxidants and therefore can be evaluated for their use as reference antioxidants.

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Oxidative Stress-Mediated Human Diseases

8

Arti Srivastava and Ashutosh Srivastava

8.1 Introduction

Oxidative process is regularly going on in the cell, and it is crucial for life and death of a cell. Living system encounters with various stresses through their interaction with environment. Endogenous productions of reactive oxygen species (ROS) and reactive nitrogen species (RNS) are major consequences activated by living organisms. To fight with the oxidative stress caused by ROS and RNS, animal and human cells have developed a ubiquitous antioxidant defense system consisting of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione reductase (GR) together with lot of other low-molecular-weight antioxidants such as ascorbate, α-tocopherol, glutathione (GSH), etc.

When there is balance in between prooxidants and antioxidants, it may have many beneficial biological functions such as apoptosis, necrosis, and phagocytosis. However, inconsistency generates a pathological condition illustrated by increased levels of intracellular ROS and perturbation of antioxidant defense system. Oxidative stress is developed when imbalance between prooxidants and antioxidants shifted in favor of oxidants which results in oxidative damage and various diseases (Monteiro et al. 2013; Saliu and Bawa-Allah 2012; Huang et al. 2010). These reactive oxygen species are involved in generation of free radicals. Free radicals are the molecules having unpaired electrons, known as electrophiles. Free radicals cause an electrophilic attack on biological macromolecules like lipids, proteins, and nucleic acids, along with small molecules and biogenic amines. Reactive oxygen species comprises hydroxyl radicals ($^{\bullet}OH$), superoxide anion radicals ($O_2^{\bullet-}$), hydrogen peroxides (H_2O_2), alkoxyl radicals (RO•), singlet oxygens (O_2^1), and lipid hydroperoxides (LOOH) in living cells (Cao et al. 2010; Huang et al. 2010; Firat et al. 2009; Ruas 2008). Oxidative stresses whether result in direct production of ROS or it acts

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indirectly by binding with cellular thiols and reducing antioxidant potential. ROS formation results in destruction of lipids, proteins, and DNA, consequently changing the structure and function of biological membranes and ultimately leading to cellular dysfunction and cell death (Ozkan-Yilmaz et al. 2014; Cirillo et al. 2012; Monteiro et al. 2010).

8.2 Production of ROS in Cell

Reaction of unpaired electrons of molecular oxygen resulted into formation of reactive oxygen species which can be produced from both enzymatic and nonenzymatic sources (Chen et al. 2012; Koevary 2012). Reactive oxygen species are highly reactive because of the presence of unpaired electrons in their outer orbit, generated by oxygen metabolism. All aerobic organisms produce ROS during their cellular metabolism. ROS, generated by enzymatic sources under subcellular levels, includes cyclooxygenases (COX), xanthine oxidase, NO synthases, mitochondrial oxidases, and lipoxygenases (LOX) (Lambeth 2004). Nonenzymatic source for generation of ROS primarily includes Fenton's and Haber's reactions.

Cellular organelles which are associated with production of ROS in cell are:

(a) Mitochondria
(b) Endoplasmic reticulum
(c) Phagocytosis
(d) Other sources

8.2.1 Generation of ROS in Mitochondria

Mitochondrion is the hub of biochemical reactions, where ROS is generated by release of electrons by electron transport chain. Normally, mitochondrial electron transport chain causes the transfer of electrons for reduction of oxygen to water, but nearly 1–3% of all electrons leak from the system and produce superoxide ($O_2^{\bullet -}$). Superoxide dismutase (SOD) causes the formation of less toxic hydrogen peroxide moiety (H_2O_2) from toxic superoxide radicals. However by Fenton's reaction, hydrogen peroxide is converted to most reactive hydroxyl radicals when it interacts with ions such as iron and copper (Kowaltowskia et al. 2001).

8.2.2 Generation of ROS by Endoplasmic Reticulum

Cytochrome P450 reductase in endoplasmic reticulum is used to detoxify hydrophilic compounds. NADPH and NADH provide electrons for reduction of cytochrome P450 and cytochrome b5 in the process of detoxification (Shafaq 2012).

8.2.3 Generation of ROS by Phagocytosis

Production of reactive oxygen species in phagocytosis occurs when bacteria engulf the phagocytic cells. NADPH gives electron to the $NADP^+$ by the enzyme NADPH oxidase via Cytochrome b_{245} and produced ROS.

8.2.4 Generation of ROS by Other Sources

Other sources for reactive oxygen species are as follows: apoptosis, auto-oxidation of small molecules, hydrogen peroxide generation by peroxisomes, and reactive oxygen species generation by lysosomes (Shafaq 2012).

8.3 Antioxidant

Usually, at normal physiological condition, organisms have a natural defensive mechanism to counteract the impact of reactive oxygen species by sustaining a balance between its generation and neutralization. This equilibrium is maintained by various antioxidants (Tanekhy 2015; Monteiro et al. 2013; Cao et al. 2010).

Antioxidants are the molecules that inhibit the oxidation of vital molecules by interacting with free radicals to terminate the chain reaction. Antioxidants of a living system may be categorized into two types. One is explained as enzymatic antioxidants such as superoxide dismutase (SOD) and catalase (CAT), and the other one is glutathione-dependent enzymes such as glutathione-S-transferase (GST), glutathione peroxidase (GPx), and glutathione reductase (GR) which convert the reactive oxygen species into less toxic form in the presence of glutathione.

Another group of antioxidants is nonenzymatic antioxidants such as vitamin C, reduced glutathione (GSH), and metallothionein (MT) which neutralizes the ROS by directly scavenging the free radicals by oxidizing themselves (Birben et al. 2012). These groups of antioxidants have usually low molecular weight and may be lipid or water soluble. In biological systems ideally the oxidation process is balanced by the presence of natural antioxidants.

