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Review Article

Antioxidants, Radical Scavengers, and Their Impact on Oxidative Stress

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ABSTRACT

Antioxidants impede free radical chain reactions and thereby retard and prevent oxidative cellular damage. The present review provides an overview of the basic mechanisms by which antioxidants scavenge free radicals and describes the basic methods for measurement of antioxidant activities against free radicals as well as the underlying chemistry and pathologies linked with oxidative stress. This review was sourced online from scientific search engines using appropriate keywords. For appropriate measurement of antioxidant capacity, with respect to reaction mechanisms involved in the free radical reduction process, exclusive application of HAT, SET methods, and/or a combination of both HAT/SET methods should be considered. The human body is equipped with an antioxidant defence system to mitigate or prevent free radical-induced cellular damage through free radical scavenging mechanisms such as inhibition of enzyme activity involved in free radical generation, activation of intracellular enzymatic antioxidant activity, metal ion chelation, as well as inhibition of protein modification, DNA damage, and lipid peroxidation. Impaired antioxidant activity is associated with chronic diseases such as cardiovascular diseases, diabetes mellitus, inflammatory disease, cancer, cataracts, Alzheimer's disease, autism, and ageing. Overexpression of enzymatic antioxidants, as demonstrated in mutant mice models, could serve as a novel alleviative measure against the development of pathological conditions associated with oxidative stress.

Keywords: Antioxidants, Enzymatic antioxidants, Free radical, Lipid peroxidation, Oxidative stress.

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INTRODUCTION

Antioxidants impede free radical chain reactions and thereby retard and prevent oxidative cellular damage. Although antioxidant defences differ in diverse species, the antioxidant defence mechanism is universal. Antioxidants are enzymatic or non-enzymatic entities present in extracellular and intracellular environments.[1] Antioxidants are seen occurring naturally in plants, animals, and microorganisms or are synthesised using chemical methods. Higher plant constituents are rich sources of natural antioxidants, such as the polyphenols and tocopherols, which occur in large quantities in fruits, herbs, vegetables, spices, cereals, oils, grains, and seeds. Marine organisms, such as fish/shellfish, algae, and marine bacteria, were also reported as sources of antioxidants.[2,3] Furthermore, food and agricultural industrial byproducts, such as skins and shells of nuts, as well as cereals, fish viscera extracts, citrus peels, etc., have been considered as rich sources of antioxidants.[3-6] It is worthwhile to note that regular intake of dietary antioxidants is essential to sustain adequate antioxidant status.[7,8]

Free radicals are referred to as atoms, molecules, or ions that possess unpaired electrons and react very actively with other molecules. Biologically, free radicals arise from oxygen, sulphur, and nitrogen molecules and form parts of groups of molecules known as ROS, RSS, and RNS.[9]

High cellular levels of ROS cause harmful effects on various biological molecules such as protein, lipid, DNA, and RNA. Evidence has shown that ROS attack on macromolecules is one of several attributes of oxidative stress. Oxidative stress contributes to the development of CVD, DM, inflammatory disease, cancer,

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cataracts, AD, autism, and aging. The ROS levels usually increase in conditions of environmental stress and cell dysfunction, eliciting considerable damage to body cells.[10-12]

The human body consists of an antioxidant defence system for the mitigation or prevention of free radical-induced cellular damage through free radical scavenging mechanisms, such as inhibition of enzyme activity involved in free radical generation, activation of intracellular enzymatic antioxidant activity, metal ion chelation, as well as inhibition of protein modification, DNA damage, and lipid peroxidation. Antioxidants neutralise free radicals by donating or accepting electron(s) to obliterate unpaired electrons of radicals. Antioxidants neutralise reactive radicals such that the radicals are less active and deleterious with longer half-lives compared with the neutralised radicals. In addition, free radicals are further neutralised by the actions of other antioxidants or through other mechanisms that annihilate their deleterious activities.[8]

Antioxidant activity assays have undergone remarkable advancement within the last few decades. Chemical assays that are made up of high sensitivity as well as automated detection technologies are used to evaluate antioxidant activity through mechanisms such as free radical scavenging activity, HAT, SET, reducing power, etc.,[13,14] A good knowledge of the basic mechanisms, usefulness, and limitations of the various assays is essential for the selection of appropriate method(s) required for measurement of antioxidant capacity of desired application.[14]

The mode of actions of radicals and mechanisms of antioxidant activities, in addition to the connecting cellular events leading to oxidative stress, relate to the pathogenesis and pathophysiology of human disorders as well as the ageing process. And in that regard, measurements of cellular redox status and perturbation levels are of immense application in prognosis/diagnosis and mitigation strategies against human pathological conditions. Therefore, the present review provides an overview of the basic mechanisms by which antioxidants scavenge free radicals and describes the basic methods for measurement of antioxidant activities against free radicals as well as the underlying chemistry. The pathophysiology of oxidative stress-induced states, as well as establishing novel scientific approaches to prevention and mitigation of oxidative stress-induced pathological conditions, were also highlighted.

Evidence Acquisition

This review was sourced online from scientific search engines, namely, ResearchGate, Google Scholar, Scopus, PubMed, Medline and SpringerLink, using keywords, namely, 'antioxidants', 'enzymatic antioxidants', 'electron transfer', 'free radical', 'hydrogen atom transfer', 'oxygen radical', 'reactive oxygen species', 'reactive sulphur species', 'reactive nitrogen species' 'metal ion chelating', 'lipid peroxidation', 'DNA damage', 'aging', 'Alzheimer's disease,' 'Parkinson's disease', 'cancer', 'diabetes mellitus', 'cardiovascular diseases', 'sickle cell disease', 'oxidative stress and pathology'. A total number of 283 references published online between 1959 and 2022 were used as information sources and cited in this review.

BASES FOR ANTIOXIDANTS CATEGORIES

Antioxidants are categorised based on their activity, solubility, size, mode of action, and occurrence. Based on activity, antioxidants can be categorised as 'enzymatic' and 'non-enzymatic' entities. The enzymatic antioxidants can convert deleterious oxidative products to H_2O_2 and finally to water, as well as possessing the capacity to break down and eliminate free radicals using cofactors such as Fe, Zn, Cu, Se, and Mn. The non-enzymatic antioxidants are ascorbic acid, α-tocopherol, carotenoids, glutathione, as well as plant polyphenols. These antioxidants act to hinder and interfere with free radical chain reactions.[1,14,15]

Based on solubility, there are 'water-soluble' and 'lipid-soluble' antioxidants. For example, ascorbic acid and α-tocopherol are water-soluble and lipid-soluble antioxidants, respectively.[15] Based on size, antioxidants are classified as 'small-molecule' and 'large-molecule.' The LMWAs include the carotenoids, ascorbic acid, α-tocopherol, glutathione, etc., The HMWA are enzymes such as CAT, SOD, and GPx, as well as sacrificial proteins (albumin), which absorb ROS and inhibit deleterious actions of radicals against important macromolecules.[1,14]

Based on mode of action, antioxidants are categorised into 6 groups, namely: (1) antioxidants that break chain reactions by reacting with $RO_2^{\bullet}$ that are composed of weak O-H or N-H bonds as characteristics of the phenols, aromatic amines, naphthols, aminophenols, and hydroquinones. (2) Antioxidants that possess the capacity to break free radical chain reactions by reacting with alkyl radicals. And these include the quinones, iminoquinones, and nitrones. (3) There are antioxidants that terminate cyclic free radical chain reactions of nitroxyl radicals, aromatic amines, and variable valence metal compounds. (4) Another form of antioxidants that disintegrates $^{\bullet}OOH$ are the phosphides, thiophosphates, and sulfides. (5) Antioxidants that deactivate hydroxyl acids, diamines, and bifunctional compounds. (6) Finally, the phenol sulphide antioxidants, whose phenolic group reacts with $RO_2^{\bullet}$ of sulphide group and $^{\bullet}OOH$ in a synergistic manner.[1,15]

Based on occurrence, antioxidants are classified as 'natural' or 'synthetic' products. The natural antioxidants are chain-breaking antioxidants that undergo reactions with radicals and convert them to more stable adducts. The natural antioxidants are phenolic in structure and include the vitamins, phytochemicals, and minerals (which are enzymatic antioxidants cofactors such like Mn, Fe, Cu, Zn, and Se. The synthetic antioxidants are also phenolic compound derivatives that trap free radicals and terminate free radical chain reactions. The synthetic antioxidants include BHT, metal chelating agent-EDTA, NDGA, BHATBHQ, and PG.[16,17]

SOURCES OF FREE RADICALS

The ROS are mainly generated in the mitochondria, through the electron transport chain events associated with aerobic respiration.[18] The ROS include $O_2^{\bullet-}$, $^1O_2$, $^\bullet OH$, $RO^\bullet$, $RO_2^{\bullet-}$, H_2O_2, LOOH.[1,19] Approximately 1-3% of electrons react prematurely with oxygen during the electron transport chain to generate $O_2^{\bullet-}$.[20]

The first mechanism involved in ROS generation is the formation of $O_2^{\bullet-}$ from oxygen and NADPH by the action of NADPH oxidase (Eqn 1). The $O_2^{\bullet-}$ is further acted upon by SOD to generate H_2O_2 (Eqn 2). The MPO in the neutrophil cytoplasmic granules converts H_2O_2 in the presence of Cl^- to HOCl (Eqn 3).[21]

$$2O_2 + NADPH \xrightarrow{\text{oxidase}} 2O_2^{\bullet-} + NADP^+ + H^+ \quad (1)$$

$$2O_2^{\bullet-} + 2H^+ \xrightarrow{\text{SOD}} H_2O_2 + O_2 \quad (2)$$

$$Cl^- + H_2O_2 + H^+ \xrightarrow{\text{MPO}} HOCl + H_2O \quad (3)$$

The $O_2^{\bullet-}$ and H_2O_2 are also involved in other ROS generation suchlike $^\bullet OH$ through respiratory burst by Fenton (Eqn 4) and/or Haber-Weiss reactions (Eqn 5).[22]

$$H_2O_2 + Fe^{2+} \longrightarrow {}^\bullet OH + OH^- + Fe^{3+} \quad (4)$$

$$O_2^{\bullet-} + H_2O_2 \longrightarrow {}^\bullet OH + OH^- + O_2 \quad (5)$$

The RNS suchlike $NO^\bullet$ is generated by eNOS (Eqn 6). The reaction between $NO^\bullet$ and $O_2^{\bullet-}$ leads to the production of a very strong oxidant- $ONOO^-$.[23]

$$\text{L-Arginine} + O_2 + NADPH \longrightarrow NO^\bullet + \text{L-Citrulline} \quad (6)$$

ROS and RNS are generated endogenously through inflammation mechanisms and immune cell stimulation, ageing, extreme exercise, mental stress, ischaemia, infectious diseases, etc. Furthermore, ROS and RNS emanate exogenously through smoking, the presence of heavy metals, and exposure to radiation, as well as the intake of alcohol, some drugs such as cyclosporine and tacrolimus, and certain organic solvents such as benzene, polluted air inhalation, polluted water consumption, etc., These compounds, often referred to as pro-oxidants, are converted to ROS in the body system.[24] The ROS are also generated via lipo-oxygenation, lipid peroxidation, and cyclo-oxygenation.[14]

Mechanisms of Action of Antioxidants Against Free Radicals

Antioxidants scavenge free radicals and impede their actions through inhibition of enzymes involved in free radical production, activation of intracellular enzymatic antioxidants, metal ion chelation, and inhibition of protein modification, DNA damage, and lipid peroxidation.

