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Oxidative Stress: Mechanisms, Quantification and its role in human aging

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ABSTRACT

Oxidative stress refers to the imbalance between the production of oxidant species and the body's ability to quench them using antioxidants, favoring the rise in oxidant levels. This leads to the damage of cellular macromolecules such as lipids, DNA, RNA, and proteins. The body's ability to manage oxidative stress and maintain it at an optimum level is crucial for overall health. Oxidative damage, if left unmitigated, contributes to the aging process characterized by the progressive deterioration of physiological functions and cellular structures. Understanding the mechanisms of oxidative stress along with its reliable quantification can enable consistency and comparability in clinical practice across diseases. While direct quantification of oxidant species in the body would be ideal for assessing oxidative stress, it is not feasible owing to their high reactivity, short half-life, and quantification challenges using conventional techniques. Quantifying oxidative damage products and antioxidants pose as appropriate markers, indicating the degree of oxidative stress in the body. This review comprehensively discusses the mechanism of generation of key oxidant species, their sources, the beneficial roles played by them at low levels and the detrimental effects exerted by their elevated levels. The review also provides insights into the effective quantification techniques for damage products of lipids nucleic acids, and proteins along with the endogenous and exogenous antioxidant markers. Effective quantification of oxidative stress may improve our understanding on the phenomenon which may aid in maintaining cellular integrity, preventing age-associated diseases, and thereby promoting optimal well-being and longevity.

Keywords: oxidative stress, reactive oxygen species, reactive nitrogen species, free radicals, antioxidants, lipid peroxidation

INTRODUCTION

'Oxidative stress' is a term that was first coined by the German physician, Helmut Sies as an imbalance between the production of oxidants and antioxidant defenses that may result in damage to biological systems [1]. Since then, the phenomenon has been extensively studied, as it has been implicated in a wide range of diseases, including cancer, neurological disorders, atherosclerosis, hypertension, ischemia, diabetes, acute respiratory distress syndrome, idiopathic pulmonary fibrosis, chronic obstructive pulmonary disease, and asthma [2]. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are the key players contributing to oxidative stress generated intrinsically from normal cellular metabolism, and extrinsically, from environmental factors such as toxins, UV radiation, or cigarette smoke [1].

Additionally, biological processes such as oxidative phosphorylation, activation of several transcriptional factors, apoptosis, immunity, cell differentiation, and amino acid synthesis produce ROS and RNS [3,4]. ROS and RNS can be divided into two groups: free radicals and nonradicals. The molecules that contain one or more unpaired electrons contributing to their reactivity are called 'free radicals.' On the other hand, when two free radicals share their unpaired electrons, then 'nonradical forms' are created [2]. The ROS that are physiologically relevant include superoxide anion radicals ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radicals ($^{\bullet}OH$), and singlet oxygen ($^1O_2$), which are generally present in cells at low levels [3]. The human body has an integrated antioxidant system comprising enzymatic and nonenzymatic antioxidants that help combat the harmful effects of ROS and RNS [2]. Superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) are the primary enzymatic antioxidants present in cells that help to protect cells from ROS-induced damage [3]. The secondary enzymatic antioxidants, such as the thioredoxin system and glutaredoxins are important in maintaining cellular redox balance and repairing oxidized products [2]. The nonenzymatic antioxidants would include low-molecular-weight compounds such as vitamins (vitamins A, C and E), b-carotene, uric acid (UA), alpha-lipoic acid, and glutathione (GSH), a tripeptide (L-g-glutamyl-L-cysteinyl-L-glycine) that comprise a thiol (sulfhydryl) group. While the primary antioxidants inhibit and scavenge oxidant formation, the other antioxidants in the body scavenge oxidants as well as repair the oxidized molecules [5].

An imbalance in the oxidant and antioxidant entities favouring the increase in oxidants, coupled with the body's inability to salvage oxidized molecules, leads to oxidative stress. It has damaging effects on various cellular structures like proteins, lipids, and nucleic acids, which ultimately lead to various pathological conditions [3]. Understanding the interplay between oxidant and antioxidant systems will help in studying oxidative stress-mediated diseases and will provide a rationale for improving therapeutic approaches to antioxidant defenses.