Mode of action of antioxidants can be divided into following categories (Symons and Gutteridge 1998):

(a) Removing oxygen or decreasing the O_2 concentrations
(b) Eradicating catalytic metal ions
(c) Neutralizing the reactive oxygen species such as $O_2^{\bullet-}$ and H_2O_2
(d) Scavenging initiating radicals such as $^{\bullet}OH$, $LO^{\bullet}$, and $LOO^{\bullet}$
(e) Breaking the chain of an initiated sequence

Antioxidants act synergistically with one another by preventing the initiation and propagation step to detoxify the effects of lipid peroxidation.

8.4 Cellular Response of ROS

Increased level of ROS can cause carbohydrate oxidation resulting in ketoamines and ketoaldehydes. ROS also attack on the protein molecule. ROS interacts on protein molecule at the specific amino acid side chains and changes the protein structure results in fragmentation of the peptide chain. ROS can also break the DNA strand. Sugars and base moieties are degraded by ROS and cause oxidation of bases and cross-linking to protein. DNA-MDA adducts is the most characteristic feature of nucleic acid oxidation (Noori 2012).

Lipids play a crucial role in various physiological functions of organisms, and due to rich in double bonds, they are more susceptible to damage by free radicals. Plentiful polyunsaturated fatty acids present in cell membrane are main targets to free radical attack. Lipid peroxidation, a well-established mechanism of cellular injury, is a consequence of oxidative damage of polyunsaturated fatty acids (PUFA) leading to formation of lipoperoxyl radical ($LOO^{\bullet}$). Lipoperoxyl radical reacts with new molecule of lipid to yield a lipid radical. Lipid radical forms peroxyl radical when reacts with oxygen. Lipid hydroperoxides are formed by peroxyl radical to initiate a chain reaction for transforming polyunsaturated fatty acids (Metwally and Fouad 2008; Faix et al. 2005; Barrera 2012). Lipid hydroperoxides are very unstable; therefore, it is decomposed to secondary products, such as malondialdehydes (MDAs) and other aldehydes such as alkanals, hydroxyalkenals, and ketones. Peroxidation of lipids interrupted with the integrity of cell membranes and results to rearrangement of membrane structure (Viarengo 1989; Barrera 2012). Likewise, increased ROS are detrimental and play a crucial role in acceleration of aging and age-related disease and also lead to cell death. Additionally elevated level of ROS also creates a stress signal which activates specific redox signaling pathways. After activation these diverse signaling pathways may have either damaging or protective effects (Finkel and Holbrook 2000).

Superoxide dismutase (SOD) is a metalloenzyme, acting as primary defensive mechanism against generation of superoxide by oxygen metabolism. Superoxide dismutase catalyzes the conversion of highly reactive superoxide anion ($O_2^{\bullet-}$) to far less reactive product hydrogen peroxide (H_2O_2) and oxygen (O_2) (Fridovich 1995; Bertini et al. 1998; Symons and Gutteridge 1998). Superoxide dismutases (SODs) are critical for the protection of the cell against the toxic reactive molecules produced by aerobic respiration of living organisms.

Glutathione (GSH) (L-γ-glutamyl-cysteinyl-glycine), a ubiquitous tripeptide, is by far the most important antioxidant in the upmost mammalian cells. Glutathione is rich in soluble cellular thiol, acts as a potent-reducing agent, and performs various cellular functions (Apel and Hirt 2004). Usually very low concentration of glutathione is present in the cell. It is extensively synthesized in cytoplasm and distributed into intracellular organelles such as mitochondria, nucleus, and endoplasmic reticulum. Most of the glutathione in mitochondria is in reduced form and comprises only 10–15% of glutathione among the total cellular glutathione content. Whereas considered to volume of the mitochondrial matrix, concentration of total mitochondrial glutathione (mGSH) is like to that of cytosolic glutathione (Mari et al. 2009;

Garcia-Ruiz et al. 1994). Nucleus contains glutathione in critical protein sulfhydryl form required for both repair and expression of DNA (Valko et al. 2007). Glutathione performs a crucial role in DNA synthesis by catalyzing the reduction of ribonucleotides to deoxyribonucleotides (Holmgren 1977). Glutathione is primarily found in its reduced form, except in the endoplasmic reticulum, where it occurs primarily as oxidized form (GSSG), because oxidized glutathione is a foremost source of oxidizing equivalents hence favors the disulfide bond formation and right folding of nascent proteins (Chakravarthi et al. 2006; Hwang et al. 1992). Glutathione participates in various processes which are essential for metabolisms of proteins, deoxyribonucleotides synthesis, regulation of several enzymes, and protection of cells against reactive oxygen species. Some of the enzymes such as glutathione peroxidase (GSH-Px), glutathione reductase (GR), and glutathione-S-transferase (GST) are considered as glutathione-dependent enzymes because they used glutathione (GSH) as a cofactor. In above mentioned enzymes, first two are antioxidant defense enzymes, while GST is a phase II biotransformation enzyme (Perendija et al. 2007). Glutathione has a major role in scavenging of the reactive oxygen species to maintain the cellular redox status. It exists both in reduced and oxidized form. In reduced state, cysteine group of glutathione is able to donate its reducing electron to reactive oxygen species and converted itself to reactive glutathione which react with another reactive glutathione and ultimately form oxidized glutathione by selenium-containing GSH peroxidase. Reduction of hydrogen peroxide in the presence of reduced glutathione and glutathione peroxidase is combined with oxidation of glucose-6-phosphate and of 6-phosphogluconate, which provides NADPH for conversion of formed oxidized glutathione into reduced glutathione. This conversion is done in the presence of glutathione reductase enzyme (Lu 2013). Hence, it constitutes a major pathway for the hydrogen peroxide metabolism in cells; therefore, glutathione peroxidase enzyme plays a key role for the protection of membrane lipids against oxidation. Generated hydrogen peroxides also can be reduced into molecular oxygen and water molecule by another important enzyme catalase (CAT). Catalases are primarily present in the peroxisomes (Scibior and Czeczot 2006; Switala and Loewen 2002). Thus, humans have evolved with important antioxidants such as superoxide dismutase (SOD), reduced glutathione (GSH), and catalase (CAT) which majorly participates in the elimination of superoxides, hydrogen peroxides, and hydroxyl radicals to protect against free radicals.