Inhibition of Enzymes Involved in Free Radical Generation

A reasonable number of enzymes are involved in various free radical generation. The plasma membrane-linked enzymes NADPH oxidases induce $O_2^{\bullet-}$ formation by transferring one electron from the cytosolic donor NADPH to a molecule of molecular oxygen.[25] Xanthine oxidase enhances $O_2^{\bullet-}$ and H_2O_2 production through the oxidation of hypoxanthine and xanthine to uric acid.[26] The $O_2^{\bullet-}$ is also produced by enzymes such as cyclooxygenases, monooxygenases, and NADH oxidase. Excessive levels of $O_2^{\bullet-}$ are deleterious. However, it offers protection to the body against invading microorganisms during oxygen-dependent pathogen obliteration mechanisms.[27]

Many natural antioxidants are potential inhibitors of enzymes that promote $O_2^{\bullet-}$ generation and other ROS. For instance, polyphenols and α-tocopherol are known inhibitors of NADPH oxidase activity.[28] In addition; the polyphenolic compound coumarin elicits an inhibitory effect on xanthine oxidase.[29]

Intracellular Enzymatic Antioxidants

The ROS, namely $O_2^{\bullet-}$, $^\bullet OH$, H_2O_2, etc., are irreversibly generated during metabolic events. Certain intracellular enzymes, such as SOD, GST, CAT, GR, GP_X, TrxR, biliverdin reductase, and heme oxygenase, extensively mitigate the levels of ROS.[8,30]

The SOD enzymes (which are metal-containing proteins) catalyse the dismutation of two molecules of $O_2^{\bullet-}$ to O_2 and the less reactive species, H_2O_2 (Eqns 7 and 8; Figure 1).[8,31] The survival rate of rats subjected to 100% oxygenation increased following intravenous injection of liposomes containing SOD and CAT before and during the exposure to oxygen.[32] According to the study by Li *et al.*[33] lack of expression of Mn-SOD in Mn-SOD knockout mice was accompanied by dilated cardiomyopathy as well as neonatal lethality. Van Loon *et al.*[34] also reported that Mn-SOD plays an important role in the natural protection of cells against oxygen toxicity in their study using yeast mutants.

$$M^{(n+1)+}\text{-}SOD + O_2^{\bullet-} \longrightarrow Mn^{+}\text{-}SOD + O_2 \quad (7)$$

$$M^{n+}\text{-}SOD + O_2^{\bullet-} + 2H^+ \longrightarrow M^{(n+1)+}\text{-}SOD + H_2O_2 \quad (8)$$

The H_2O_2 can be further neutralized to form water via the reactions catalyzed by CAT or GP_X. Thus, CAT and/or GP_X catalyze the eradication of H_2O_2, and thereby prevent $^\bullet OH$ generation through the Fenton Reaction.

$$2H_2O_2 \xrightarrow{\text{CAT}} 2H_2O + O_2 \quad (9)$$

The GP_X uses hydrogen from two molecules of GSH to nullify H_2O_2 to two water molecules, forming GSSG in the process (Eqns 9 and 10; Figure 1).[8,31,35]

$$2GSH + H_2O_2 \xrightarrow{\text{GPX}} GSSG + 2H_2O \quad (10)$$

The GR further catalyzes the reduction of the GSSG back to GSH, thereby completing the cycle (Eqn 11; Figure 1).[31,36]

$$GSSG + NADPH + H^+ \xrightarrow{GR} 2GSH + NADP^+ \quad (11)$$

Metal Ion Chelating

Transition metals, such as copper and iron, exacerbate oxidative stress. Fe^{2+} and Cu^+ ions undergo a reaction with H_2O_2 generated from dismutation of $O_2\bullet^-$ by SOD to form a very reactive free radical, ▪OH (Eqn 12). In comparison with iron, copper produces more singlet oxygen than ▪OH when reacted with H_2O_2. During this process, Fe^{2+} and Cu^+ ions undergo oxidation to Fe^{3+} and Cu^{2+} ions, respectively. Furthermore, the Fe^{3+} and Cu^{2+} ions formed can undergo reduction back to Fe^{2+} and Cu^+ ions in the presence of reducing agents such as $AscH^-$. Thus, Fe^{2+} and Cu^+ ions can again react with another H_2O_2 molecule to produce more ▪OH (Eqn 13).[8,15]

The ▪OH generated can directly react vigorously with proteins and other macromolecules to form carbonyls, namely, aldehydes and ketones, leading to cross-linking and lipid peroxidation. Thus, the formation and action of ▪OH and ROS is impeded through chelation of metal ions, especially iron and copper.[8]

$$Fe^{2+} \text{ (or } Cu^+) + H_2O_2 \longrightarrow Fe^{3+} \text{ (or } Cu^{2+}) + \text{▪OH} + OH^- \quad (12)$$

$$Fe^{3+} \text{ (or } Cu^{2+}) + AscH^- \longrightarrow Fe^{2+} \text{ (or } Cu^+) + Asc\bullet^- + H^+ \quad (13)$$

Metal chelating is often regarded as the most acknowledged and common antioxidant action/mechanism. Antioxidants bind to metal ions through their functional groups, as observed in Fe binding to an antioxidant such as taxifolin. The chelating sites for the Fe^{2+} ion to taxifolin is the 4-oxo and 5-OH groups that are found between the heterocyclic and the A rings, the catechol moiety of the B ring, and the 3-OH groups and 4-oxo group in the heterocyclic ring (Figure 2A). Thus, taxifolin can chelate more than one Fe^{2+} ion through its functional hydroxyl and carbonyl groups.[37] Other functional groups through which antioxidants chelate metal ions, such as Fe^{2+} and Cu^{2+} ions, include -OH and $-OCH_3$ groups as observed in curcumin and kaempferol.[38] This metal-chelating capability of antioxidants was reported in the study carried out by Kazazica *et al.*[39] in which kaempferol was bonded to Fe^{2+} and Cu^{2+} ions through -OH and $-OCH_3$ groups (Figure 2D). Another antioxidant, usnic, was reported to chelate Fe^{2+} ions through its -OH and -COOH groups bonded to the phenolic ring (Figure 2B).[40] In another study, the antioxidant capability of resveratrol was linked to its metal-chelating property, whereby Fe^{2+} ions are chelated through their -OH groups at the meta positions (Figure 2C).[41] *In vitro* studies on metal ion chelating efficacy by antioxidants showed that quercetin, (+) catechin, and rutin efficiently chelated Fe^{2+} ions.[42,43]

Other functional groups through which antioxidants chelate metal ions, such as Fe^{2+} and Cu^{2+} ions, include -OH and $-OCH_3$ groups as observed in curcumin and kaempferol (Figure 2D).[38,39] Another antioxidant, usnic, has been reported to chelate Fe^{2+} ions through its -OH and -COOH groups bonded to the phenolic ring (Figure 2B).[40] The antioxidant capacity of resveratrol has been linked to its metal-chelating capacity, as they have been reported to chelate Fe^{2+} ions via their -OH groups at meta positions (Figure 2C).[41]

Generally, compounds made up of two or more -COOH, -C=O, -OH, $-OCH_3$, -SH, $-PO_3H_2$, -O-, $-NR_2$, and -S- functional groups in the suitable structural conformation can effectively chelate Fe^{2+} ions.[44-47] However, the interaction between antioxidants and metal ions can disrupt the biological actions and properties of antioxidants.[48,49] Metal chelation is also an important aspect of the antioxidant mechanism that mitigates the concentration of metals involved in lipid peroxidation catalytic activities.[50]

Inhibition of Protein Modification

Free radicals initiate the modification of proteins through amino acid nitration or chloration. The RNS, namely, $ONOO^-$, is a known powerful oxidant and nitrating agent.[52] The $ONOO^-$ has the capacity to initiate considerable damage to cellular proteins and DNA. Both $ONOO^-$ and its protonated form, ONOOH, modify proteins via one- or two-electron oxidation processes.[53] The $ONOO^-$ undergoes reaction with carbon IV oxide yielding $ONOOCO_2^-$. The $ONOOCO_2^-$ decomposes, leading to $CO_3^{\bullet-}$ as well as $\text{▪}NO_2$ generation. The $\text{▪}NO_2$ is a RNS that has the capacity to nitrate Tyr to nitrotyrosine. Thus, these radicals are known to elicit $ONOO^-$-related cellular damage (Figure 3). Furthermore, $ONOO^-$ can directly undergo reactions with electron-rich groups such as iron-sulphur centres, zinc-thiolates, sulfhydryls, as well as the sulfhydryl active site that occurs in tyrosine phosphatases.[52]

An alternative pathway of protein nitration or chloration involves heme protein MPO-generated HOCl, which undergoes reaction with NO_2^- to generate NO_2Cl. The NO_2Cl may further disintegrate spontaneously to yield NO_2^- and Cl▪. The NO_2^- and Cl▪ are probably responsible for the nitrating and chlorinating capacities of NO_2Cl.[54] Chlorotyrosine and nitrotyrosine are biomarkers for disorders caused by RNS.[55]

These modifications usually lead to changes in the function and structure of proteins, especially inhibition of enzyme activities. Tissue proteins made up of nitrotyrosine residues have been reported to be present in various pathologic conditions such as hypertension, atherosclerosis, and DM and are associated with increased $ONOO^-$ generation.[56]

Non-enzymatic antioxidants and enzymatic antioxidants suppress protein modification elicited by $ONOO^-$ and HOCl. Elimination of H_2O_2 by antioxidants, such as the enzymatic antioxidants, CAT, will inhibit HOCl generation. Furthermore, antioxidants such as polyphenols, curcumin, and the enzymatic antioxidant SOD suppress $ONOO^-$ formation through $O_2^{\bullet-}$ scavenging (Figure

3).[57] Both *in vivo* and *in vitro* studies on the antioxidant efficacy of S-allylcysteine revealed the capability of the garlic-derived compound to scavenge HOCl, $O_2^{-\bullet}$, H_2O_2, $^{\bullet}OH$ and $ONOO^-$.[58] Furthermore, Fernandes *et al.*[59] reported that $O_2^{-\bullet}$, $^{\bullet}OH$, $NO^{\bullet}$, and $ONOO^-$ are readily scavenged by sulindac and sulindac sulfone, whereas sulindac sulphide scavenged HOCl, $O_2^{-\bullet}$, $^{\bullet}OH$, $NO^{\bullet}$, and $ONOO^-$.

Inhibition of DNA Damage

The $^{\bullet}OH$ and $ONOO^-$ react with plasmid DNA and nick or cleave one DNA strand, resulting in the unwinding of the supercoiled plasmid DNA, which promotes oxidative DNA damage. DNA damage is accompanied by cell death and mutation, as well as associated CVD, neurodegenerative diseases, ageing, and cancer. The $NO^{\bullet}$ and $O_2^{\bullet-}$ are the precursors of $^{\bullet}OH$ and $ONOO^-$, whereas Fe^{2+} and Cu^+ ions are also known to generate $^{\bullet}OH$ by reacting with H_2O_2. Antioxidants impede $^{\bullet}OH$ and $ONOO^-$-induced DNA damage by scavenging $O_2^{\bullet-}$ and $^{\bullet}OH$.[27,60-65]

According to Kang *et al.*[63] three polyketides, which include cyathuscavins A, B, and C isolated from mycelium culture of *Cyathus stercoreus*, elicited antioxidant activities against DNA damage and thereby protect the supercoiled plasmid DNA from Fe^{2+}/H_2O_2-induced breakage. Melatonin, an antioxidant, protected DNA from oxidative damage in a study carried out by Zhao *et al.*[64] in which each molecule of melatonin trapped approximately two radicals. The inhibitory effect of polyphenols on DNA damage acts by binding to Fe^{2+} ions, as reported by Perron *et al.*[65] According to Zhao *et al.*[64] icariin (flavonoid) exhibited antioxidant activity against 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH)-induced oxidative DNA damage in a dose-dependent manner.

Lipid Peroxidation Inhibition

Lipid peroxidation is the oxidative deterioration of lipids made up of carbon-carbon double bonds present in phospholipids, unsaturated fatty acids, glycolipids, cholesterol esters, and cholesterol. The destructive mechanism of ROS is initiated by electrophilic attack of unsaturated fatty acids that are made up of multiple double bonds and $-CH_2-$ groups (Figure 4).[7,66] Antioxidants, such as vitamins, flavones, and polyphenols, act directly to quench peroxide radicals to obliterate free radicals' chain reactions. Lipid peroxidation leads to various deleterious health outcomes, such as cancer and atherosclerosis. However, ROS and peroxide radicals are scavenged by antioxidants and thus prevent the presentation of certain pathologic conditions.[7] Polyphenols from green tea, α-tocopherol, and ascorbic acid acted synergistically to inhibit peroxidation of linoleic acid in SDS micelles.[67] Likewise, α-tocopherol at μM concentrations inhibited copper-induced peroxidation of liposomal palmitoyllinoleoyl-phosphatidylcholine as reported by Gal *et al.*[68]

MEASUREMENT OF ANTIOXIDANT ACTIVITIES

Antioxidant activities are measured either *in vivo* or *in vitro* using standard experimental methods. Also, standard methods are available for measurement of actions of pro-oxidants. Generally, antioxidant activity is not measured directly but is based on the ability of antioxidants to control the rate of oxidation. There are different established methods that are used for measuring antioxidant capacity.[69]

During measurement of antioxidant activity, it is necessary to take into consideration the source of ROS and target substrate. For instance, a given antioxidant that inhibits oxidative damage to lipids, on the contrary, could promote oxidative damage of another biological molecule.[70]

Most of the methods used in the measurement of antioxidant activities accelerate the oxidation of the substrate in the presence of a promoter under regulated experimental variables such as pH, temperature, concentration of the antioxidant, etc., of the test system. Nevertheless, changes may occur in the oxidation mechanisms when some of the variables are altered. Thus, to obtain an authentic result, it is crucial to estimate the time interval within which the antioxidant activity quantification was carried out.[69]

The analysis of antioxidant capacity is categorised into two main groups with regards to the reaction mechanisms involved in the free radical reduction process. The first and second groups are based on SET and HAT, respectively. The kinetics and secondary reactions that are involved in the methods vary. The SET-based

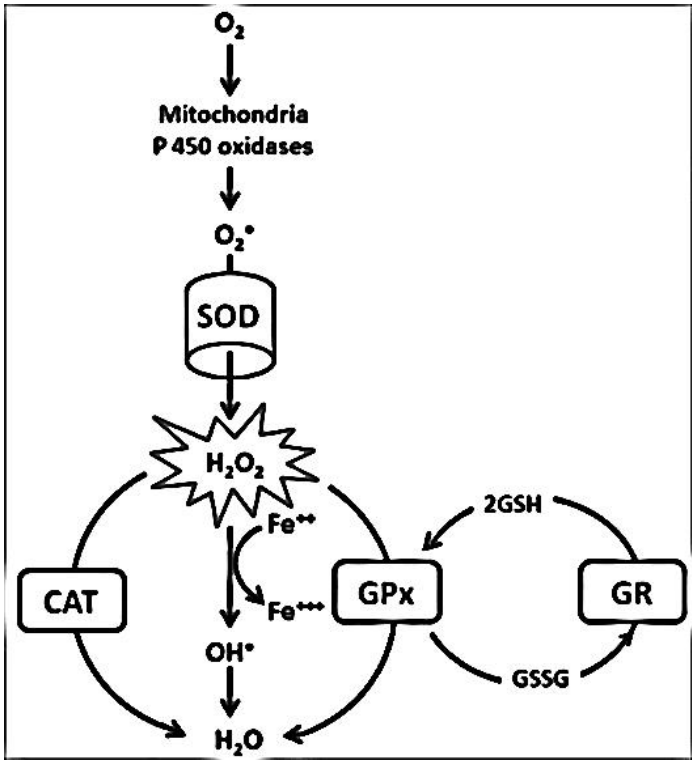

Figure 1: Action of enzymatic antioxidants on free radicals.[31]

methods detect the ability of a potential antioxidant to transmit chemical species such as metals, carbonyls, and radicals. In addition, the SET-based methods are expressed by a change in colour that occurs because of the reduction of the oxidant by an antioxidant.[71] The HAT-based methods measure the capacity of an antioxidant to deactivate free radicals through a hydrogen atom donation mechanism. The reactions of HAT do not theoretically depend on solvent nature and pH and occur at a very fast rate (less than a few minutes). However, HAT testing is complicated, and significant errors may occur due to the reducing agents present in the samples in conjunction with the antioxidants under study.[13]

The techniques used in the measurement of antioxidant activity must be fast, reproducible, and carried out using small amounts of the compound being analysed and must not be affected by the physical properties of the compound being analyzed. Since the outcomes of *in vitro* assays can be used as a direct indicator of antioxidant activity *in vivo*, a compound that has low antioxidant capacity *in vitro* will also exhibit low antioxidant capability *in vivo*.[70] Nevertheless, there may be variations in the results obtained depending on the oxidation conditions used and various factors such as temperature, metal catalysts, and the oxygen concentration in the reaction medium that can influence oxidation. The tests employed for the measurement of substrates or products can also cause variable results depending on their specificity.[72] The methods used in the measurement of free radical scavenging capacity of antioxidants *in vitro* and *in vivo* are described below.