OXIDANT SPECIES

The fundamental process of energy production in the mitochondria is known to generate free radicals. When oxygen is used to produce adenosine triphosphate (ATP) in the body, ROS and RNS are produced as by-products owing to the cellular redox process [6]. ROS and RNS are collectively called 'free radicals.' Free radicals have one or more unpaired electrons in their outer shells. They are formed via the breakage of chemical bonds in a molecule such that each fragment keeps one electron, by cleavage of a radical to form another radical, and via redox reactions [6]. Free radicals are highly unstable molecules that have unpaired electrons readily available to react with various organic substrates such as lipids, proteins, and DNA [6]. Free

radicals include $O_2^{\bullet-}$, $^{\bullet}OH$, peroxyl ($ROO^{\bullet}$), nitric oxide ($^{\bullet}NO$), and nitrogen dioxide ($^{\bullet}NO_2$) [6]. On the other hand, the non-free radical species include H_2O_2, hypochlorous acid (HOCl), hypobromous acid (HOBr), ozone (O_3), $^1O_2$, nitrous acid (HNO_2), nitrosyl cation (NO^+), nitroxyl anion (NO^-), dinitrogen trioxide (N_2O_3), dinitrogen tetraoxide (N_2O_4), nitronium (nitryl) cation (NO_2^+), hydroperoxides (ROOH), aldehydes (HCOR), and peroxynitrite ($OONO^-$) [7]. These species can lead to free radical reactions in living organisms [7].

Production mechanisms of oxidant species

Free radical oxidants

Free radicals can be generated via enzymatic and non-enzymatic reactions. The **superoxide anion radical ($O_2^{\bullet-}$)** is a major ROS formed enzymatically via the action of xanthine oxidase, lipooxygenase, cyclooxygenase, and NADPH-dependent oxidase [6,7]. It can also be produced by nonenzymatic electron transfer reactions, in which an electron is transferred to molecular oxygen (O_2) [7]. It can exist in two forms, $O_2^{\bullet-}$ at physiological pH or hydroperoxyl radical (HO_2) at low pH [7]. The HO_2 form is the most important one, as it can easily penetrate the phospholipid bilayer compared to the charged form ($O_2^{\bullet-}$). The $O_2^{\bullet-}$ can react with another $O_2^{\bullet-}$ in a dismutation reaction (Eq. 1), in which one radical is oxidized to O_2 and the other is reduced to H_2O_2 [7].

$$O_2^{\bullet-} + O_2^{\bullet-} + 2H_2O \xrightarrow{\text{Cu, Zu, and Mn-SOD}} H_2O_2 + O_2 \ldots \text{(Equation 1)}$$

The **hydroxyl radical ($^{\bullet}OH$)** is the neutral form of the hydroxide ion and is a highly reactive free radical [7]. It is produced via a Fenton reaction (Eq. 2), wherein H_2O_2 reacts with the metal ions Fe^{+2} or Cu^+. These metal ions are often bound in complexes with different proteins, such as ferritin (an intracellular protein that stores iron), ceruloplasmin (a plasma copper-carrying protein), or other molecules [7]. Under physiological stress, excess $O_2^{\bullet-}$ releases free iron from ferritin. The released free iron participates in the Fenton reaction to form $^{\bullet}OH$. $^{\bullet}OH$, can also be formed by the reaction between $O_2^{\bullet-}$ and H_2O_2 in a reaction called the Haber-Weiss reaction (Eq. 3) [7]. $^{\bullet}OH$, can strongly react with both organic and inorganic molecules, including DNA, proteins, lipids, and carbohydrates [2,7].

$$Fe^{+2} + H_2O_2 \rightarrow Fe^{+3} + {}^{\bullet}OH + OH^- \text{ (Fenton reaction) } \ldots \text{ (Equation 2)}$$

$$O_2^{\bullet-} + H_2O_2 \longrightarrow {}^{\bullet}OH + OH^- \text{ (Haber-Weiss reaction) } \ldots \text{ (Equation 3)}$$

The **peroxyl radical ($ROO^{\bullet}$)** is derived from O_2 in living systems. Its simplest form is the per hydroxyl radical ($HOO^{\bullet}$), which is formed by the protonation of $O_2^{\bullet-}$. It can induce lipid peroxidation (Eq. 4) [7].

$$O_2^{\bullet-} + H_2O \rightleftharpoons HOO\bullet + OH^- \ldots \text{(Equation 4)}$$