8.5 Oxidative Stress-Mediated Human Diseases

There are several human diseases such as atherosclerosis, cancer, myocardial infarction, rheumatoid arthritis, and neurodegenerative diseases such as Huntington's disease, Alzheimer's disease, and Parkinson's disease which are associated with oxidative stress (Chaitanya et al. 2010; Zhang et al. 1999). The role of oxidative stress for the pathogenesis of these diseases was monitored by evaluation of production of reactive oxygen species (ROS), reactive nitrogen species (RNS), and antioxidant defense level (Yudoh et al. 2005). Irradiation and hyperoxia also induce oxidative stress and are

likely to be responsible for age-related development of cancer. Vasavidevi et al. (2006) found the production of reactive oxygen species and nitrogen species with infection by *Helicobacter pylori* in the human stomach is an important factor for the development of gastric cancer. Several other diseases also have been reported by various investigators caused by generation of reactive oxygen species (Haydent and Tyagi 2002; Peña-Silva et al. 2009; Vasavidevi et al. 2006; Verzola et al. 2004).

8.5.1 Oxidative Stress-Mediated Neurodegenerative Diseases

Neurodegenerative diseases comprise a condition where nerve cells from the brain and spinal cord are functionally lost or there is sensory dysfunction (dementia). Parkinson's disease (PD), Alzheimer's disease (AD), multiple sclerosis (MS), and amyotrophic lateral sclerosis (ALS) are the neurodegenerative disease in humans.

8.5.1.1 Parkinson's Disease (PD)

Parkinson's disease (PD) is a neurodegenerative disease characterized by the loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) (Dias et al. 2013) and deposition of intracellular inclusion bodies (Lewy bodies) of α-synuclein.

It has been observed by various authors that oxidative damage and mitochondrial dysfunction contribute to the cascade of events leading to degeneration of these dopaminergic neurons (Schapira and Jenner 2011; Zhu and Chu 2010; Parker et al. 2008; Jenner and Olanow 2006). Dopamine is a neurotransmitter and also a very good metal chelator and electron donor that set in vivo conditions for redox metal chemistry to generate free radicals. Mutation in α-synuclein protein has a role in modulating the dopamine activity in a negative way.

A characteristic feature of the neurons within the substantia nigra is the age-dependent accumulation of neuromelanin. Neuromelanin is a dark brown pigment that accumulates metal ions, particularly iron. It is known that it consists primarily of the products of dopamine redox chemistry.

8.5.1.2 Alzheimer's Disease

Characteristic feature of Alzheimer's disease (AD) is deposition of amyloid plaques chelating amyloid-β peptide (Aβ) with transition metal ions (Cu^{2+}, Zn^{2+}, and Fe^{3+}). Binding of Cu^{2+} and Fe^{3+} results in the production of OH free radicals. 4-Hydroxy-2,3-nonenal (HNE), acrolein, malondialdehyde (MDA), and F2-isoprostanes are important breakdown products of lipid peroxidation. Increased HNE level has been observed in AD (Selley et al. 2002; Butterfield et al. 2002).

8.5.1.3 Multiple Sclerosis (MS)

MS causes the demyelination of central nervous system. Unregulated iron metabolism and ROS generation are responsible for the pathogenesis of disease. High lipid content generated by myelin and oligodendrocytes invites massive accumulation of iron and other metals as redox metals function as catalytic center for the lipid. Iron

plaque deposited over myelin sheath invokes as inflammatory response causing substantive damage and demyelination to CNS (Uttara et al. 2009).

8.5.1.4 Amyotrophic Lateral Sclerosis (ALS)

ALS is characterized by the loss of the lower motor neurons of the spinal cord and upper motor neurons in the cerebral cortex due to deposition of misfolded protein in neural tissue in relation with mutated SOD enzyme associated with Cu/Zn redox metallobiology (Bruijn et al. 1998). Mutation in SOD is responsible for the loss of active sites for Cu binding that leads to conversion of SOD itself in prooxidant protein that participate in ROS generation.

8.5.2 Oxidative Stress-Mediated Cardiovascular Diseases

Several studies reported that oxidative stress plays an important role in the parthenogenesis and development of cardiovascular diseases, including hypertension, dyslipidemia, atherosclerosis, myocardial infraction, angina pectoris, and heart failure (Rahman et al. 2012).

8.5.2.1 Coronary Heart Disease (CHD)

Hyperlipidemia (cholesterol, LDL, etc.), hypertension, cigarette smoking, diabetes, overweight, physical inactivity, etc. are some of the traditional vascular risk factors, but many studies also support the role of oxidative stress in disease pathogenesis. Paradoxically, regular endurance exercise results in the improved cardiovascular function and a reduction in traditional CHD risk factors due to activation of signaling pathways that lead to increased synthesis of intracellular antioxidants and antioxidant enzymes and decreased ROS production during exercise.

8.5.2.2 Atherosclerosis

Atherosclerosis is a complex process involving the development of plasma lipoproteins and the proliferation of cellular elements in the artery wall. This chronic condition advances through a series of stages leading to the atherosclerotic plaques formation. Studies suggest that free radical-mediated oxidative processes and its specific products are responsible for atherogenesis.

Stroke and *obesity* are also linked with oxidative stress. Various hypotheses for these linkages have been strongly supported by various studies (Rahman et al. 2012). Since chronic hyperglycemia is more prevalent on obese individuals, oxidative stress is also believed to play a major role in the development of obesity-related disorders including *diabetes* and *hypertension*.

8.5.3 Asthma

Several studies suggest that oxidative stress plays an important role in the pathogenesis of asthma. Uncontrolled oxidants may cause airway allergic inflammation in

the initial phase. Furthermore, enhanced oxidative stress may contribute to the progression of existing airway inflammation through enhanced airway hyperresponsiveness, stimulation of mucin secretion, and induction of various proinflammatory chemical mediators, all of which are believed to be linked with severe asthma (Rahman et al. 2012). Controlling intracellular oxidative stress is important for effectively managing bronchial asthma.