IN VITRO METHODS

Total Oxyradical Scavenging Capacity Assay

Total oxyradical scavenging capacity assay TOSCA is based on HAT. The TOSCA evaluates antioxidant activity in the gaseous phase and involves the introduction of KMBA to powerful oxidising agents such as ROO•, •OH, and $ONOO^-$ (Figure 5),[13] which causes the conversion of KMBA to ethylene. The basis of the TOSCA assay is the ability of antioxidants to inhibit the formation of ethylene from KMBA, whereby the antioxidants compete with KMBA for ROS. The capacity of antioxidants is assessed by comparing the extent of ethylene formation with a control reaction using HS-GC. The TOSCA is not suited for a high-performance analysis due to the multiple injections of each sample needed for the estimation of ethylene production.[13,69] A major limitation of this method is that the concentration of the antioxidants cannot be measured by the percentage inhibition of KMBA oxidation.[73]

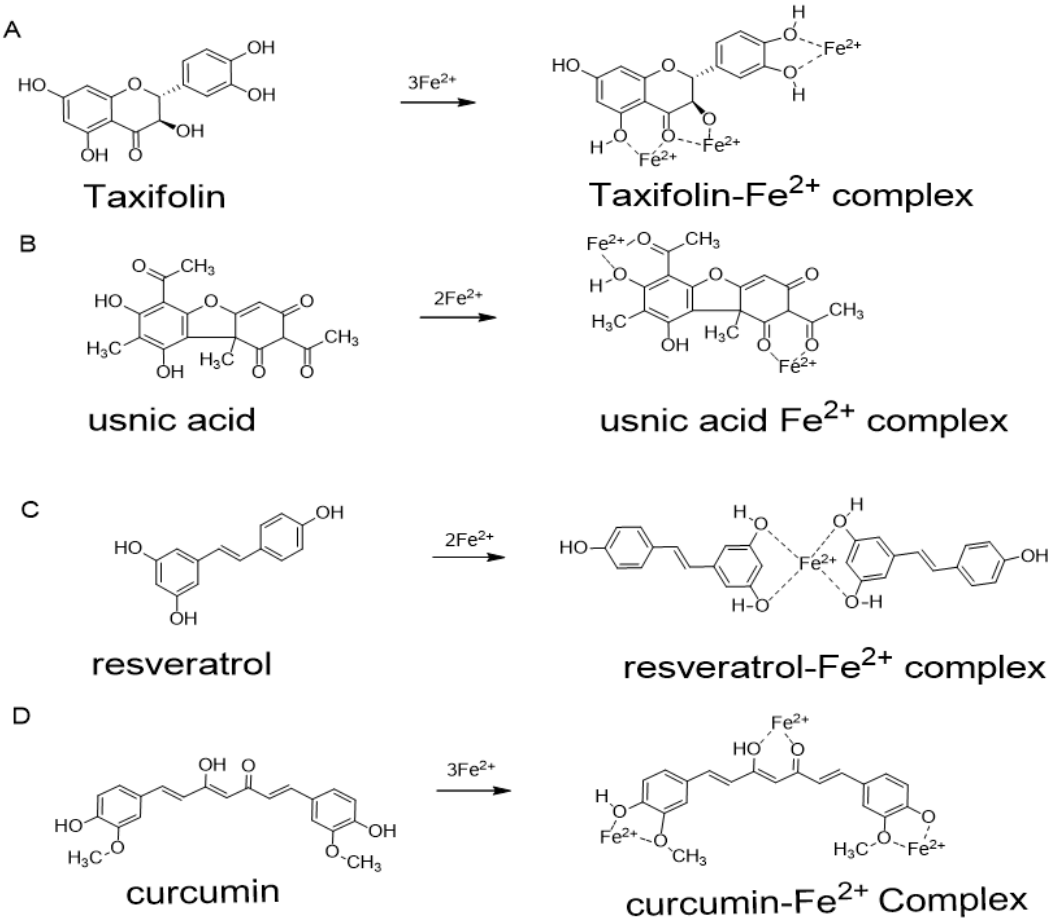

Figure 2: Fe^{2+} binding mechanisms of antioxidants (A) taxifolin, (B) usnic acid, (C) resveratrol and (D) curcumin.[51]

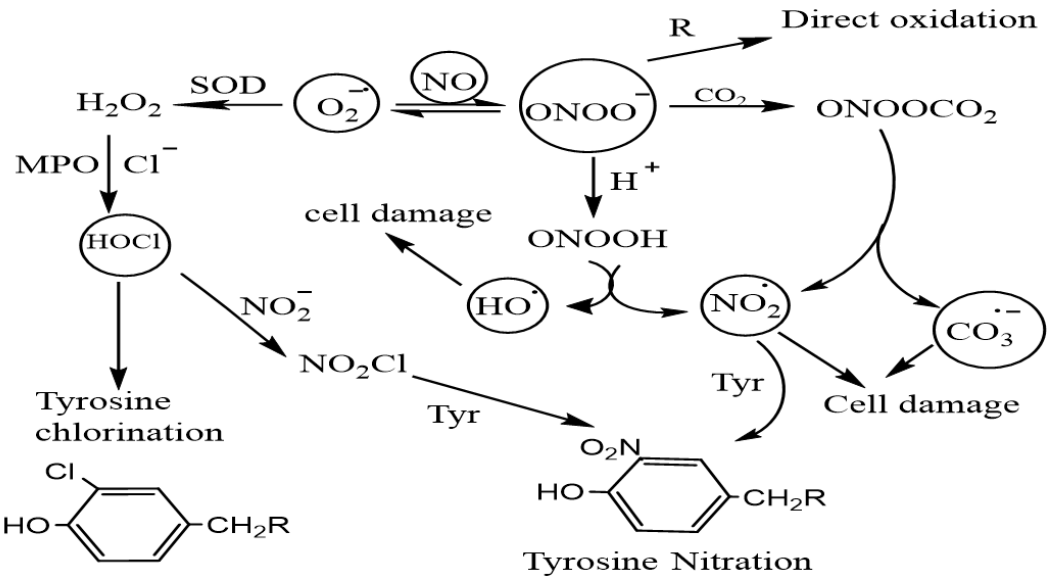

Figure 3: The nitration and chloration of tyrosine.[7]

2,2-Diphenyl-1-picrylhydrazyl (DPPH) Radical Scavenging Assay

The DPPH radical scavenging assay is based on SET, with HAT mechanism as the only marginal reaction pathway.[13] The DPPH is a stable chromogen radical characterised by a deep purple colour. The basis of the DPPH scavenging assay is the donation of electrons by antioxidants for the neutralisation of DPPH radicals, and the antioxidant activity is measured by the colour change of DPPH at λ_{max}=517 nm.[74] The DPPH assay is a very simple process that involves only an ultraviolet spectrophotometer or an EPR spectrometer. The EPR spectroscopy directly estimates DPPH radical concentration at sub-micromolecular levels. This assay is majorly dependent on the assumption that the antioxidant electron-donating power (or reducing ability) is directly proportional to the antioxidant activity. Although this method is very simple and does not need special sample treatment, the sensitivity of this assay may be influenced by certain factors such as the type and quantity of the solvent used, the availability as well as the concentration of hydrogen and metal ions, and the viability of DPPH reagent.[75-77] The main limitation of the spectrophotometric DPPH assay is the overlapped spectra of compounds that absorb at the same wavelength range as DPPH. A good example is anthocyanins that elicit strong absorption over an equivalent wavelength range as DPPH (λ_{max}=500-600 nm), which interferes with the results and affects their interpretation. To overcome this drawback, the use of ultraviolet spectrophotometry should be substituted with EPR spectroscopy. The EPR spectroscopy is more effective than the classic spectrophotometric method for both highly coloured samples and samples that appear turbid in the chosen solvent.[78]

The DPPH radical possesses an unpaired electron, producing a characteristic and distinguishable EPR spectrum in a magnetic field under microwave spectrum. The peak intensity in the EPR spectrum is equal to DPPH concentration, and the presence of antioxidants causes its decline with time. The decline in DPPH signal intensity is used as an indicator of antioxidant scavenging capacity against DPPH radical.[79] Antioxidants scavenging capacity of DPPH radical can also be measured using amperometric detection. The DPPH radical produces a constant electric current at an applied voltage. However, the amperometric signal decreases when DPPH radical concentration drops due to scavenging action by antioxidants. The antioxidants are then quantified using amperometric detection of residual concentration of unreacted DPPH radical.[80,81] The DPPH radical scavenging assay can also be used in conjunction with other methods for purposes. For instance, DPPH assay can be used in concert with online HPLC to enhance the screening of large quantities of antioxidant samples and thereby decrease the number of antioxidants that will be lost through the purification processes.[75]

Empirically, the methods by Manzocco *et al.*[82] measure DPPH radical scavenging percentage of sample extract diluted in methanol mixed with DPPH solution. The absorbance is read after 30 min at 517 nm. The percentage inhibition of DPPH radical scavenging in the presence of the sample is calculated (Eqn 14):

$$\% \text{ inhibition of DPPH radical} = ([Abr - Aar]/Abr) \times 100 \quad (14)$$

Where A_{br} and A_{ar} are the absorbance before and after the reaction has occurred respectively.

Total Radical-Trapping Antioxidant Parameter

TRAP measures the status of a secondary antioxidant in plasma and functions via HAT. The TRAP is based on the measurement of the rate of uptake of oxygen during a controlled peroxidation reaction following thermal decomposition of ABAP, which is associated with the release of ROO• at a constant rate (Figure

6). This process is carried out by first adding ABAP to human plasma. The parameter being evaluated is the 'delay time' within which oxygen is absorbed in the plasma that is influenced by antioxidants present in the medium. The delay time is estimated through oxygen concentration data in plasma diluted in a buffer solution observed using an electrode. In the absence of ABAP, other free radical initiators, namely, luminol, ABTS, phycoerythrin, and dichlorofluorescein diacetate can be used.[83-85] The results of this method are reported in μmol of ROO• trapped per litre of plasma.[69] The major limitation of TRAP is that error may arise during detection of the end point due to instability of the oxygen electrode, which at this point can take up to 2 hr for oxygen to stabilize. However, this problem can be overcome by carrying out electrochemical detection of oxygen using chemiluminescent detection based on the use of luminol and horseradish peroxidase.[69]

The TRAP of diluted samples in phosphate buffer, mixed with bi-distilled water, R-phycoerythrin, and ABAP, is measured according to the methods of Ghiselli *et al.*[83] using a luminescence spectrometer. The TRAP is computed using the length of the lag phase due to the sample compared with the standard.