Nitric oxide synthases (NOS) convert L-arginine to L-citrulline in tissues to yield a small molecule called **nitric oxide ($^{\bullet}NO$)** (Eq. 5). The reaction involves the oxidation of one of the terminal guanido nitrogen atoms to give $^{\bullet}NO$ [7]. There are three isoforms of NOS, including neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS). All three forms aid in the formation of the $^{\bullet}NO$. As $^{\bullet}NO$ is water and lipid-soluble, it can readily diffuse through the cytoplasm and plasma membrane [7]. $^{\bullet}NO$ is known to be a multifaceted molecule capable of having pro-oxidant as well as oxidant-protective effects. It is a crucial signalling molecule

as it is a vasodilator that helps maintain endothelial function [8]. It also has important immune functions, which will be discussed in detail in the later sections. The underlying oxidative status of a tissue is a key for determining $^{\bullet}NO$ function. If $^{\bullet}NO$ is in excess among other oxidants, then lipid oxidation and monocyte margination into the vascular wall will be attenuated, producing antiatherogenic effects. However, when endogenous tissue oxidant levels are high, $^{\bullet}NO$ can react with them to produce secondary oxidizing species that can promote membrane and lipoprotein lipid oxidation, which may further have proatherogenic effects [8].

L-Arginine + O_2 + NADPH ⟶ L-Citrulline + $^{\bullet}NO$ + $NADP^+$… (Equation 5)

Nitrogen dioxide ($^{\bullet}NO_2$) is not produced in the body as a free radical. It is a common atmospheric pollutant produced from external sources such as combustion processes and by bacterial action [9]. It is also a constituent of tobacco smoke. It can be produced in aqueous systems by the acid decomposition of nitrite (NO_2^-) and by exposure of nitrate (NO_3^-) or NO_2^- solutions to ionizing radiation [9]. $^{\bullet}NO_2$ is a strong oxidizing free radical and a toxic agent, owing to its capability to induce lipid peroxidation. It is also believed to lead to cell damage, followed by cell death [9].

Non-free radical oxidant species

Hydrogen peroxide (H_2O_2) is formed via the dismutation reaction catalysed by the enzyme, SOD (Eq. 1). As it is uncharged, it can easily penetrate the biological membranes and can cause cellular damage. It has no direct effect on DNA but can damage DNA by producing $^{\bullet}OH$ in the presence of transition metal ions [7].

The powerful oxidant **ozone (O_3)** is formed by the antibody-catalysed water oxidation pathway; an integral process occurring in all antibodies which is associated with inflammation [7]. O_3 can form other reactive species and can lead to lipid peroxidation. It can oxidize different functional groups in proteins and nucleic acids, including amine, alcohol, HCOR, and sulphydryl [7]. O_3 or O_3-mediated free radicals can cause chromosomal aberrations [7].

Singlet oxygen ($^1O_2$) is an electronically excited and meta-stable state of O_2 [7]. The activation of neutrophils and eosinophils (Eq. 6) or the enzymatic reactions catalysed by the enzymes, lipoxygenases, dioxygenases, and lactoperoxidase can lead to the formation of $^1O_2$ [7]. It is formed when the O_2 is excited to first state, $^1\Delta_g$, which is an extremely reactive state compared to the other higher electronically excited states [7]. It is a strong oxidizing agent, leading to DNA and tissue damage [7].

$HOCl + H_2O_2 \rightarrow {}^1O_2 + H_2O + Cl^-$… (Equation 6)

Peroxynitrite ($OONO^-$) is generated from the reaction between $O_2^{\bullet-}$ and $^{\bullet}NO$ [7,10] (Eq.7). Its reaction with carbon dioxide (CO_2) forms the reactive nitroso peroxo carboxylate ($ONOOCO_2^-$) or peroxynitrous acid (ONOOH) [7]. Homolysis of ONOOH forms both $^{\bullet}OH$ and $^{\bullet}NO_2$. It may also rearrange to form NO_3^-. $OONO^-$ oxidizes lipids, methionine, and tyrosine residues in proteins. Nitrotyrosine is a marker of $OONO^-$ [7]. $OONO^-$ also oxidizes DNA to form 8-nitroguanine, which is a marker of RNS-induced nitrative DNA damage [7]. These markers are discussed in the following sections.

$O^{\bullet-}2 + {}^{\bullet}NO \rightarrow OONO^-$ … (Equation 7)

The reaction of $^{\bullet}NO$ with O_2 and H_2O gives NO_3^- and NO_2^- ions. An electron oxidation of $^{\bullet}NO$ leads to the formation of a nitrosonium cation ($NO_2^{\cdot}+$), while an electron reduction results in $^{\bullet}NO$. These ions can react with $^{\bullet}NO$ to yield N_2O and $OH^{\cdot}$. $^{\bullet}NO$ reacts with radicals such as H_2O_2 and HOCl to give N_2O_3, $^{\bullet}NO_2$, and NO_3^- [7].