8.5.4 Lung Cancer

Some studies suggest that ROS stimulate the oncogenes such as Jun and Fos and overexpression of Jun is directly responsible for lung cancer (Szabo et al. 1996; Volm et al. 1994). In lung cancer p53 is often mutated and defective in inducing apoptosis. Mutated p53 accumulates in the cytoplasm and acts as oncogene (Stewart and Pietenpol 2001).

8.5.5 Other Diseases

Oxidative stress has been associated in the pathogenesis of several eye conditions such as *cataract, macular degeneration, diabetic retinopathy, retinitis pigmentosa, corneal disease,* etc. (Rahman et al. 2012). *Skin diseases* are also associated with oxidative stress. UV-induced generation of ROS in the skin develops oxidative stress and causes many skin diseases.

Oxidative stress has been proven as one factor affecting *fertility* status. Several studies have examined the role of oxidative stress and *pregnancy complications* (reviewed by Rahman et al. 2012). ROS have been linked with the development of premature rupture of the fetal membranes (Plessinger et al. 2000; Bilodeau and Hubel 2003). Oxidative stress is also responsible for blood disorders like *beta-thalassemia* and *acute lymphoblastic leukemia* (ALL) and joint disorder like *rheumatoid arthritis*.

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Potential Applications of Antioxidants from Algae in Human Health

9

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9.1 Introduction

Ageing is a very complex process that leads to various physiological changes due to multiple environmental and lifestyle changes. Initially it is harmless as our body is trained to tackle all these small damages, but it does not have the potential to handle these impacts for infinite period. Oxidative stress and production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) are the major reasons for various age-related disorders such as neurodegenerative disorders, types of cancers and diabetes (Collier et al. 1990; Boynes 1991). Oxidative stress plays a significant function in their pathogenesis, and this may bring about certain changes in the oxidative stress biomarkers such as malondialdehyde (MDA), superoxide dismutase (SOD), glutathione, etc. These biomarkers are excellent tool that can be used as an indicator to analyse the pathological or control conditions. Different biomarkers have varied characteristics according to disease state, disease trait and rate of the disease. As per global ageing index, approximately 22% of the total population will be 60+, i.e. 2031 million (http://www.helpage.org/global-agewatch/population-ageing-data/global-ageing-data/). This will have a huge impact on healthcare and needed infrastructure cost (http://www.nia.nih.gov/sites/default/files/global_health_and_aging.pdf).

The age-related disorders come with pain and suffering. The fundamental regulators of age-related diseases are characterised by biochemical and cellular deregulation, environmental toxins, telomerase activity in cancer cells, intracellular as well as extracellular junk, false/weak antioxidant defence mechanism, oxidative stress, etc. Recent advancements in antioxidant technology and its potential to inhibit ageing through many pathways have increased the lifespan to some limit. Imbalance between free radicals and antioxidants creates instability in most of the physiological functions. So to provide the balance between reactive species and

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antioxidants, thus intensive research is being conducted on external antioxidant supplementation to tackle oxidative stress/reactive species/free radicals (Harman 2003). Advancements in free radical theory and antioxidant production technologies are providing with revolution in safe health management (Aruoma 2003). Enough evidence has been provided to use natural antioxidants that can be used in chemotherapy and as oxidative inhibitors (Halliwell and Gutteridge 2007; Guerin et al. 2003; Kohen and Nyska 2002; Xue et al. 2004). In already synthesised antioxidant molecule like butylated hydroxyanisole (BHA), butylated hydroxytoluene is in use to inhibit oxidative stress, but it comes along with many side effects such as liver injury and carcinogenesis (Namiki 1990). Therefore, it is very important to develop the natural and safe antioxidant that has free radical scavenging activity without any negative effects (Li et al. 2007). Antioxidant market has grown in billion dollars due to its huge potential in controlling age-related problems. Natural antioxidant substances, as a nutritional supplement, signify a new boundary in prevention and cure of various diseases including cardiovascular diseases. Scientific fraternity encourages the use of phytochemicals and naturally occurring antioxidants against some inflammatory and chronic diseases. Many researchers are supporting the use of algae for producing natural antioxidants, those are non-toxic to human and can be easily administered (Natrah et al. 2007; Lee et al. 2010). Algae can be found everywhere in the surface with different and harsh climate zones; basically it is found where the process of photosynthesis is possible (Dixon 1973). They are the most versatile photosynthetic organism with great diversity and many shapes. Algae are known to contain many antioxidants like ascorbate, glutathione (GSH) and secondary metabolites, for example, carotenoids, catechins, tocopherols, etc. (Spolaore et al. 2006; Takaichi 2011). The capacity to produce wide range of antioxidant molecules has raised the interest of algae in food, health and medicine industries (Pulz and Gross 2004). Algal antioxidants have prominent role in regulating the carcinogenesis at later stages, regression of premalignant cancer, cardiovascular diseases, enhancing eye health, and increasing muscle strength and are also an excellent anti-inflammatory and immune protective agent. The regular metabolic process such as oxidative metabolism in alga generates large amount of by-products in the form of ROS such as superoxide anion, H_2O_2 and singlet oxygen possessing big threat to these aerobic organisms. These elements are responsible for damage of various cellular parts and inactivate enzymes. The imbalance between free radicals and antioxidant system creates oxidative stress that leads to changes in the biomolecules like lipids, proteins and nucleic acid. Excess of ROS has been linked with progression of many chronic disorders such as atherosclerotic processes, myocardial and cerebral ischemia, renal failure, rheumatoid arthritis, inflammatory bowel disease, retinopathy of prematurity, asthma, Parkinson's disease, kidney damage, preeclampsia, etc. It has also been reported that some of the species function as signalling molecules to alter gene expression and activate defence proteins (Vranová et al. 2002). To overcome negative effects of this global phenomenon, algae and other organisms have evolved and developed the antioxidant defence system to neutralise the active oxygen species. The clear understanding of the enzymes and working of antioxidant defence system are not yet proved; therefore, comparative study of different types of transgenic algae can provide detailed information. There is

requirement to understand the route for the generation of antioxidants, their effects and applications in treatment of diseases.