Crocin-Bleaching Assay

The CBA is used to measure the rate at which RO• generation is inhibited. The reaction mechanism of this assay is based on HAT. The CBA is carried out by evaluating the protective effect elicited by antioxidants on crocin (a carotenoid that gives an intense red colour upon encountering RO•) (Figure 7). This is made possible using an ultraviolet-visible spectrophotometer for a measure of the reaction kinetics. The absorbance is measured at a wavelength of 440 nm, whereby the relative velocity constants are obtained. The antioxidant capacity of the reference compounds is estimated through the relative velocity constants. The absolute bleaching velocity of crocin is dependent on the kind of radical that attacks the polyene structure of crocin. Crocin shows a high selectivity for RO• formed from •OOH photolysis with $RO_2^{\bullet-}$ formed after the thermolysis of azo initiators.[86]

Oxygen Radical Absorbance Capacity (ORAC) methods

The ORAC methods are centered on the prevention of oxidation initiated by $RO_2^{\bullet-}$ and simultaneously measure the time effect as well as the degree of inhibition. The reaction mechanism of action of this test is based on HAT and utilises the ability of antioxidants to prevent the degradation of fluorescein (fluorescence probe) by promoting the elimination of radicals.[87] The ORAC starts with the thermal degradation of azo compounds, such as AAPH. This degradation leads to the release of free radicals. The free radicals enhance the degradation of fluorescein. However, the antioxidant activity promotes the removal of $RO_2^{\bullet-}$ and thereby prevents the degradation of fluorescein. The dilapidation in fluorescence caused by radicals and antioxidants protective effects results in a curve. The area under the fluorescence decrease curve is used to estimate the extent of the antioxidant effect. The standard used in this assay is Trolox. And therefore, the antioxidant activity is measured in terms of Trolox equivalents. The ORAC has been used extensively to measure the antioxidant capacity of vegetables, fruits, supplements, and beverages.[13,88,89] The ORAC is performed at about pH 7.4, which is adjusted using a phosphate buffer with the antioxidant, AAPH, and fluorescein at 37°C. Fluorescence is observed at 1 min interval for a period of 35 min at an excitation wavelength and emission wavelength of 485 nm and 520 nm, respectively. Furthermore, the modification of this method involves the use of fluorescein as a probe to estimate lipophilic and hydrophilic compounds as well as the total antioxidant capacity of the compound.[90]

According to the method by Prior *et al.*[91] the ORAC assay is performed in polypropylene fluorescence plates. The hydrophilic and lipophilic assays in the presence of AAPH are carried out using Trolox in standard phosphate buffer. The fluorescence is measured at excitation and emission wavelengths of 485 nm and 520 nm, respectively. Wallac Workout 1.5 software is used for the computation of the area under the curve. The results are gotten from the difference of the areas-under-the-decay curves between blank and sample and/or standard (Trolox), expressed as μM TE/g (μM of Trolox equivalents, TE per g dry weight of sample).

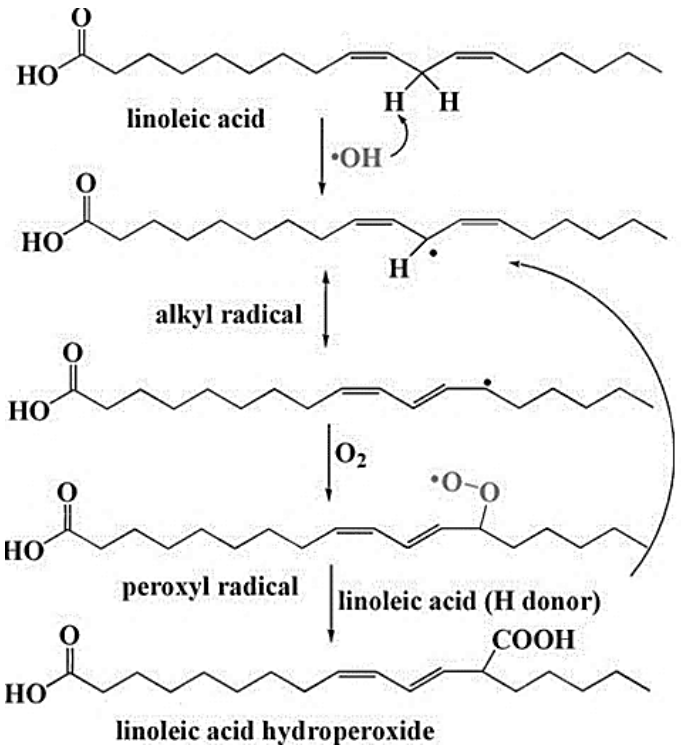

Figure 4: Linoleic acid peroxidation mechanism by hydroxyl radical initiation.[8]

IN VIVO METHODS

For all *in vivo* methods, blood or tissues collected from animals such as rats, mice, etc., treated with the samples whose antioxidant efficacy are to be tested, are used for the assay.[92] The levels of lipid peroxidation, FRAP, GSH, and antioxidant enzymes such as GP_X, GST, SOD, CAT, GGT, and GR are estimated.

Lipid peroxidation

MDA is used as an indicator of lipid peroxidation. MDA is among the end products of lipid peroxidation, and it is generated during oxidative degeneration as a product of free oxygen radicals.[92] The methods by Ohkawa *et al.*[93] for the estimation of lipid peroxidation have been reported elsewhere.[94,95]

Ferric reducing ability of plasma

Antioxidant efficacy is evaluated through the measurement of the rise in absorbance that occurred by the generation of ferrous ions from FRAP reagent. The absorbance is measured at 593 nm using a spectrophotometer.[92,96]

Reduced glutathione

GSH is an intracellular reductant that guides the cell from damage by free radicals, peroxides, as well as other toxic substances.[97] The measurement of GSH in tissue homogenate using Ellman's reagent was described.[98] The values of the absorbance at λ_{max}=412 nm are compared with a standard curve gotten from known GSH.

Glutathione peroxidase activity

Cytosolic GP_X activity is measured by a coupled reaction involving the conversion of NADPH to NADP in the presence of glutathione reductase. The change of absorbance at λ_{max}=340 nm of the sample mixture is measured at regular intervals of 1 min for 5 min.[99] The GP_X activity is expressed as mg of proteins.

Glutathione S-transferase activity

GST activity is measured by the conjugation of CDNB with GSH (Eqn 15) at λ_{max}=340 nm at 37°C.[100,101]

$$CDNB + GSH \rightarrow DNPSG(15)$$

The increase in absorbance is measured for 10 min against a blank solution using a spectrophotometer.

Superoxide dismutase activity

According to McCord and Fridovich,[102] SOD activity is measured in erythrocyte lysate prepared from the 5% erythrocyte suspension. The sample mixture consists of Tris-HCl buffer (pH 8.2), EDTA, and pyrogallol. The increase in absorbance is measured at λ_{max}=420 nm for 3 min against a blank solution using a spectrophotometer. One unit of SOD activity is equivalent to the auto-oxidation rate of pyrogallol with regards to the change in absorbance/min. SOD enzyme activity is expressed as units/mg protein.

Catalase activity

CAT activity of erythrocyte lysate is measured in phosphate buffer (pH 7.0). The enzyme activity is evaluated using the molar extinction coefficient of H_2O_2 (Σ=43.6 M cm^{-1}). One unit CAT activity is equivalent to 1 mmol of H_2O_2 consumed per min. Catalase activity is expressed as units per milligram of protein.[103]

γ-Glutamyl transpeptidase activity

The γ-GGT activity is measured according to the methods previously described.[104] The serum sample is pre-mixed with $MgCl_2$, glycylglycine and g-Glutamyl-p-nitroanilide in tris buffer (free base) (pH 8.2) incubated for 1 min at 37°C. The absorbance is measured at regular interval of 1 min for 5 min at λ_{max}=405 nm.

Glutathione reductase activity

The oxidation rate of NADPH by GSSG at 30°C is used as a standard method for the measurement of GR activity. The reaction system is made up of NADPH, GSSG, EDTA and sodium phosphate buffer (pH 7.6). The Unit of GR activity is derived from the oxidation of NADPH/min. The specific activity of GR is stated as Unit per mg of protein.[105]

OXIDATIVE STRESS AND PATHOLOGY

Pathological conditions such as CVD, DM, tissue inflammation, sickle cell anaemia, cancer, cataracts, AD, autism, and ageing are provoked by increased free radical generation in conjunction with alteration in system antioxidant defence capabilities. Thus, indicating the contributive role of ROS in pathological on set, escalation, and complications. Increasing evidence in both experimental and clinical studies suggests that oxidative stress contributes largely to the pathogenesis of various disease conditions.[106-108]

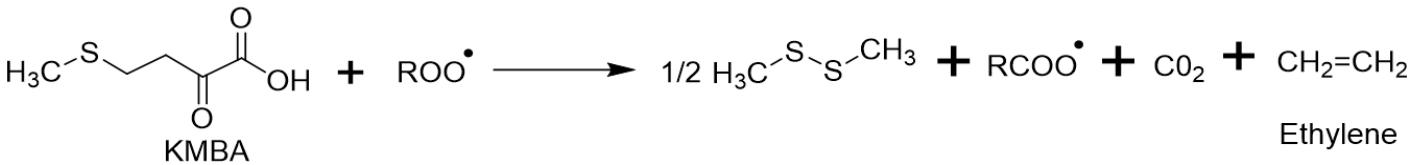

Figure 5: Reaction between KMBA and ROO• to form ethylene.

It's conventional to integrate antioxidant formulations into therapeutic interventions, whereby natural antioxidants administered through dietary supplementation are recommended for a holistic approach to the management and prevention of oxidative stress-induced pathological conditions and complications. An earlier proposed unique therapeutic strategy, whereby overexpression of enzymatic antioxidants was provoked in a mutant mice model in a tissue-specific manner, could serve as a novel alleviative measure against the development of pathological conditions associated with oxidative stress.[109,110] Similar novel approaches have been adduced against human inflammatory pathways associated with diabetic complications.[111] Specifically, one of such approaches is the activation of transcription nuclear factor, Nrf2, which in turn induces several enzymatic antioxidants and detoxification genes against oxidative stress-induced pathology such as lung cancer.[110,112]

Cardiovascular Disease

The ROS plays a dual role in cardiovascular physiopathology. Low levels of ROS provide significant benefits to the cardiovascular system, whereby they exhibit pro-angiogenesis, anti-atherosclerotic, and endogenous cardio-protective effects.[113] On the contrary, high levels of ROS engender deleterious actions, through oxidative stress, in the cardiovascular system by provoking CVD, such as atherosclerosis, heart failure, endothelial dysfunction, arrhythmias, and myocardial ischaemia/reperfusion.[113,114] In addition, oxidative stress causes alteration in gene expression in CVD.[115] At high levels of ROS, one of the foremost occurrences is oxidative modification of LDL in atherogenesis and other CVD associated with endothelial dysfunction.[116,117] Furthermore, LDL and cell membranes are composed of large amounts of phospholipids, which are sensitive to oxidative modification. Through receptor-mediated or receptor-independent pathways, oxidised phospholipids stimulate endothelial cells, enhance the expression of endothelium adhesion molecules, attract monocytes, elicit endothelium cytotoxic effects, and promote the activities of pro-inflammatory genes and cellular growth factors. All these events elicit aggregation of platelets, expression of metalloproteinase, and promote thrombogenesis.[118]

Generally, endothelial dysfunction initiated by excessive ROS generation overwhelms antioxidant defence systems, causing damage to endothelium-mediated vasodilation, impaired vascular reactivity, and vasospasm, as well as increased expression of chemotactic and adhesive molecules. And thereby, enhances activation of platelet and thrombus generation, increased permeability of the endothelium, leucocyte adhesion, as well as monocyte movement into the vascular wall. The regeneration of endothelial cells, accompanied by the proliferation and migration of smooth muscle cells, is impaired, and in the process, engenders vascular damage (Figure 8).[119-121]

The major risk factors of CVD, namely, hypercholesterolaemia and hypertension, promote ROS generation, which eventually leads to oxidative stress.[122] Higashi *et al.*[123] investigated the level of 8-OHdG in urine as well as serum MDA-modified LDL as markers of oxidative stress in patients suffering from reno-vascular hypertension. Higher levels of these oxidative stress indicators were observed in hypertensive patients in comparison with the non-hypertensive control group. Another study by Lip *et al.*[124] also reported elevation of biomarkers of oxidative stress in hypertensive individuals.

Oxidative stress induces the rupture of atherosclerotic plaque and eventually causes thrombosis by stimulating the expression of matrix metalloproteinase.[125] The action of NF-κB is upregulated by oxidised LDL in atherosclerosis.[116] The study carried out by Guzik *et al.*[126] showed a correlation between endothelial dysfunction and elevated vascular $O_2^{\bullet-}$ generation in human atherosclerosis. Azarsiz *et al.*[127] reported the relationship between oxidative stress and atherosclerosis, in which they noted increased erythrocyte levels of TBARS with corresponding severity of blockage of the arteries.

Because cardiac myocytes possess higher amounts of mitochondria in comparison with other cells, they consume relatively higher levels of oxygen to generate energy in the form of ATP. The relatively high level of non-neutralised ROS generated as a byproduct of tissue metabolism causes cardiac injury in the myocytes, with concomitant oxidation of essential proteins required for the excitation-contraction process and mitigation of the bioactivity of NO.[128]

In myocardial ischaemia, there is an imbalance in mitochondrial electron transport with concomitant ATP level reduction, acidosis, and depolarisation of the mitochondria, intracellular Ca^{2+} overload, and apoptosis.[129] Oxidative stress generated in the mitochondria causes impaired mitochondrial DNA and presentation of CVD. Furthermore, in myocardial ischaemia, free radical generation is enhanced in the cardiac tissue by hypoxia

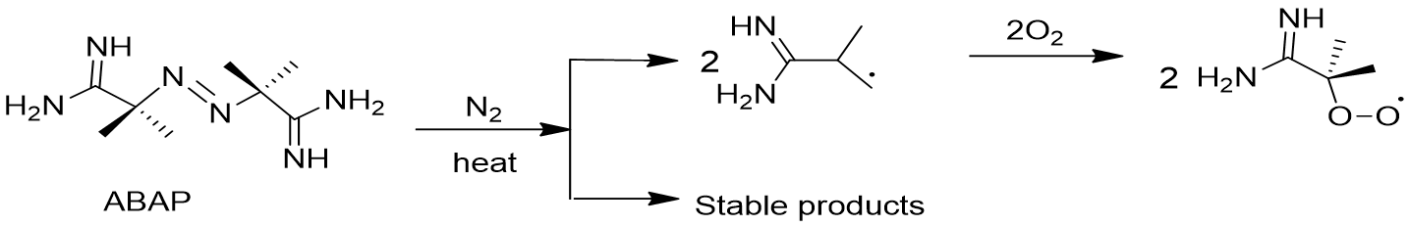

Figure 6: Peroxyl radical formation from 2,2′-azobis-(2-amidopropane).