The halide oxidants **hypochlorous acid (HOCl) and hypobromous acid (HOBr)** are produced from H_2O_2, and the corresponding halide ions (Cl^- and Br^-) catalysed by the leukocyte-derived heme peroxidase enzymes myeloperoxidase (MPO) and eosinophil peroxidase (EPO), respectively [11, 12]. HOCl has important antibacterial properties and aids in immune function [13]. It takes part in a vital immune process called 'respiratory burst.' However, as HOCl is highly reactive, it can oxidize thiols and other biological molecules, including ascorbate, urate, pyridine nucleotides, and tryptophan. It chlorinates several amines to give chloramines; tyrosyl residues to give ring chlorinated products; cholesterol; and unsaturated lipids to give chlorohydrin. It can also chlorinate DNA [7]. HOBr can also readily react with amino acids, proteins, antioxidants including thiols, carbohydrates, lipids, and DNA [14].

Sources of oxidant species

Oxidant species can be produced from "endogenous" or "exogenous" sources. The endogenous sources are different cellular organs such as mitochondria, peroxisomes, and endoplasmic reticulum, where oxygen consumption is high, followed by the cytosol and plasma membrane [4]. Exogenous sources include external entities such as toxins, UV radiation, alcohol, tobacco smoke, certain medications, and so on [5].

Endogenous sources

Production of ROS

Metabolism

Mitochondria

The mitochondria are the organelles that produce the highest amount of intracellular ROS. They contribute to approximately 90% of cellular ROS generated in the body [15]. 0.2-2.0% of the O_2 consumed by mitochondria is reduced to $O_2^{\bullet-}$ [15]. Complex I (NADH dehydrogenase) and complex III (ubiquinone cytochrome c reductase) are the two major sites in the electron transport chain that produce super $O_2^{\bullet-}$. When electrons are transferred from complex I or II to coenzyme Q or ubiquinone (Q), the reduced form of coenzyme Q (QH_2) is formed. This reduced form of QH_2 regenerates coenzyme Q via an unstable intermediate semiquinone anion ($^{\cdot}Q^-$) in the Q-cycle. An immediate transfer of electrons from the formed $^{\cdot}Q^-$ to O_2 yields $O_2^{\bullet-}$. As this generation of $O_2^{\bullet-}$ is non-enzymatic, it has a higher metabolic rate, which leads to a greater production of ROS [16].

The other mitochondrial components that contribute to the formation of ROS include monoamino oxidase, α-ketoglutarate dehydrogenase, glycerol phosphate dehydrogenase, and p66shc [7]. p66Shc is a member of the adaptor protein family and is involved in lifespan regulation and apoptosis [17]. p66Shc is mostly located in the cytoplasm, with a small fraction localized in the mitochondrial intermembrane space; nevertheless, it can initiate the production of ROS in the mitochondria. During oxidative stress, p66Shc translocates to mitochondrial intermembrane space, where it associates with cytochrome-c, leading to ROS generation [7].

Peroxisomes

The respiratory pathway in peroxisomes involves the transfer of electrons from various metabolites to O_2, which leads to the formation of H_2O_2. The β-oxidation of fatty acids is the major process producing H_2O_2 in the peroxisomes [7]. The β-oxidation enzymes, acyl CoA oxidases, D-amino acid oxidase, L-α-hydroxy oxidase, urate oxidase, and D-aspartate oxidase produce H_2O_2 while xanthine oxidase produce H_2O_2, $O_2^{\bullet-}$, and $^{\bullet}NO$ [7,18]. The H_2O_2 inside peroxisomes may give rise to $^{\bullet}OH$ through the Fenton reaction. The presence of $^{\bullet}NO$ and $O_2^{\bullet-}$ kinetically and thermodynamically favours their reaction to form $OONO^-$ in the peroxisomes [18].

Endoplasmic Reticulum

In the endoplasmic reticulum, metabolic enzymes including cytochrome p-450 and b5 and diamine oxidase contribute to the formation of ROS. The thiol oxidase enzyme, Erop1p, catalyses the transfer of electrons from dithiols to O_2, resulting in the production of H_2O_2 [7].