9.2 Metabolic Generation of ROS in Algae and Defence Mechanism

All aerobic cells produce various forms of free radicals or reactive oxide species (ROS) such as superoxide anion, hydroxyl radical, singlet oxygen and hydrogen peroxide as by-products of oxidative stress. In some of the reports, it has been reported that ROS also plays a significant role in signalling and cell adaptation by regulating gene expression and inactivating the defence proteins (Vranová et al. 2002) but if generated in large amount leads to adverse conditions like organelle dysfunction, mutagenesis and changes in cell structure (Halliwell and Gutteridge 1999). In a photosynthetic system such as plants and algae, ROS are produced due to extensive environment stress such as high light, drought, high salt concentration, temperature or heavy metal stress, etc. It is very important to understand the mechanism of ROS generation and its defence mechanism in algae because it is the primary producer in oceans and inland waters that plays a significant role in securing man's future and life on earth. Its presence in wide range of environments offers a very unique model to study that can be used to understand various problems related to evolution, physiology, genetics, biotechnology, sustainable products, etc. (Butow et al. 1997; Raven et al. 1999). With time, algal cells have increased the strength of its defence and antioxidant mechanism system via enzymatic and non-enzymatic strategies. Enzymes such as SOD, POD, CAT, etc. contain several mettaloisoenzymes that can neutralise the harmful effects of ROS. The presence of trace metals such as Mn-SOD in chloroplasts and Fe-SOD in mitochondria is very important for defence mechanism as chloroplasts and mitochondria are quite susceptible to oxidative damage. Catalase is also present in algae in large amount and has been found to be an effective mechanism in degrading the effects of H_2O_2. In a study of Rady et al. (1994), the algae growth was noticed at 36 °C, 20 °C and 43 °C to understand the trend of SOD activity. They observed that at 20 °C there was prominent increase in SOD and catalase activity whereas decrease in SOD activity at higher temperature. Similar trend was observed in catalase activity of cyanobacterium *Synechocystis* PCC 6803 (Lesser and Stochaj 1990). There are many low molecular weight compounds such as carotenoids that are naturally occurring in various aquatic microbes, animals and humans that have shown antioxidant potential. (Britton et al. 1995). They are light-harvesting proteins that protect the photosynthetic machinery from excess radiations by energy dissipation (Frank and Cogdell 1996; Krinsky 1989; Pinto et al. 2003, Woodall et al. 1997). Fucoxanthin and peridinin located in chloroplasts protect the light-harvesting pigments by inhibiting the photochemical damage. Peridinin neutralise the mutagenic and DNA-damaging effect of excited molecules of O_2 by quenching (Di Mascio et al. 1990; Hollnagel et al. 1996). It has been reported that quenching is directly proportional to conjugated double bonds which holds true for peridinin whose quenching efficiency is ten times less than B-carotene (Foote et al. 1970). Pinto et al. (2003) analysed the

pigments by HPLC in dinoflagellates *Lingulodinium polyedrum* which showed that total concentration of peridinin was more than B-carotene and therefore contributes significantly in quenching the free radicals (Pinto et al. 2000). Ascorbate is an important enzyme electron donor to OH (radical) as there is no alternative neutralising mechanism. The degradation of H_2O_2 in chloroplasts is done by the activity of ascorbate peroxidase as there is absence of catalase enzyme (Tanaka et al. 1982). Ascorbate is an important antioxidant to protect the photosynthetic apparatus and to maintain the continuity of cycling of ascorbate between oxidised and reduced forms (Hardeland et al. 1995; Rodriguez et al. 2004; Hardeland et al. 2003). A study in a fresh water alga dinoflagellate *Peridinium gatunense* observed that the ascorbate level was doubled when cells were treated with irradiance from 60 to 600 J.l.mol photon m-2 S-1 (Barros et al. 2001; Yan et al. 1999).

9.3 Ways to Generate Oxidative Stress in Algae and Their Possible Effects

Several ways can be utilised to generate the oxidative stress in photosynthetic microbes like algae, and the effects of these parameters are discussed as given in Fig. 9.1.

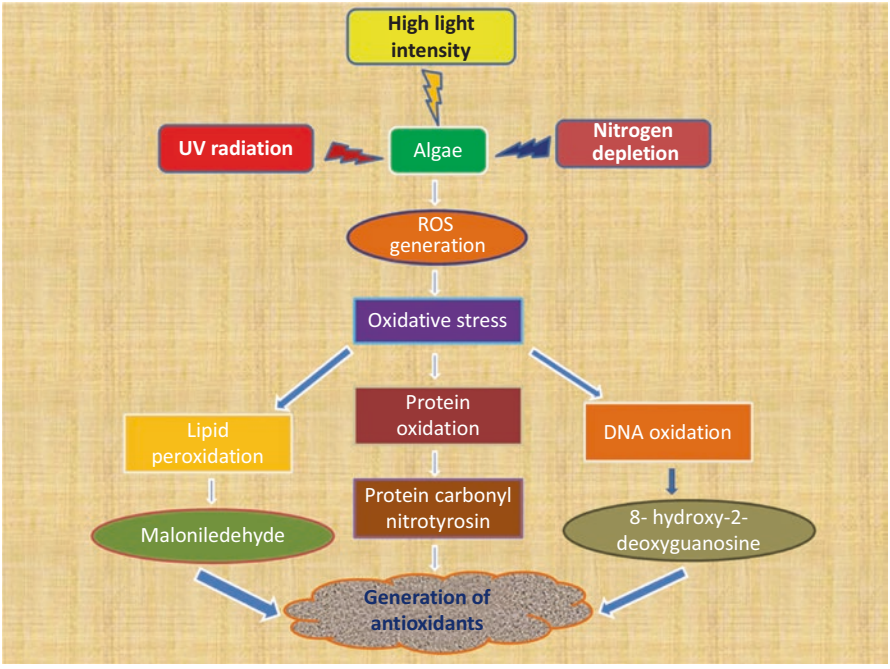

Fig. 9.1 Flow chart describes generation of ROS in algae and consecutively production of antioxidants