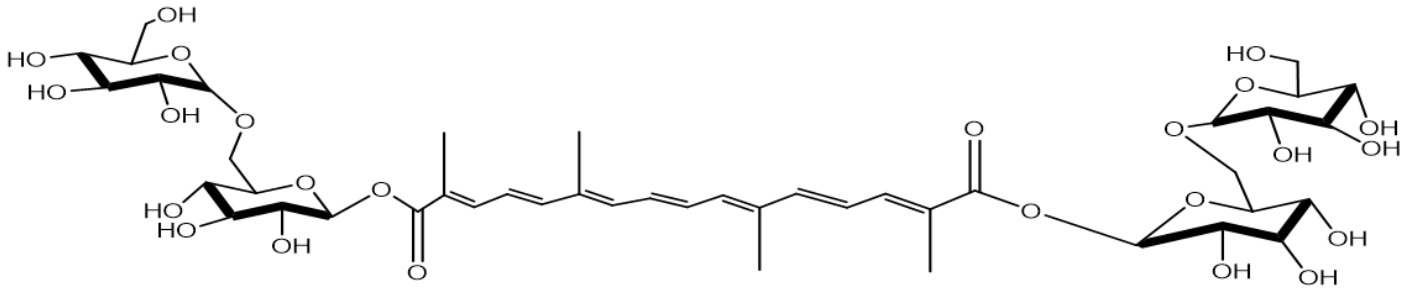

Figure 7: Molecular structure of crocin.

and re-oxygenation.[115] The ROS generated via re-oxygenation initiates direct oxidative damage to cellular components and indirect impairment by provoking localised inflammation.[130] The increased ROS generation in heart failure is dependent on the elevated activity of xanthine oxidoreductase and NADPH oxidase. High levels of ROS are accompanied by long-term endoplasmic reticulum stress as well as mitochondrial-derived oxidative stress in cardiometabolic disorders.[114]

Diabetes Mellitus

Experimental and clinical studies suggest that oxidative stress contributes significantly to the pathogenesis and development of DM complications. Nevertheless, the mechanism through which oxidative stress contributes to and enhances DM complications is not fully understood and needs to be fully clarified.

Hyperglycemia activates free radical generation and alters the endogenous antioxidant defence system in diabetic individuals.[131] There are eight main mechanisms that lead to free radical generation through uncontrolled hyperglycemia, and they include an increase in the flux of the polyol pathway, glucose autoxidation, an increase in the rate of glycolysis, increased AGEs generation, increased expression of the receptor for AGEs as well as its activating ligands, non-enzymatic glycation of proteins, NAD(P)H oxidase activation through the protein kinase C-dependent pathway, and increased activity of the hexosamine pathway.[132-136,110] The formation of oxidants in DM and the concomitant oxidative stress leading to oxidative cellular damage is described in Figure 9.

RAGE: Receptor for AGEs

Furthermore, the eight major pathways/mechanisms contributing to the pathogenesis of diabetic complications are induced by excessive $O_2^{\bullet-}$ generation via hyperglycaemic intracellular setting (Figure 10).[135,138-141] Specifically, there is an increase in the level of pyruvate produced from glucose catabolism with high intracellular glucose concentration, thereby making more pyruvate available for oxidation via the TCA cycle, which increases the flow of NADH and $FADH_2$ (electron donors) into the electron transport chain. This process causes a continuous increase in voltage gradient across the mitochondrial membrane until a critical threshold is reached. At the threshold point, the transfer of electrons in the mitochondrial electron transport chain causes $O_2^{\bullet-}$ generation through the leakage and transfer of one electron to molecular oxygen (Figure 10).[141]

In addition, hyperglycemia also activates GAPDH, which eventually leads to a high accumulation of glycolytic intermediates that are upstream of this enzyme.[142] The highly accumulated glycolytic intermediate, glyceraldehyde-3-phosphate, activates the PKC pathway (because diacylglycerol, which is the activator of PKC, is formed from glyceraldehyde-3-phosphate) as well as the pathway that leads to AGE generation (the chief intracellular precursor of AGE, methylglyoxal, is produced from glyceraldehyde-3-phosphate). The flux of the hexosamine pathway is also increased through the accumulation of another glycolytic intermediate, fructose-6-phosphate, which is upstream of GAPDH. Also, an increase in the level of glucose through the inhibition of GAPDH by hyperglycemia increases the polyol pathway flux (Figure 10).[141,143-146]

A study carried out by Davison *et al.*[147] suggested that a high level of oxidants in type I DM male individuals was because of the autoxidation of glucose and/or an increase in the rate of oxidation of the major lipid-soluble antioxidant, α-tocopherol, due to low exercise. Hyperglycemia was observed to induce overproduction of oxygen free radicals in children with chronic insulin-dependent DM in a study carried out by Ramakrishna and Jailkhani.[148] According to the study carried out by Godin *et al.*[149] using streptozotocin-induced diabetic rats, it was reported that elevated and uncontrolled oxidative activities promoted the pathogenesis and complications that are associated with chronic diabetic conditions. The increase in susceptibility of erythrocyte lipids to oxidation was directly proportional to the severity of diabetic complications. Additionally, certain changes, such as elevation in GR activity, reduced vulnerability to decreased oxidative glutathione (which is associated with hyperglycemia), and elevation in malondialdehyde generation (which is an indirect measure of lipid peroxidation), were observed in the diabetic state. Yakubu *et al.*[107] were of the view that the pathogenesis of glucose-induced vasculopathy correlated with PKC-coupled oxygen free radical generation, which suppressed $NO^{\bullet}$ generation and $NO^{\bullet}$-dependent vascular relaxation. The previous report by Ohkuwa *et al.*[150] suggested that $^{\bullet}OH$ generation was dependent on the amount of streptozotocin injected into experimental rats.

And therefore, they concluded that •OH generation is involved in the pathogenesis of DM.

The production of $O_2^{\bullet-}$ was reported by Ceolotto *et al.*[151] to be induced by insulin through an NAD(P)H-dependent mechanism, which involved the activation of PI-3'-kinase and activation of proliferative ERK-1 and -2 dependent pathways. An elevation in oxidative stress was observed to be accompanied with a marked reduction in the level of GSH as well as alterations in enzymatic antioxidant activities such as catalase, GPx, and Cu-Zn-superoxide dismutase in the heart, liver, and kidneys of streptozotocin-induced diabetic rats.[106] Similar changes in the enzymatic antioxidants in rat brain were also reported.[108]

Cancer

Oxidative stress in cancerous cells arises when ROS generation overwhelms the antioxidant defence system, leading to DNA, protein, and/or lipid damage; chromosomal instability; genetic mutation; as well as cell growth modulation, which eventually results in cancer (Figure 11).[152]

Free radicals cause chromosomal defects as well as the activation of oncogenes, which eventually leads to the emergence and progression of cancer.[153] High levels of free radicals have been reported to cause cell damage and apoptosis. Free radicals and DNA reactions have been linked to being associated with various forms of cancer, leading to mutations that have a deleterious effect on the cell cycle and eventually result in neoplasia.[154,155]

Excessive ROS generation promotes the proliferation of cancer cells, metastatic potential, as well as invasiveness and poor prognosis.[156] ROS promotes the migration of cancer cells by supporting matrix degradation, which facilitates contact among cells, cytoskeleton remodelling, as well as regulates gene expression and invadopodia formation.[154] For example, mitochondria-derived ROS influences initial extracellular matrix contact; NOX-derived ROS are associated with invadopodia formation, and an increase in the level of ROS in the cytosol contributes to cytoskeleton remodelling.[157]

The ultraviolet component of sunlight, within wavelengths of 320-400 nm, has the potency to initiate oxidative stress in tissues. And continuous exposure of human skin to ultraviolet radiation is accompanied by skin carcinogenesis as well as photoaging through DNA damage. The physiological concentration of ultraviolet components of sunlight affects the expression of certain genes, such as nuclear oncogenes, collagenase, and heme oxygenase 1, whose effects can be significantly increased through the removal of intracellular GSH or by prolonging the half-life of molecular oxygen.[158,159] The study by Sander *et al.*[160] reported that oxidative stress contributed to the pathogenesis of human skin cancers and suggested that in non-melanoma skin cancer, the reduction in antioxidant defence system induced by exposure to chronic ultraviolet radiation may promote multistep carcinogenesis, while melanoma cells exhibit increased oxidative stress that can initiate tissue damage and promote metastasis.

The •OH-attach to DNA reacts with deoxyGTP in the nucleotide pool to generate 8-OHdG, which causes mutation in mammalian and bacterial cells (Figure 12).[152]

The 8-OHdG also induces the transformation of G-C pairs to T-A pairs during the process of DNA replication, which eventually leads to the emergence of cancer-inducing mutagenesis.[161] Thus, 8-OHdG can serve as a biomarker for the detection of the presence of free radicals during DNA mutagenesis.[162] Many studies have reported high levels of 8-OHdG in various human cancers[163-166] as well as in animal models of tumours.[167,168] Furthermore, ROS accumulation is associated with an increased proliferation of cancer cells.[114]

There is continuous oxidative stress in cancer cells, which is enhanced by mitochondrial dysfunction as well as metabolic changes. Under normal conditions, high ROS levels induce cell death, but this does not occur in cancer cells due to their ability to activate numerous oncogenes, which in turn activates the expression of NRF2. The NRF2 is the primary regulator of cell survival that increases cancer progression by defending cancer cells from the damages caused by ROS on DNA.[114,169] The ROS are implicated in the progression of cancer through the promotion of cyclin D1 expression, ERK, and the phosphorylation of JNK.[159] The investigation carried out by Aikawa *et al.*[170] reported the role of ROS in the activation of ERK in cultured cardiac myocytes of neonatal rats.

Alzheimer's Disease

AD is one of the most encountered neurodegenerative diseases that lead to dementia among the elderly. The AD is characterised by gradual loss of memory as well as other cognitive functions, which finally leads to the complete incapacity and death of the affected person within a period of 3 to 9 years following diagnosis.[171] The presence of senile plaques, NFTs, and neuronal cell death are the main pathological characteristics of AD brains.[172]

Cytochrome c oxidase deficiency is the major and common impairment in the mitochondrial ETC in AD, which results in increased ROS generation.[173] The ROS triggers the accumulation of Aβ protein, which leads to lysosome membrane degradation as well as neuronal death.[174] Furthermore, ROS suppresses PP2A, leading to the activation of GSK 3β and probably the hyperphosphorylation of tau and neurofibrillary lesions.[175,176] Elevated ROS initiates low mitochondrial membrane potential ($\Delta\Psi_m$) and ATP generation by unfavourable mitochondrial energy stores, disrupting energy metabolism, as well as compromised dynamics and mitophagy. These events further trigger caspase activity and apoptosis (Figure 13).[177] Studies have shown that mitochondrial dysfunction is a major factor that is associated with the pathogenesis of AD.[178,179] Many mitochondrial and

metabolic abnormalities occurred in the hippocampal neurones in AD compared with age-matched controls.[178,179] Morphometric analysis of biopsies from AD brains reported a substantial reduction in mitochondria, whereas mitochondrial DNA and protein located in the cytoplasm and vacuoles associated with lipofuscin showed significant increase.[178-180] These mitochondrial abnormalities were followed by oxidative damage marked by raised levels of 8-OHdG and nitrotyrosine, which showed that mitochondrial impairment occurred during AD progression.[178]

Many hypotheses are in support of oxidative stress as one of the factors involved in the pathogenesis of AD. However, the mechanism through which the redox balance is altered, and the sources of free radicals have not been established.[181]

Studies have also reported possible correlation between oxidative stress and Aβ protein-induced toxicity. Increased H_2O_2 and $NO^{\bullet}$ generation coupled with enhanced oxidative modifications of proteins and lipids were directly proportional to the age-associated Aβ protein accumulation in various AD transgenic mouse models carrying mutants of APP and PS-1, which suggested that Aβ protein promoted oxidative stress.[182-185] In the study carried out by DeFelice *et al.*[186] using hippocampal neuronal cell cultures, ROS activated by soluble Aβ protein oligomers triggered NMDA receptor, which was accompanied by a sudden rise in the levels of neuronal calcium. The report therefore suggested a possible role of soluble Aβ protein oligomers as proximal neurotoxins and the active role of oxidative stress in synaptic damage and neuronal loss initiated by soluble Aβ protein oligomers.