Cytosol

In the cytosol, ROS can be formed via NADPH activity and can influence metabolic processes including glycolysis and downstream oxidative phosphorylation, pentose phosphate pathway activity, and autophagy [19].

Plasma membrane

The plasma membrane made up of the lipid bilayer is also crucial in producing free radicals as it is generally exposed to an oxidizing environment [4]. The production of $O_2^{\bullet-}$ by phagocytic cells occurs via the plasma membrane-localized, NADPH oxidase (NOX) [20]. Free radicals formed from the plasma membrane can, in turn, attack the fatty acyl chain or the head group of phospholipids in the lipid bilayer. ROS can also target the side chains of membrane proteins. ROS abstracting hydrogen from membrane lipids further leads to the formation of ROS, which, upon reaction with O_2, gives rise to peroxide-containing products. Hydrogen abstraction of unsaturated acyl chains can initiate a chain reaction that propagates to other lipids present in a bilayer. This reaction is generally amplified and can result in the formation of many lipid peroxides [21].

Inflammation

Inflammation is the primary response mounted by the immune system against invading pathogens or foreign substances. In the innate immune system, macrophages play a crucial role in eliminating pathogens via the generation of ROS, including $O_2^{\bullet-}$, H_2O_2, $^{\bullet}OH$, $^{\bullet}NO$, $OONO^-$, and HOCl. This process lasts until the pathogens are eliminated and the repair mechanisms have been completed. However, continued active inflammation can lead to cell damage or cellular hyperplasia caused by ROS overproduction from inflammatory cells. Chronic inflammation allows ROS to interact with DNA in mitotic cells, leading to recurrent DNA damage, which can increase the frequency of genomic mutations [22]. Additionally, these ROS also actively damage lipid and protein entities in the body.

Other sources of endogenous free radicals can be mental stress, excessive exercise, ischemia, cancer, and aging [6].

Production of RNS

The enzymes NOS catalyse the conversion of L-arginine into L-citrulline and $^{\bullet}NO$ by 5-electron oxidation of the guanidine nitrogen of L-arginine [4]. NOS has various isoforms and is present in numerous cell types, particularly in the plasma membrane or cytosol of these cells. To date, there are 3 known isoforms of NOS: nNOS; type I NOS, eNOS; type III NOS, and iNOS; type II NOS [4]. nNOS synthesizes $^{\bullet}NO$ in neurons where it aids in communication between nerve cells, whereas $^{\bullet}NO$ generated by iNOS in macrophages and smooth muscle cells contributes to their killing action [4]. The endothelium, brain, and heart also produce $^{\bullet}NO$ via eNOS, where $^{\bullet}NO$ regulates blood pressure [4].

Exogenous sources of ROS and RNS

Cigarette smoke and alcohol

Cigarette smoke contains many free radicals, including $O_2^{\bullet-}$ and $^{\bullet}NO$. Additionally, the inhalation of cigarette smoke into the lungs also activates various endogenous mechanisms, such as the accumulation of neutrophils and macrophages, which further contribute to oxidant injury [2]. Alcohol (chemically known as ethyl alcohol or ethanol) is commonly consumed across the globe. A deleterious effect of ethanol metabolism is its implications in oxidative stress. Ethanol is broken down in the liver in two steps: first, it is metabolized to acetaldehyde. Next, the enzyme aldehyde dehydrogenase converts acetaldehyde to acetate. Both reactions produce a molecule of NADH. This provides more starting material for the respiratory chain reaction and, therefore, increased production of $O_2^{\bullet-}$ [23]. Systems producing $O_2^{\bullet-}$ will subsequently result in the formation of H_2O_2 [23].

Ozone (O_3)

O_3 exposure can lead to lipid peroxidation. It can also induce an influx of neutrophils into the airway epithelium, which accelerates oxidant injury [2]. Even short-term exposure to O_3 can result in the release of inflammatory mediators such as MPO, eosinophil cationic proteins, lactate dehydrogenase, and albumin. These factors can contribute to oxidative stress [2].

Ionizing radiation

In the presence of O_2, ionizing radiation converts $^{\bullet}OH$, $O_2^{\bullet-}$, and organic radicals to H_2O_2 and $ROO^{\bullet}$. These $ROO^{\bullet}$ species then react with the active redox metal ions, Fe and Cu, via Fenton reactions, leading to oxidative stress [2]. Oxidative reactions are triggered by ultraviolet A (UVA) photons owing to the excitation of endogenous photosensitizers, such as porphyrins, NOX, and riboflavin. 8-Oxo-7,8- dihydroguanine (8-oxoGua) is the main UVA-mediated DNA lesion product formed by the oxidation of $^{\bullet}OH$, 1-electron oxidants, and $^1O_2$ that mainly reacts with guanine [2]. Ionizing radiation can effectively bring about the formation of the guanine radical cation [2].