9.3.1 UV Radiation

The stratospheric ozone layer acts as a cover over the earth's atmosphere and is the most important agent to absorb the ultraviolet radiation. However, the increase in human and industrial development led to depletion of ozone layer and enhanced the level of UV radiation on the earth surface (Seckmeyer and McKenzie 1992). Exposure of UV-B stimulates the generation of ROS and enhances the oxidative stress in the cell. Generally the oxidative stress results the formation of superoxide, hydrogen peroxide and hydroxyl radicals, respectively, by transferring electrons (Halliwell and Gutteridge 1989). This ROS generates the oxidative destruction of the cell components through oxidative damage of membrane lipids, proteins and nucleic acids like DNA (Imlay and Linn 1998). Antioxidants like ascorbate, glutathione (water soluble), alpha tocopherol and carotenoids (water insoluble) have been considered to be non-enzymatic agent for searching ROS. Furthermore, the enzymatic ROS scavenging pathways produce superoxide dismutase (SOD) which converts O_2^- to H_2O_2 and then ascorbate peroxidase and glutathione reductase in the ascorbate glutathione cycle utilised for H_2O_2 removal. Antioxidant defence mechanism is the first line of defence strategy of algae and plants to inhibit the harmful effects of free radicals. It has been reported that the stress tolerance in algae increases with antioxidant content and antioxidant enzyme activity (Butow et al. 1994).

9.3.2 Nitrogen Depletion

To the best of our knowledge, the stress condition enables the reduction of biomass productivity but increases the lipid accumulation in algal cells (Li et al. 2008). So, it is quite obvious that the biomass production and lipid content are negatively co-related. However, this problem has been solved in *Oocystis sp*. and *Amphora sp*. by addressing the nitrogen stress in a biphasic reactor system where, in first phase, the algae were supposed to grow in an optimum culture condition for better biomass production, and in second phase, a nitrogen stress was given to those algal species for better lipid production (Csavina et al. 2011). Hence, nitrogen depletion strategy can be considered for enhancing the algal lipid accumulation (approx. 90%) till date.

However, the possible mechanism has not been explained in terms of its physiological and molecular aspects. Although oxygen is not harmful for algal cells, but the presence of ROS leads to oxidative damage and creates incompatible cellular environment (Alscher et al. 1997). The oxidative stress arises due to the redox reactions of the reactive oxygen species like H_2O_2, O_2^- or OH^- radicals with lipids, proteins, DNA, etc. It has been reported earlier that the ROS and lipid accumulation in algae are co-related with each other under nitrogen-depleted condition. Furthermore, the interconnection between the levels of ROS and lipid content has also been investigated in green microalgae. When the microalgae are exposed to various stress conditions, it results in an increase in ROS production that further enhances the cellular lipid content.

The integrity of algae life cycle is dependent on the rate of photosynthesis and cellular density; therefore it is essential to secure them against oxidative stress; otherwise cells are not able to survive the extreme conditions and ultimately lead to cell death. It can be concluded that ROS is well demonstrated to modify cellular responses against different stress in corresponding cell signalling pathways. So, the levels of ROS are increased in microalgal cells with various environmental stress conditions.

9.3.3 High Light Intensity

The survival and productivity of photosynthetic microbes like algae are majorly dependent on light intensity. Hence, under the high light stress, the photosystems imitate the balance of photoprotective mechanisms against photoinactivation and photo damage to the algal cells. High light intensity comprises the reduction of the antennae and both photosystem I (PSI) and II (PSII), while the quantities of antioxidants like zeaxanthin and various photo-repair mechanisms like amount of the D1 protein of PSII are increased. The light-harvesting antennae contain chlorophyll and carotenoid pigment – protein complexes that absorb photons and deliver excitation energy to photosystems. The enhanced deep oxidation state (DPS) of the xanthophyll cycle pigments in high light plays a major protective role and is often correlated with induction of non-photochemical fluorescence quenching (NPQ) (Li et al. 2000; Niyogi 1999). The role of xanthophylls and carotenoids like zeaxanthin is not only limited to the quenching of NPQ while they also help in the protection against lipid peroxidation (discussed in detail later).

9.3.4 Chemicals

Algae cells are highly susceptible by some chemicals like hydrogen peroxide, azides, cyanides, toluene, etc. These chemicals are responsible for degeneration of enzymes like superoxide dismutase, and as a result there is increase in synthesis of carotenoids in the algal cells against reactive oxygen species. The inhibition of SOD has been extensively noticed in *H. pluvialis* by the incorporation of HCN and H_2O_2 that triggers the synthesis of astaxanthin as defence mechanism (Kobayashi et al. 1997).

9.4 Role of Algal Antioxidants as Food Supplements and Human Health

The Table 9.1 shows a diverse range of antioxidants produced from algae and their applications in various industries that help to understand their emergence and functions properly.