Alterations in metal homeostasis have been shown to relate to AD. Abnormal levels of Cu, Zn, and Fe were reported to occur in the hippocampus and amygdala regions with severe histopathologic alterations in AD.[187] Transition metals such as Cu, Zn, and Fe are known to play important roles in neural functions. However, metal levels and transportation are stringently regulated because abnormal metal homeostasis can lead to neurotoxic free radical generation. For instance, Fe or Cu at very high levels can directly interact with oxygen, generating $O_2^{\bullet-}$, H_2O_2, and $^{\bullet}OH$ and possibly resulting in oxidative stress as well as a cascade of biochemical alterations that eventually leads to neuronal cell death.[188] Furthermore, studies have also reported the presence of these transition metals within the amyloid deposits of patients suffering from AD and transgenic mouse models.[189,190]

Parkinson's Disease

PD is one of the major neurological disorders that are very prevalent among individuals above 65 years of age.[191] The PD is a progressive neurodegenerative disorder that affects, primarily, dopamine neurones that originate from the midbrain and extend to the putamen and caudate regions (the striatum), which are regions that are responsible for the control of motor movements.[181] The PD is characterised by loss of dopaminergic neurones in the SNpc. Oxidative stress promotes dopaminergic neurotoxicity. Complex I deficiencies of the respiratory chain contribute

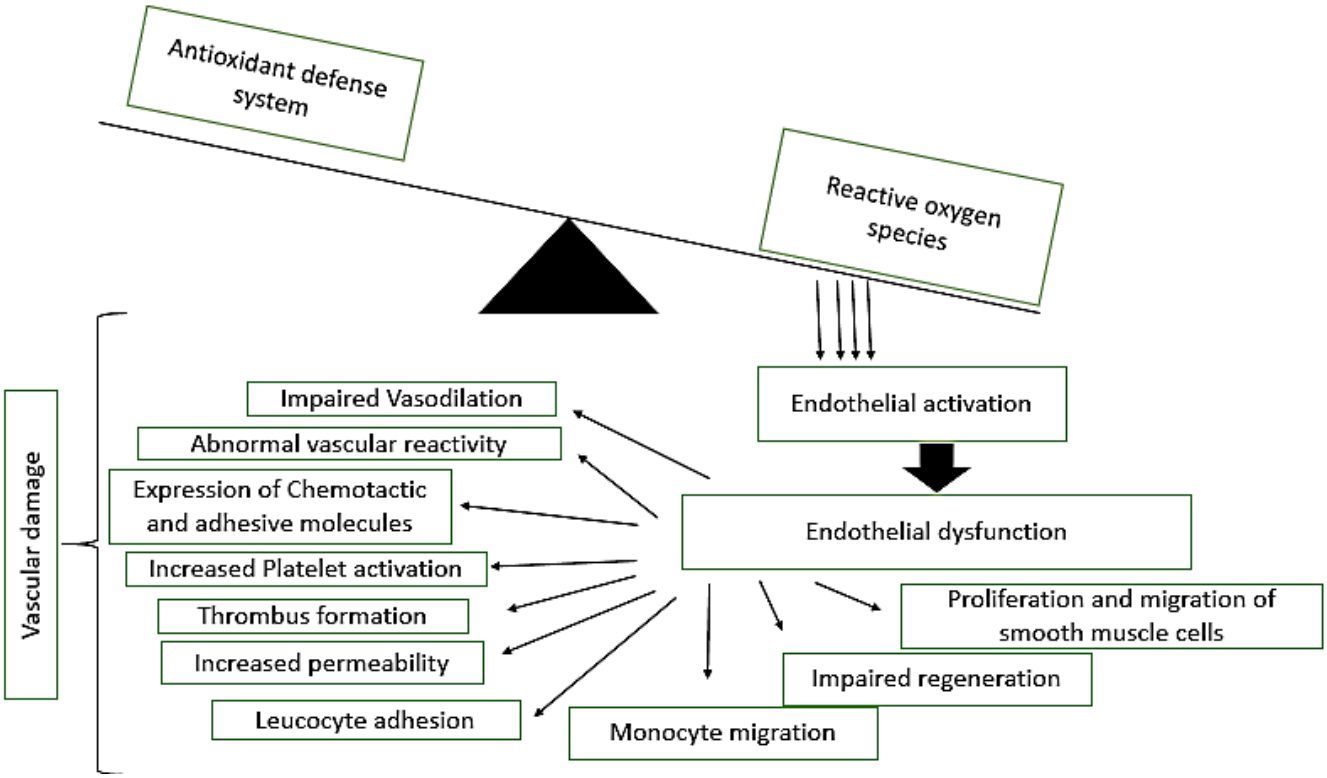

Figure 8: Endothelial dysfunction leading to vascular damage initiated by oxidative stress.[121]

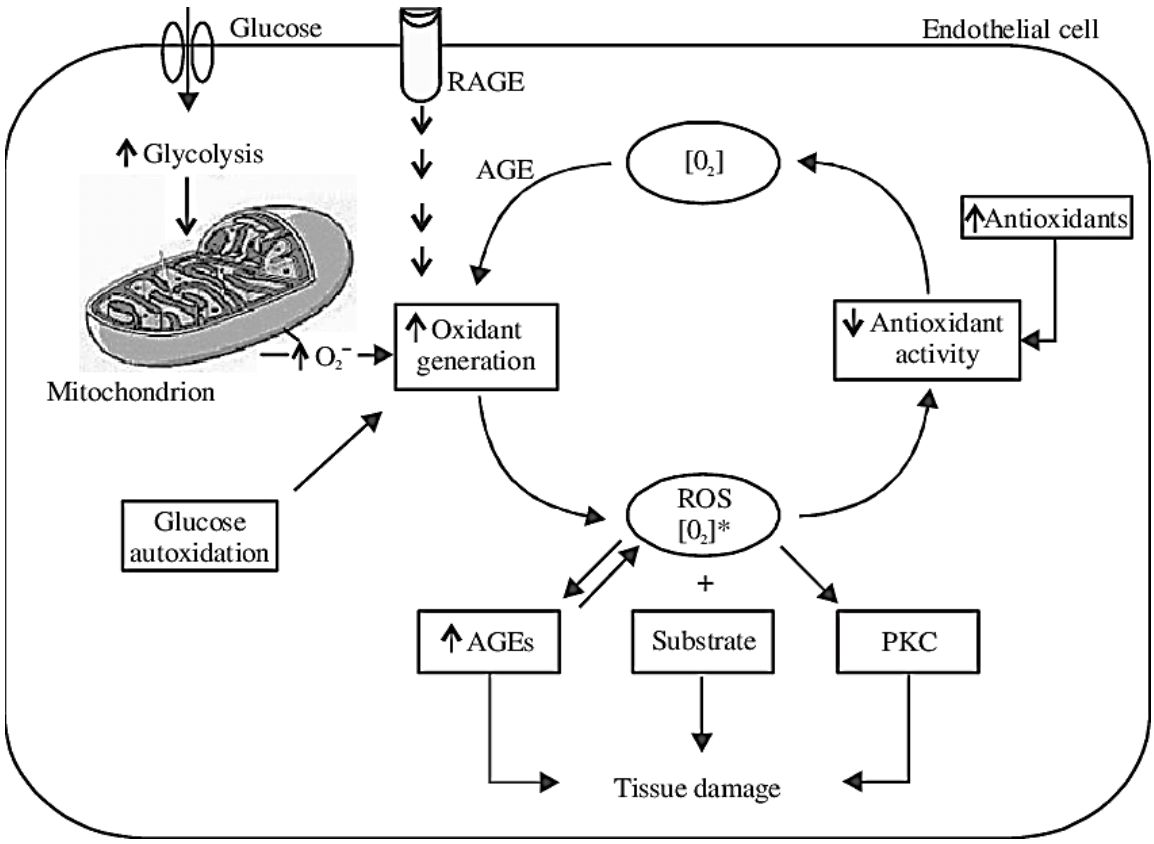

Figure 9: Oxidant formation in diabetes mellitus and the concomitant oxidative stress leading to oxidative cellular damage.[110,137]

significantly to most of the adverse neuronal degeneration in PD. Dopamine, neurotoxins, and mutations of genes in PD-associated proteins play a major role in mitochondrial dysfunction, which leads to ROS generation (Figure 14).[192]

The metabolism of dopamine, which is catalysed by monoamine oxidase, is enhanced in PD, and excess H_2O_2 is generated in the process (Figure 14). The polymerisation of the auto-oxidative products of dopamine can possibly initiate the production of the characteristic pigmentation of the substantia nigra. These free radicals generated might be responsible for the loss of dopaminergic neurons.[181]

Mitochondrial dysfunction enhances the formation of ROS in PD.[193] Oxidative phosphorylation supplies most of the energy required to trigger neural activity in which the mitochondria use their structure, enzymes, and energy generated through the oxidation of nutrients to produce ATP.[194] However, this metabolic pathway that occurs in the mitochondria is the major source of $O_2^{\bullet-}$ and H_2O_2.[192]

Complex I deficiencies of the respiratory chain contribute significantly to unfavourable neural apoptosis and are seen as one of the major sources of ROS in PD. The inhibition of complex I results in increased ROS generation. Reports showed a decreased activity of complex I in the SNpc of sporadic PD patients.[107,195,196] Furthermore, studies have reported mitochondrial complex I deficiency in various brain regions, skeletal muscle, fibroblasts, lymphocytes, and blood platelets of PD patients.[197-202]

Other evidence that supports the relationship between mitochondrial dysfunction and oxidative stress as well as dopamine cell damage arises from discoveries that mutations in genes of proteins such as parkin, PINK, α-syn, or DJ-1 are associated with familial forms of PD.[203,204] Generally, these findings suggested that mutations in these genes altered the function and integrity of the mitochondria and triggered oxidative stress (Figure 14).[205]

According to Wang *et al.*[206] PINK-1 plays a major role in the maintenance of normal morphology of mitochondrial SNpc dopamine neurones and eliciting a neuroprotective effect by suppressing ROS generation. Studies carried out using animal models showed that deficiency of PINK-1 resulted in impaired mitochondrial morphology, decreased activity of complex I, loss of SNpc dopamine neurones, and increased oxidative stress.[207-209] The accumulation of α-syn as intracellular filamentous aggregates serves as a pathological indicator of sporadic and familial PD.[210] Also, accumulation of α-syn wild-type in dopamine neurones decreased mitochondrial complex I activity with increased ROS generation, and cell death ensues.[211] Dendritic mitochondrial oxidative stress was increased by α-syn inclusions in dopamine neurones in the study carried out by Dryanovski *et al.*[212] Oxidative stress has been reported to promote influx, assemblage, and oligomerisation of extracellular α-syn in oligodendrocytes and

promote posttranslational modifications of α-syn, which may lead to increased dopamine toxicity.[213,214]

Aging

Ageing is a gradual and complex event that is associated with progressive and permanent deterioration of the cells, tissues, organs, and the whole organism itself and can be referred to as pathological conditions that have effects on the individual's quality of life.[215]

According to the free radical theory of ageing, which was postulated about 60 years ago, ROS is recognised as the main cause of impairments of the cells and responsible for ageing and ageing-associated degenerative diseases.[216] Although there are many theories of ageing today, oxidative stress remains a causative factor.[217] In the study by Kumsta *et al.*[218] Caenorhabditis elegans was subjected to oxidative stress via sub-lethal short treatment of H_2O_2 stress. The report showed that most of the worms exhibited symptoms of severe but reversible behavioural changes that are evidence of common age-related changes, such as reduction in body movement, decreased pharyngeal pumping, low reproduction rate, morphological changes, and decline in metabolic activity. These findings were in accordance with the Harman free radical theory of ageing.[218]

The mechanism through which oxidative stress elicits cell aging process is as follows: (1) the metabolism of the cell causes the formation of ROS, which is followed by oxidative damage; (2) the macromolecule mostly affected by oxidative stress are proteins as they undergo various modifications in order to prevent being properly degraded and recycled by the proteasome and protein function is altered in the process; (3) cytoskeletal proteins are also affected by oxidative stress, leading to structural impairment and signaling alterations; (4) oxidative stress further disrupts energy generation in the mitochondria; (5) the peroxisomes are affected by oxidative stress, which is accompanied by alteration of normal metabolic functioning; (6) the cellular membrane is also affected. (7) Finally, all these processes disrupt the cell's transcriptional activity and cause altered gene expression, which leads to the cell ageing process with concomitant degenerative diseases (Figure 15).[215]

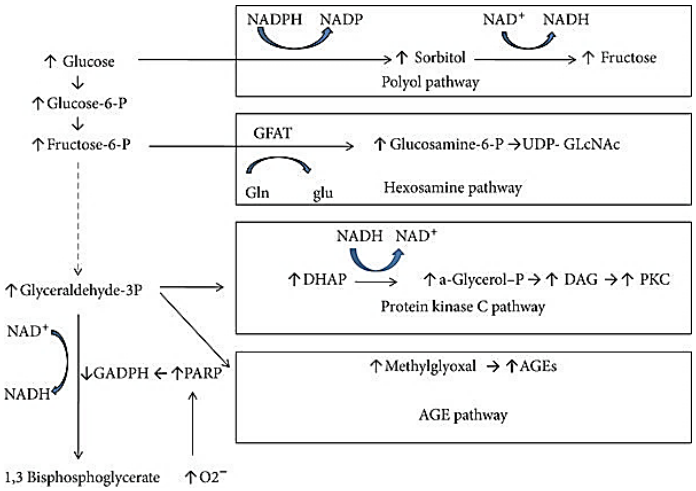

Figure 10: High levels of superoxide generation in the mitochondria under hyperglycemic conditions leading to the activation of four major pathways associated with hyperglycemic damage through the suppression of GAPDH.[141]