Xenobiotics

Oxidative stress is believed to be the most common mechanistic feature in toxicology [24]. The physio-chemical properties of various xenobiotics, including heavy metals, environmental toxins, and per- and polyfluoroalkyl substances (PFAS), are known to induce oxidative stress [24]. Heavy metals, including iron, copper, cadmium, mercury, nickel, lead, and arsenic, can generate free radicals, resulting in cellular damage. Generally, metal-mediated free radical production is brought about by the Fenton or Haber-Weiss reactions (Eqs. 8 and 9). Due to these reactions, metals like iron and copper can react with H_2O_2 and $O_2^{\bullet-}$ to give $^{\bullet}OH$ [2].

$Metal^{3+} + O_2 \longrightarrow Metal^{2+} + O_2$ (Haber-Weiss) (Equation 8)

$Metal^{2+} + H_2O_2 \longrightarrow Metal^{3+} + OH^- + {}^{\bullet}OH$ (Fenton reaction) (Equation 9)

Apart from these reactions, certain metal ions directly react with cellular molecules to generate free radicals, such as thiol radicals [2]. These radicals may also react with other thiol molecules to generate $O_2^{\bullet-}$. $O_2^{\bullet-}$ can further be converted to H_2O_2. Some metals, such as arsenite, induce ROS production indirectly by activating the radical-producing systems in cells [2]. Arsenic is a highly toxic element as it not only generates a variety of oxidants (${}^{\bullet}OH$, $^1O_2$, $ROO^{\bullet}$, ${}^{\bullet}NO$, H_2O_2, and dimethylarsinic peroxyl radicals) but also inhibits numerous antioxidant enzymes (including the GSH-dependent enzymes, such as glutathione-S-transferases (GST), GPx, and glutathione reductase (GR), via binding to their sulfhydryl (-SH) group) [2]. The metal lead can cause lipid peroxidation. It is known to significantly decrease the activity of tissue SOD and brain GPx [2].

Environmental toxins such as bisphenol A (BPA) are known to give rise to oxidative stress-mediated metabolic and hormonal disturbances [25]. The chemical, once inhaled or ingested from the environment or common consumer products, mainly gets metabolized into bisphenol A glucuronide (BPAG) or bisphenol A sulfate (BPAS) and is eliminated through urination [20]. However, a portion of the remaining free BPA in the body can produce ROS via the enzymatic (H_2O_2/peroxidase and NADPH/CYP450) and non-enzymatic ($OONO^-/CO_2$ and ^-OCl/ HOCl) formation of phenoxyl radicals. Subsequently, these radicals react with NADPH or intracellular GSH to produce a variety of radical species, including $O_2^{\bullet-}$, peroxides, and ${}^{\bullet}OH$, thereby leading to oxidative stress [25].

PFAS are commonly found in a wide range of consumer goods. These goods release PFAS, and they persistently remain in the environment [26]. PFAS can be ingested from contaminated food and water. This can increase the burden of PFAS in the body, leading to oxidative stress [26]. Exposure to PFAS is believed to overwhelm and destabilize the mitochondria, which limits its effectiveness in managing ROS, thereby resulting in oxidative stress [26].

Medications

Certain immunosuppressant drugs, such as cyclosporine, tacrolimus, and gentamycin, are known to contribute to oxidative stress as they increase free radical levels via lipid peroxidation [3]. The drug, Doxorubicin (Dox), is an anthracycline antibiotic used as a chemotherapeutic agent. The drug can react with mitochondrial reductases to readily reduce O_2 to $O_2^{\bullet-}$, and H_2O_2. The reactions between Dox and iron can also produce ROS, and this reaction can subsequently generate an iron II-Dox free radical capable of reducing O_2 [27]. The antineoplastic agent, cisplatin used in the treatment of testicular, bladder, lung, gastrointestinal, and ovarian cancers is also seen to increase oxidative stress by increasing levels of $O_2^{\bullet-}$, H_2O_2, and ${}^{\bullet}OH$ [27]. A class of drugs called ‘pro-oxidants’ use their ability to induce oxidative stress to kill cancer cells. It is known that cancer cells are more sensitive to oxidative stress than normal cells. Therefore, pro-oxidant cancer drugs dramatically increase intracellular ROS and thus, induce oxidative stress by interfering with ROS homeostatic regulators such as glutathione S-transferase pi 1 (GSTP1) [28]. Figure 1 indicates the major endogenous and exogenous sources that can give rise to oxidative stress, resulting in damage to biological components.