Table 9.1 List of antioxidants, their algal source and potential function

Algal species	Types of antioxidants	Functions	References
Dunaliella salina, Chlorella ellipsoidea, Chlorella vulgaris	β carotene	Health food, natural colouring agent, additives to food, protecting tissues from chemical damage, cancer, other age-related disorders, inhibition of colon cancer	Raja et al. (2007), Halliwell and Gutteridge (2007) and Mattson (2004)
Haematococcus pluvialis, Chlorella sp.	Astaxanthin	Improving eye health, enhancing muscle strength and protecting skin from UV damage	Baker and Gunther (2004)
Chlorella sp.	Canthaxanthin, astaxanthin and lutein	Animal feed (aquaculture, cattle feed)	Plaza et al. (2009) and Cysewski and Lorenz (2004)
Cystoseira crinita	Prenyl toluquinones	Radical scavenging activity	Fisch et al. (2003)
Ecklonia stolonifera, Ecklonia cava	Phlorotannins	Radical scavenging activity	Kang et al. (2003) and Ham et al. (2007)
Hijikia fusiformis, P. tricornutum and Isochrysis	Fucoxanthin	Antiangiogenic and improves retinol deficiency	Sangeetha et al. (2009) and Yan et al. (1999)
Spirulina maxima, Taonia atomaria, Chlorella Vulgaris	Phenolic acids, tocopherols	Anti-proliferative	Nahas et al. (2007), Yuan et al. (2005), Miranda et al. (1998), Thomas and Kim (2011) and Hajimahmoodi et al. (2010)
Spirulina, Porphyridium	Phycocyanin (blue), phycoerythrin (red)	Natural colours and additives to cosmetics	Yabuta et al. (2010)
Chaetomorpha linum	Phenolics and flavonoids	Medicine, dietary supplements, cosmetics and food industry	Farasat et al. (2013)
Chaetomorpha aerea			
Chaetomorpha crassa			
Chaetomorpha brachygona			
Haematococcus pluvialis	Secondary xanthophylls like lutein and its stereo isomer zeaxanthin	Act as nutraceuticals against macular degeneration and maintaining normal visual function	Jin et al. (2003)

9.4.1 Carotenoids

Carotenoids are the fat-soluble fractions that are available in abundance which functions majorly as photoprotective agent to protect the algal photosynthetic machinery against high light stress conditions. These pigments are of high market value used as natural colouring agent, additives for cosmetics and health food (del Campo and García-González 2007). Carotenoids are distinctively divided in two types; primary carotenoids are the xanthophylls, which are essential functional and structural component of photosynthetic apparatus required for cell survival, whereas secondary carotenoids are produced under environment stress conditions via carotenogenesis pathways (Eonseon et al. 2003, Grossman et al. 1995). Many researchers have shown strong interest in carotenoids from microalgae for large-scale industrial application as the cost required for producing 1 kg of algal carotenoids is half of its synthetic counterparts. They constitute the largest fragment of algal cell that has distinctive chemical properties and functions such as light absorption. Carotenoids are generally hydrophobic in nature; therefore they are mostly located in membrane regions, bound to proteins of photosynthetic apparatus, or the synthesised carotenoids may be transported to cytoplasm. Carotenoids have shown incredible antioxidant potential especially against cancer and cardiovascular diseases that have made them the most popular health supplements. (Van Den Berg et al. 2000). Carotenoids absorb light and thus responsible for protection against high light intensity and photo-oxidative damage not only to phytoplanktons but also to the skin and eye. β-carotene and lycopene are responsible for skin protection whereas xanthophylls, zeaxanthin, and lutein ensure the protection of macula. About 700 carotenoids are developed from natural sources with diverse structures, and application of these in pharmaceutical can be a promising research in medical science. Many novel marine carotenoids, such as fucoxanthin, astaxanthin, zeaxanthin, and rare marine carotenoids such as sioxanthin, saproxanthin, myoxol and siphonaxanthin have gained the attention of academic and industrial research to exploit their potential in developing materials for pharmaceuticals and nutrition supplements to support healthy lifestyle and human welfare. (Gammone et al. 2015).

9.4.1.1 Fucoxanthin

Fucoxanthin is an important carotenoid mostly found in brown algae which has shown potential biological activities such as anti-obesity factors, anticancer role in prostrate and liver cancer, antitumour, antidiabetic and anti-inflammatory properties (Heo et al. 2010). The known sources of fucoxanthin are *Undaria pinnatifida*, *Hijikia fusiformis*, *Sargassum fulvellum*, etc. It has been confirmed by in vitro studies that fucoxanthin and their metabolites have free radical scavenging activities and inhibiting single oxygen (Sachindra et al. 2007). Presently, the researchers are trying to identify the major biosynthetic metabolic pathways of fucoxanthin synthesis which are very essential to understand the biological activities (Mikami and Hosokawa 2013).

9.4.1.2 Astaxanthin

It is a red ketocarotenoid belonging to xanthophyll category, known to prevent lipid peroxidation in cell membrane and support human health. It has been included in list of nutraceuticals by the United States Food and Drug Administration since year 1999 (Guerin et al. 2003). *Haematococcus pluvialis* is a very popular producer of astaxanthin, and the antioxidant has been commercialised as nutritional supplement (Hu et al. 2008). High antioxidant property was expressed in *Haematococcus pluvialis* cells (astaxanthin rich) against O_2^--imbedded cells, but the activity was not found in astaxanthin free cell extract. The results suggested that the level of astaxanthin gets an increase as it functions like an antioxidant against excessive oxidative stress. The quantity of oxidative enzyme superoxide dismutase is also enhanced against both permeabilised cells and cell extracts from vegetative cells. Astaxanthin provides protection against UV radiation and age-related disorders, also provides protection for the eyes and joints and protects phospholipids from peroxidation (Naguib 2000).

9.4.1.3 Zeaxanthin

Zeaxanthin is a 40-carbon hydroxylated compound identical to lutein, and it belongs to non-provitamin A carotenoid found in many algae such as *Rhodophyta* and *Spirulina sp.* (Holden et al. 1998; Hameed et al. 2014). Along with lutein, the photopigment zeaxanthin is involved to maintain the macular pigment density of the eye and also helps in inhibiting the age-related macular degeneration (AMD). Zeaxanthin helps to lower the oxidative damage of the eye as well as filter the damaging blue light (Mares-Perlman et al. 2002). Its function is very similar to α-tocopherol, reduces oxidised glutathione (GSSG) and supplements intracellular-reduced glutathione (GSH) level to overcome the deleterious effects of oxidative stress (Giblin 2000). Hence, zeaxanthin regulates the synthesis and level of GSH.