NADH: Nicotinamide adenine dinucleotide; NADPH: Nicotinamide adenine dinucleotide phosphate; AGE: Advanced glycation end-product; GFAT: Glutamine: fructose-6-phosphate amidotransferase; GAPDH: Glyceraldehyde-3 phosphate dehydrogenase; PARP: Poly-ADP-ribose polymerase; DAG: Diacylglycerol; UDP-GlcNAc: UDP-N-acetylglucosamine

Sickle Cell Disease

Oxidative stress contributes to multiple pathophysiologic mechanisms, such as endothelial damage,[219,220] hypercoagulability,[221] reduced NO• bioavailability,[222] and increased haemolysis[223] in SCD, which eventually trigger SCD-related vaso-occlusion and chronic organ damage (Figure 16).[224-226] A cross-sectional study carried out by Atiku *et al.*[227] to compare the levels of oxidative stress biomarkers in plasma samples from SCD and healthy control participants showed elevated levels of the ROS biomarker malondialdehyde in SCD individuals in comparison with the healthy control subjects. Higher levels of •OH and NO• were also identified in the plasma of SCD children than those of control groups by Biswal *et al.*[228]

In SCD, the endothelium is the main target of oxidative stress due to its nearness to cell-free haemoglobin and regions of ischaemia-reperfusion injury. Many oxidative stress-related mechanisms are involved in endothelial damage, which contributes significantly to SCD-related chronic organ complications such as pulmonary hypertension, cerebral vasculopathy, and retinopathy (Figure 16).[220,225,226] The ROS incur direct damage on endothelial cells through oxidative reactions with (membrane) lipids, peptides, and nucleic acids.[226,229] The oxidative events lead to abnormal enlargement of the endothelial cells, resulting in their separation from the underlying basement membrane and exposure of sub-endothelial structures as well as proteins and TF, which supports hyper-coagulation associated with SCD.[230] Furthermore, by inducing NF-κB, ROS triggers gene expression and pro-inflammatory cytokine generation, such as IL-1, IL-6, and TNF-α, as well as adhesion molecules, namely, VCAM-1 and ICAM-1,[231] which eventually enhances a pro-inflammatory state. The enhanced expression of endothelial adhesion molecules might play a role in vaso-occlusive events in SCD through the attachment of sickle erythrocytes, leukocytes, as well as platelets to the endothelium.[232]

Decreased bioavailability of NO• is a major feature of SCD, and this component is essential for vaso-dilatative, anti-inflammatory, and antithrombotic functions.[233-236] Oxidative stress plays a major role in the reduced bioavailability of NO• in SCD, which takes place as follows: the $O_2^{\bullet-}$ binds to NO•, leading to the formation

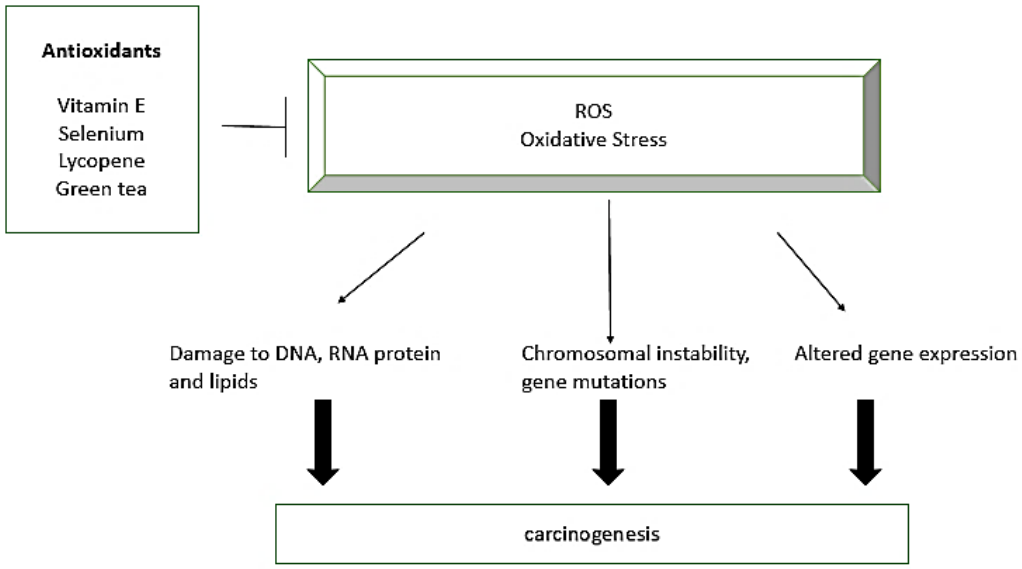

Figure 11: The role of reactive oxygen species in the development of human cancer.[152]

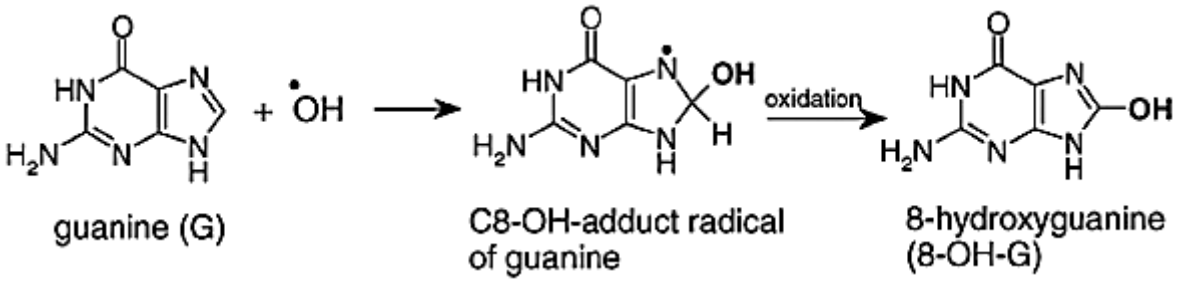

Figure 12: 8-OHdG formation by hydroxyl radicals.

of the potent oxidant $ONOO^-$ and its conjugate, ONOOH, and thereby further reduces the bioavailability of $NO^•$ and aggravates oxidative stress.[237] Oxidative stress also reduces $NO^•$ bioavailability by contributing to the formation of the eNOS inhibitor ADMA, which is involved in SCD-related pulmonary hypertension and early death.[222]

Increased intra- and extra-erythrocytic oxidative stress enhances intravascular haemolysis by triggering lipid peroxidation as well as membrane instability.[238] High OOH levels cause the degradation and deformity of the erythrocyte membrane.[239] Oxidative stress also plays a role in Ca^{2+}-dependent scramblase-mediated disruption of normal asymmetrical distribution of membrane phospholipids, which causes phosphatidylserine to be exposed at the outer surface of sickle erythrocytes.[240] Sickle erythrocytes whose phosphatidylserines are exposed are hydrolysed by secretory phospholipase A2, leading to increased intravascular haemolysis.[241]

Chronic Respiratory Diseases

CRDs refer to a group of chronic diseases that affect the airways and connecting regions of the lungs. Some of the most common CRDs that are associated with oxidative stress include bronchial asthma, COPD, pulmonary fibrosis, and lung cancer.[242] The sources and pathophysiological roles of ROS in CRDs are shown in Figure 17.

Bronchial asthma is a chronic airway disease that is associated with airway eosinophilia, goblet cell hyperplasia accompanied by mucus hypersecretion, and hyper-responsiveness to inhaled allergens and non-specific stimuli, which promote increased vascular permeability leading to plasma exudation.[244-246] Raised levels of ROS play a significant role in the contraction of airway smooth muscle, alteration in normal functioning of β-adrenergic receptor, reduction in the amount of the epithelial cilia and its function, increased generation of mucus, impairment in the release of inflammatory mediators, inflammatory cells influx, as well as an increase in vascular permeability.[247] Additionally, raised levels of ROS initiate bronchial hyperreactivity associated with asthma. Empirical studies using animal models reported that ROS play a significant role in airway hyper-responsiveness by inducing an increase in vagal tone due to the impairment of oxidant-sensitive β-adrenergic receptors and reduction of mucociliary clearance.[248,249] The study by Henricks and Nijkamp,[247]

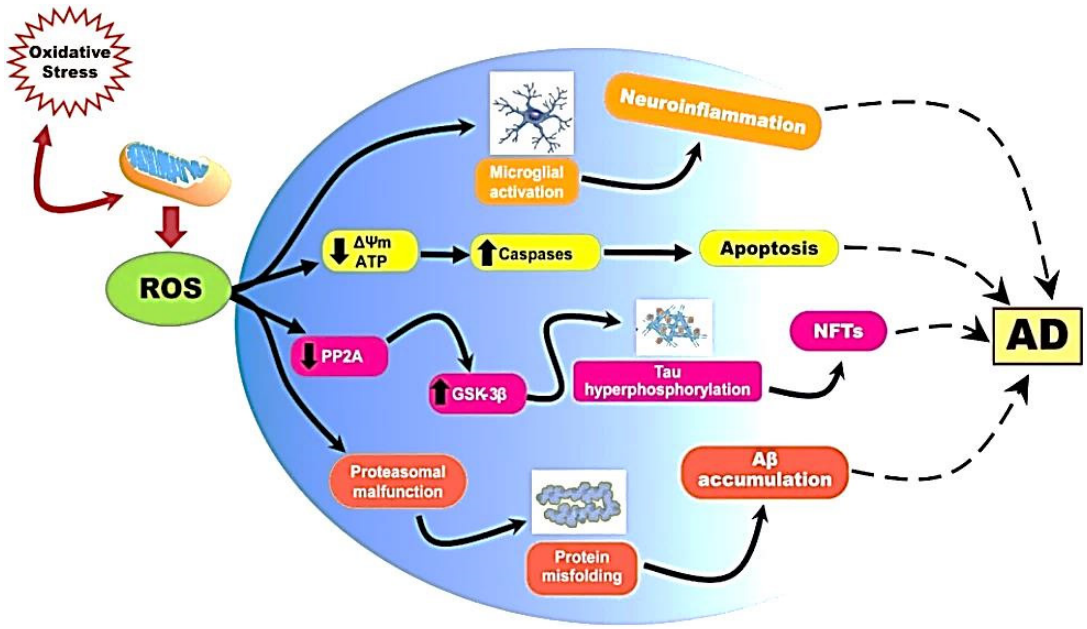

Figure 13: Impairments induced by reactive oxygen species in Alzheimer's disease.[177]

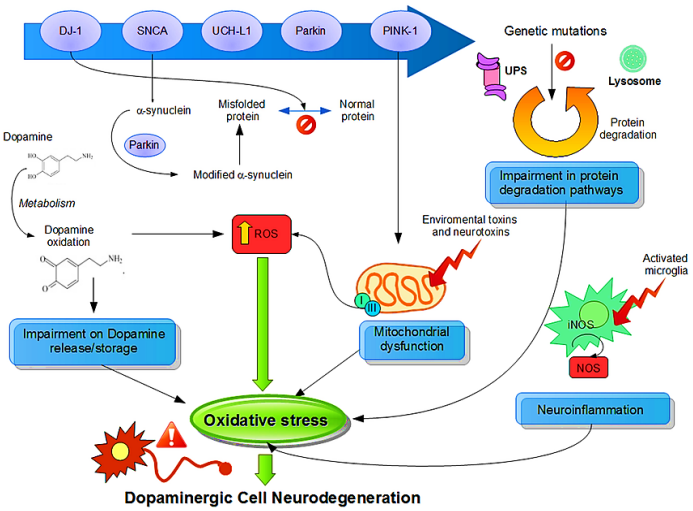

Figure 14: Different pathways and their dysfunctions resulting from dopamine metabolism and genetic modifications in Parkinson's disease-related genes and their relationship with oxidative stress.[192]

reported that ROS are involved in endothelial barrier dysfunction and increased permeability to fluid, macromolecules, and inflammatory cells. Previous studies on alveolar macrophages of asthmatic subjects reported higher generation of $O_2^{\bullet-}$ as well as other ROS when compared with those of the healthy controls.[250,251] More ROS was produced from the eosinophils, neutrophils, and alveolar macrophages of asthmatic patients than those of the normal control subjects.[252,253]

A COPD is typically associated with progressive, irreversible limitation in the expiratory airflow as well as abnormal inflammation of the lung, which is characterised by chronic inflammation of the airway's parenchyma and pulmonary vasculature, in conjunction with elevated levels of neutrophils, macrophages, and T lymphocytes (especially CD8+).[254-256] The pathophysiology of oxidative stress in COPD is complex.