MEASURING OXIDATIVE STRESS

Direct quantification of oxidant species

ROS and RNS are the key players responsible for the deleterious effects of oxidative stress. Direct quantification of their levels is one approach of determining oxidative stress [29].

H_2O_2, $^{\bullet}OH$ and $ROO^{\bullet}$

These reactive species can be measured following staining with 5-(and -6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (DCFDA). This membrane-permeable fluorogenic probe diffuses into the cells where it becomes hydrolysed by intracellular esterase to 2',7'-dichlorodihydrofluorescein (DCFH). DCFH remains trapped within the cells and reacts with H_2O_2, generating the fluorescent, 2′,7′-dichlorofluorescein (DCF). The amount of cellular H_2O_2 can be estimated by the fluorescence intensity of DCF ($\lambda_{excitation}$ = 488 nm and $\lambda_{emission}$ = 530 nm) which be analyzed by flow cytometry or via a fluorescence plate reader [29]. However, it has been observed DCFH is not only oxidative by H_2O_2 to give DCF, but also by other ROS. This makes the probe non-specific to H_2O_2 [30]. Additionally, this reaction is sensitive to local O_2 levels and pH, implying that the fluorescence yield may not be linear with increased ROS levels [30].

$O_2^{\bullet-}$

$O_2^{\bullet-}$ can be quantified from staining with the fluorescent probe, dihydroethidium (DHE). Sodium borohydride, which is the reduced form of ethidium bromide is permeable to viable cells. Inside the cells, DHE is directly oxidized to ethidium bromide by $O_2^{\bullet-}$, which then fluoresces. A flow cytometer or a fluorescence plate reader can then measure the red fluorescence ($\lambda_{excitation}$ = 488 nm and $\lambda_{emission}$ = 585 nm) which is proportional to the intracellular $O_2^{\bullet-}$ levels [29]. However, this quantification can be misleading if the detection is carried out along the mitochondria-targeted dihydroethidium (MitoSOX) probe. This is because both probes form ethidium bromide and the $O_2^{\bullet-}$ -specific product, 2-hydroxyethidium. As the 2 products have overlapping fluorescence spectra, it is difficult to differentiate the contribution of non-specific oxidation and $O_2^{\bullet-}$-dependent oxidation (if any) to the overall fluorescence [30].

Direct quantification of ROS levels with high accuracy and precision in biological species is tedious owing to their short lifespan. While H_2O_2 (chemically stable) and $ROO^{\bullet}$ (7s) are relatively stable molecules with half-lives of seconds to minutes, the other oxidant species such as $^{\bullet}OH$ (10^{-9} s), $O_2^{\bullet-}$ (10^{-6} s), alkoxyl anions (10^{-6} s), and $^1O_2$ (10^{-6} s) are very reactive having half-lives of less than a nanosecond [29, 31]. This makes it difficult to measure them in biological samples. Although the levels of oxidant species are high during oxidative stress, their levels are still lower than those of other cellular components, which makes their quantification difficult using conventional methods [30]. ROS are highly reactive and are continuously reacting with cellular components to yield new molecules, such as lipid peroxidation products or protein carbonyls, which are now studied as indirect markers of oxidative stress. Also, the body is bestowed with antioxidants, which constantly aim at quenching free radicals. Therefore, it becomes challenging to measure ROS directly without considering the impact of antioxidant systems. Attempts have been made to quantify ROS using complex techniques such as electron spin resonance, spin trapping, or pulse radiolysis [32]. However, these techniques can be labour-intensive, time-consuming, and may require sophisticated instrumentation, which limits their general use [31]. The simpler

spectrophotometric techniques are unable to measure various ROS; they are non-specific to individual ROS and can only measure the relatively stable ROS [31].