9.4.1.4 Other Rarely Found Carotenoids

Other carotenoid pigments like siphonaxanthin and saproxanthin are nowadays popular under antioxidant substances produced from green algae mainly marine. Edible green algae such as *Codium fragile*, *Caulerpa lentillifera* and *Umbraulva japonica* are rich in ketocarotenoid content such as siphonaxanthin that has light-harvesting function in underwater habitats (Sugawara et al. 2014; Wang et al. 2013). Recently, it has been found that it has pro-apoptotic effect and can efficiently inhibit human leukaemia HL-60. Further it is complemented with low level Bcl-2 expression, stimulation of caspase-3 and upregulation of death receptor 5 (DR5) expression (Ganesan et al. 2011). Saproxanthin was initially extracted from the bacterium *Saprospira grandis* but recently isolated from *Anabaena variabilis* and has inhibitory activity against lipid peroxidation induced by free radicals and is neuroprotective against l-glutamate toxicity on the neuronal hybridoma cell line (Shindo et al. 2004).

9.4.2 Phenolic Compounds

Phenols represent one of the major class of antioxidants with large number of variations in size and structures that offer protection against diseases like cancer, diabetes, neurodegenerative disorders, etc. These phenolic compounds are present in terrestrial plants, but enough data is not present in microalgae. Although, it has been reported that the content of these compounds increases with rise in UV stress period (Duval et al. 2000). Along with that, these compounds also help in reproduction, pigmentation and growth of plants. They are present in algal cell walls that are made of two aromatic rings with one or more hydroxyl groups and have reported to regulate reproduction, secondary ecological activities, therapeutic properties, etc. The market demand of phenolic compounds has increased tremendously because of its multifunctional properties. Even though, algae food products have high amount of total antioxidants, but there is very scarce data concerning the phenolic compounds. Recently, the comparison of extraction process of phenolic compounds from algae was done, and it was verified that extraction using distilled water is much better than extraction in different concentrations of methanol. Furthermore, it was reported that content of phenolic compounds varies extensively according to type of cultivation, species, geographical origin and other physiochemical variations (Ludmila et al. 2015).

9.4.3 Phycocyanins

Phycocyanin is one of the most studied pigment in algal antioxidants scientific research. It is blue coloured and water-soluble protein found in wide range of species but is mostly extracted from *Spirulina*, *Porphyridium cruentum* and *Synechococcus*. Phycocyanin has various applications in nutrition, artificial colour and cosmetics industry (Gupta and Sainis 2010; Viskari and Colyer 2003; Bermejo Román et al. 2002). With growing awareness against the harmful effect of synthetic colours, industries are choosing phycocyanins from algae and have also studied their impact on various products such as milk products, ice creams, chewing gums and deserts (Branen et al. 2001). These fluorescent pigments have also become popular choice among researchers for their extensive use in flow cytometry, fluorescence-activated cell sorting, histochemistry, etc. as fluorescent probes (Ayyagari et al. 1995). It has been reported by Nemoto-Kawamura et al. (2004) that phycocyanins inhibit the allergic inflammation and enhance the immune system to strengthen the biological defence system. It has also been reported to show significant results as anticancer, antidiabetic, anti-thromboembolic, hypolipidemic and antihypersensitive (Chiu et al. 2006; Ou et al. 2012; Han et al. 2006). In the last two decades, detailed study has been conducted from different angles such as chemical stabilization, protein engineering, recombinant proteins and purification methods (Dasgupta 2015).

9.4.4 Other Natural Pigments

Algae are rich in various other natural compounds and pigments such as chlorophyll, polysaccharides, vitamins and minerals. Chlorophyll is used as natural colouring agent usually extracted from spinach. El-Baky et al. (2007) reported that spirulina has 1.15 mg/g of chlorophyll whereas spinach has only 0.06 mg/g. Chlorophyll production varies according to composition of medium, light intensity and other physiochemical parameters. Research studies have shown that chlorophyll content increases with low light intensity and high nitrogen concentration. It is suggested to use the open ponds for fermentation to make it economically viable for industries. In addition to its role as natural colours, consumption of chlorophyll-rich diet prevents age-related disorders and inhibits the free radicals (Konícková et al. 2014). Algae have the potential to exhibit broad range of bioactive molecules including sulphated polysaccharide (SP). Considering the multivalue health prospects of SP, it has become a hot topic of research in different fields. Till now, its role in lipid lowering, nano medicine application, antiviral, antibacterial, antiprotozoan and hyperplasia prevention has been extensively studied. Researchers from worldwide are convinced to use the bioactive molecules from marine algae for various therapeutic potential (Senni et al. 2011). Many research findings have revealed that natural vitamins are more applicable than synthetic; therefore, it is very essential to focus on extraction of vitamins from natural sources (Kelman et al. 2012). Algae represents significant and balanced composition of vitamins (A, B1, B2, B6, B12, C, E, nicotinate, biotin folic acid and pantothenic acid) and mineral content (Na, K, Ca, Mg, Fe, Zn and trace minerals) (Baker and Gunther 2004). It has been proved that algae are rich in essential vitamins as compared to our traditional food (Jaime and Concepcion 1999). The use of these vitamins, as potential antioxidants, has already been studied extensively. Hence, algal cells rich in vitamins could be a great substitute in the reduction of oxidative stress in the prevention of various diseases.

9.5 Final Consideration

Algae bioactive compounds act as a clinical and therapeutic agent, and it helps in protection of the cells against oxidative stress. Algae carotenoids (astaxanthin, lutein), β-carotene, phycocyanins, phycobiliproteins and other pigments have opened the new path to deal with the cytoprotection and anticipation of various degenerative diseases. The progress and optimization of technologies based on antioxidant-producing algae require a thorough knowledge of physiology, biochemistry and genetic engineering. In this aspect, the genes which are involved for the synthesis of respective enzymes that are associated with antioxidant production will be more extensively investigated in the future.

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