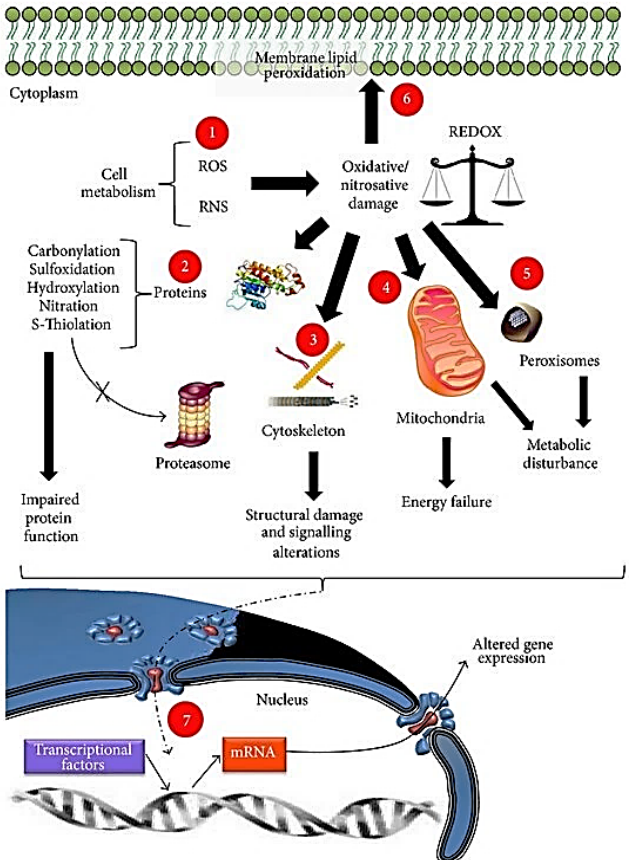

Figure 15: Impairments triggered by oxidative stress that lead to cell aging and age-related diseases.[215]

Persistent oxidative stress was observed in the lungs of COPD

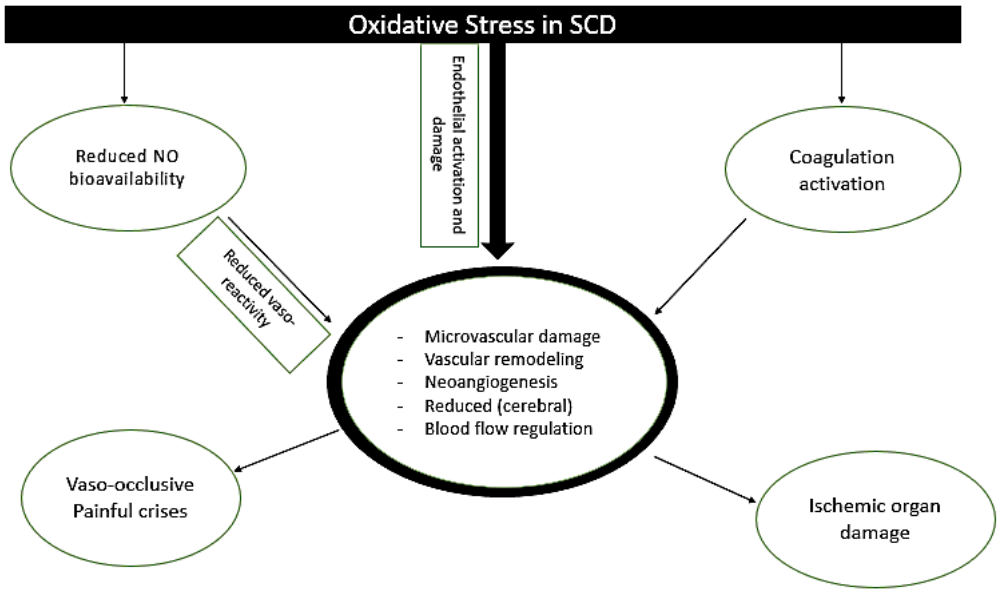

Figure 16: Pathophysiological effect of oxidative stress on sickle cell disease complications.[226]

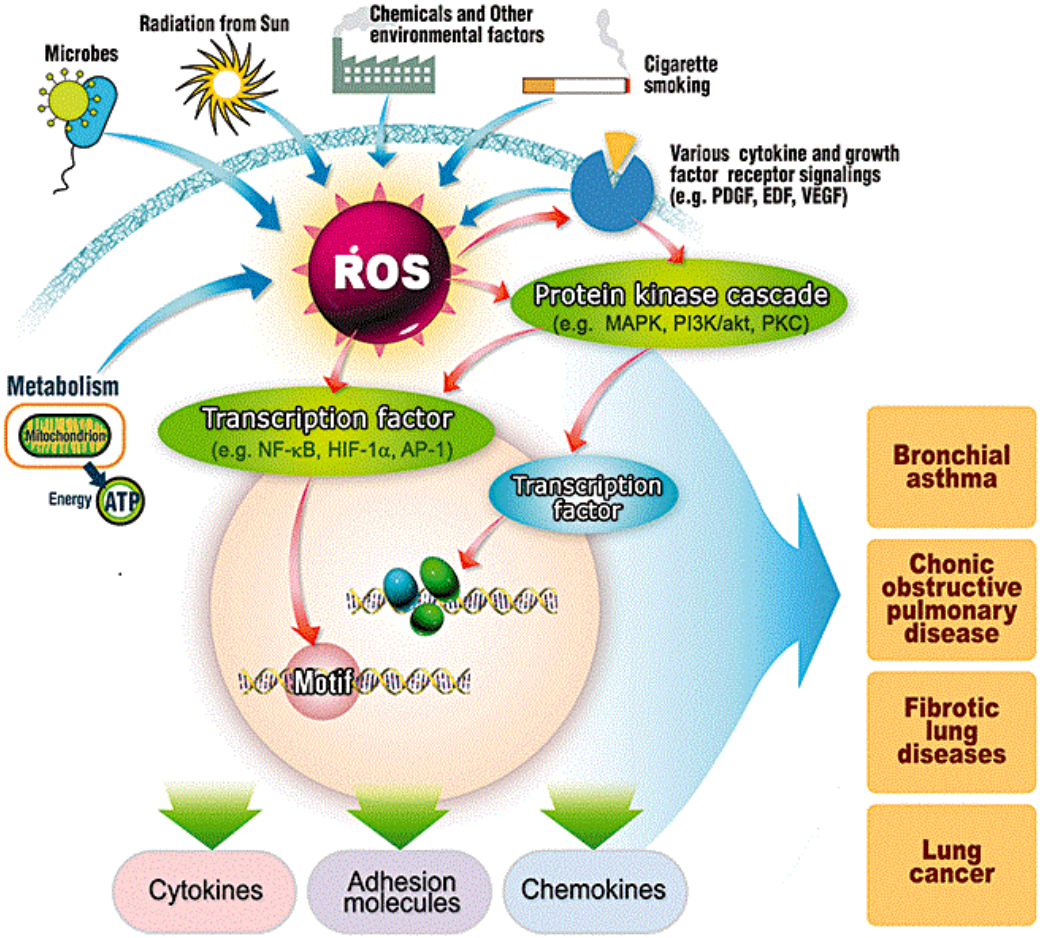

Figure 17: Sources and overall pathophysiological role of reactive oxygen species in chronic respiratory diseases.[243]

patients with higher levels of pro-inflammatory cytokine levels, CD4 and CD8 cells, and proteases, as well as increased apoptosis and senescence.[257-263] Oxidative stress-activating mechanisms are involved in COPD initiation and progression.[264] Oxidative stress biomarkers such as H_2O_2 and NO were seen to be present in the epithelial lining fluid, urine, and breath of the smokers of cigarettes and patients suffering from COPD.[254,165-167] An increase in the generation of $O_2^{\bullet-}$ and upregulation of adhesion molecules were seen in the circulating neutrophils of patients suffering from COPD.[268,269]

Pulmonary fibrosis is the outcome of a diverse group of lung impairments. There are suggestions that fibrotic stimuli of unknown origin initiate a state of disequilibrium between the generation of oxidants and antioxidant protection, leading to ROS accumulation.[270] Oxidants may contribute to the progress of pulmonary fibrosis through the generation of cytokines as well as growth factors such as TGF-β, which is a major regulator of aberrant repair mechanisms that are characteristic of various fibrotic diseases such as IPF. TGF-β reduces the rate of generation of the natural cellular antioxidant by ameliorating the expression of both CAT and mitochondrial SOD, which further encourages the production of ROS through the activation of NADPH oxidases and/or mitochondrial dysfunction.[157,271-274] Studies on human subjects suffering from IPF showed increased levels of oxidised proteins.[275-277] The protective efficacy of antioxidant enzyme systems against lung fibrosis has been reported.[278,279] The study by Rahman *et al.*[280] reported lower antioxidant efficacy in IPF subjects in comparison to healthy subjects.

The ROS have been reported to induce oncogenes such as Jun and Fos. Overexpression of Jun is significantly associated with the occurrence of lung cancer.[281,283] For instance, in lung cancers, p53, which is involved in the generation of ROS, undergoes mutation, and becomes defective in the activation of apoptosis. The p53 accumulates in the cytoplasm and functions as an oncogene after the mutation.[283]

CONCLUSION

The roles of free radicals in pathogenesis are irrefutable and well established. And therefore, the neutralisation and/or scavenging of intracellular free radicals are sustainable therapeutic strategies for the prevention and amelioration of oxidative stress and associated pathological conditions and their complications. Antioxidants prevent cellular damage caused by free radicals by inhibiting the generation and scavenging of radicals or promoting their degradation. Some actions of free radicals impair cellular activities that elicit chronic diseases such as CVD, DM, cancer, AD, PD, and aging. The proposed therapeutic strategy, whereby overexpression of enzymatic antioxidants was provoked in a mutant mice model in a tissue-specific manner, could serve as a novel alleviative measure against the development of pathological conditions associated with oxidative stress.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

AAPH: 2,2'-azobis (2-amidinopropane) dihydrochloride; **ABAP:** 2,2′-azobis-(2-amidopropane); **AD:** Alzheimer's disease; **ADMA:** Asymmetric dimethylarginine; **AGEs:** Advanced glycation end products; **AscH$^-$:** Ascorbic acid; **BHA:** Butylated hydroxyl anisole; **BHT:** Butylated hydroxyl toluene; **CAT:** Catalase; **CBA:** Crocin-bleaching assay; **-CH_2-:** Methylene; Cl^-: Chloride ion; **$CO_3^{\bullet-}$:** Carbonate radical; COPD: Chronic obstructive pulmonary disease; **CRDs:** Chronic respiratory diseases; **Cu:** Copper; **CVD:** Cardiovascular diseases; **DAG:** Diacylglycerol; **DPPH:** 2,2-Diphenyl-1-picrylhydrazyl; **eNOS:** NO$^\bullet$ synthase; **ERK:** Extracellular signal-regulated kinase; **ETC:** Electron transport chain; **Fe:** Iron; **GAPDH:** Glyceraldehyde-3-phosphate dehydrogenase; **GFAT:** Glutamine: fructose-6-phosphate amidotransferase; **GPx:** Glutathione peroxidase; **GR:** Glutathione reductase; **GSH:** Glutathione; **GSK:** Glycogen synthase kinase; **GSSG:** Glutathione disulphide; **GST:** Glutathione *S*-transferase; **H_2O_2:** Hydrogen peroxide; **HAT:** Hydrogen atom transfer; **HMWA:** High molecular weight antioxidants; **HOCl:** Hypochlorous; **HS-GC:** Headspace gas chromatography; **ICAM-1:** Intercellular adhesion molecule-1; **IPF:** Idiopathic pulmonary fibrosis; **JNK:** JUN N-terminal kinase; **KMBA:** α-keto-γ-methylthiobutyric acid; **LDL:** Low-density lipoprotein; **LMWA:** Molecular weight antioxidants; **LOOH:** Lipid hydroperoxide; **MDA:** Malondialdehyde; **Mn:** Manganese; **Mn-SOD:** Manganese-superoxide dismutase; **MPO:** Myeloperoxidase; **MPO:** Myeloperoxidase; **NADH:** Nicotinamide adenine dinucleotide; **NADPH:** Nicotinamide adenine dinucleotide phosphate; **NDGA:** Nor-dihydroguaiaretic acid; **NFTs:** Neurofibrillary tangles; **NF-κB:** Nuclear factor-κB; **NMDA:** N-methyl-D-aspartate; **$^\bullet NO_2$:** Nitrogen dioxide radical; **NO$^\bullet$:** Nitric oxide; **NO_2Cl:** Nitryl chloride; **Nrf2:** Nuclear factor-erythroid 2-related factor 2; **$^\bullet$OH:** Hydroxyl radical; **$^\bullet$OOH:** Hydroperoxide; **$^1O_2$:** Singlet oxygen; **$O_2^{\bullet-}$:** Superoxide anion radical; **$ONOO^-$:** Peroxynitrite; **$ONOOCO_2^-$:** Nitrosoperoxycarbonate; **ONOOH:** Peroxynitrous acid; **ORAC:** Oxygen radical absorbance capacity; **PARP:** Poly-ADP-ribose polymerase; **PD:** Parkinson's disease; **PG:** Propyl gallate; **PKC:** Protein kinase C; **`PP2A:** Phosphatase 2A; **RAGE:** Receptor for AGEs; **RNS:** Reactive nitrogen species; **RO$^\bullet$:** Alkoxyl radical; **$RO_2^{\bullet-}$:** Peroxyl radicals; **ROS:** Reactive oxygen species; **RSS:** Reactive sulphur species; **SCD:** Sickle cell disease; **SDS:** Sodium

dodecyl sulfate; **Se:** Selenium; **SET:** Single electron transfer; **SNpc:** Substantia nigra pars compacta; **SOD:** Superoxide dismutase; **TBARS:** Thiobarbituric acid reactive substance; **TBHQ:** Tertiary butyl hydroquinone; **TCA:** Tricarboxylic acid; **TGF-β:** Transforming growth factor-β; **TOSCA:** Total oxyradical scavenging capacity assay; **TRAP:** Total radical-trapping antioxidant parameter; **TrxR:** Thioredoxin reductase; **Tyr:** Tyrosine; **UDP-GlcNAc:** UDP-N-acetylglucosamine; **VCAM-1:** Vascular cell adhesion molecule-1; **Zn:** Zinc; **γ-GGT:** γ-Glutamyl transpeptidase; **8-OHdG:** 8-hydroxy-2′-deoxyguanosine.

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