As the direct quantification of ROS is fraught with various limitations and challenges, indirect means of detecting oxidative stress have been utilized. The indirect markers include markers of lipid peroxidation, nucleic acid, and protein damage, which will indicate the level of oxidative stress based on the damage done to these cellular components. Additionally, the quantification of antioxidants in the body is also quantified to assess the body's ability to counteract oxidative stress, with insufficient antioxidant levels being indicative of oxidative stress. The markers under either category will be discussed in the later sections.

BENEFICIAL FUNCTIONS OF OXIDANT SPECIES

Oxidant species are seen to play dual roles by benefiting the body at lower levels and being harmful at higher levels [6]. The finding that the $^{\bullet}OH$ radical helps stimulate the production of cyclic guanosine monophosphate (cGMP) (a signalling messenger molecule) has led to an understanding of the dual nature of ROS and RNS in biological systems. It then became clear that the human body not only adapted to a coexistence with free radicals but also developed means to utilize these toxicants to their own advantage by using them in critical physiological processes [7]. This has been supported by the fact that at low or moderate concentrations, ROS regulate cell growth and apoptosis at the cellular level [7]. ROS can contribute toward cell survival in two ways: by either acting on transcription factors that directly interact with specific DNA motifs on promoters of target genes or via the activation of mitogen-activated protein kinases (MAPK), phosphoinositide 3-kinases (PI3Ks), phosphatase and TENsin homolog (PTEN), and protein tyrosine phosphatases that initiate signalling in several cellular processes, including proliferation and survival [4].

At the system level, ROS contributes to complex functions, such as immune function. Phagocytes such as neutrophils, macrophages, and monocytes release free radicals to destroy invading pathogens [6]. During bacterial infection, these cells identify and engulf bacteria, leading to the formation of a vesicle called the phagosome. This process activates the otherwise dormant enzyme present in the cytosol and plasma membrane, NOX. This activation is brought about by cytochrome b558 and the translocation of the cytosolic components to the phagosome membrane [4]. Phagosome maturation is mediated by the successive fusion and fission interactions between the new phagosome and early endosomes, late endosomes, and finally lysosomes, leading to the formation of the 'phagolysosome.' The phagolysosome is the final microbicidal organelle, and it contains hydrolytic enzymes (cathepsins, proteases, lysozymes, and lipases) and scavenger molecules, including NOX [33].

At this stage, the catalytically activated NOX undergoes a 'respiratory burst' wherein it uses up enormous amounts of O_2 to produce $O_2^{\bullet-}$. This $O_2^{\bullet-}$ then dismutates to H_2O_2, which can in turn react with $O_2^{\bullet-}$ to generate more-complex ROS such as $^{\bullet}OH$ and $^1O_2$ [33]. Additionally, H_2O_2 can be combined with Cl^- ions to give HOCl via the enzyme, MPO [33]. These ROS being highly reactive, damage the bacterial proteins, lipids, and nucleic acids, thereby disrupting the bacterium's vital functions. HOCl particularly has antimicrobial functions and can further damage bacterial components, leading to bacterial death [34]. The critical role of ROS in immune function has been supported by their absence in granulomatous disease patients. These patients have an impaired membrane-bound NOX system which makes them unable to produce the $O_2^{\bullet-}$, resulting in persistent infections [35].

The respiratory burst is the only physiological mechanism that produces HOCl, which can then react with tyrosyl residues in proteins to give 3-chlorotyrosine [36]. Therefore, 3-chlorotyrosine has emerged as a specific marker for the oxidant activity of MPO-containing cells [36]. As 3-chlorotyrosine results from phagocytic activity only, a rise in its levels could also be indicative of increased phagocytosis owing to persistent infection. This may justify the elevated levels of 3-chlorotyrosine observed in infants who had lung infections or were *Ureaplasma urealyticum* positive [36]. From this, we suggest that 3-chlorotyrosine not only serves as a biomarker of the oxidant activity of MPO-containing cells but also as a marker of infection.

Interestingly, ROS are also involved in the expression of antioxidants. This is mediated by the expression of the transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2), which regulates the expression of several antioxidant and detoxifying genes by binding to promoter sequences containing a consensus antioxidant response element [4]. ROS initiate the Nrf2-Keap1 (Kelch-like ECH-associated protein 1) pathway by modifying critical cysteine residues of Keap1 and Nrf2. This results in the activation of the Nrf2-controlled genes that encode detoxification enzymes NQO1 (NAD(P)H quinone oxidoreductase 1), antioxidant enzymes (GPx2, Srx1 (Sulfiredoxin 1)), and enzymes that synthesize low-molecular-weight antioxidants (GSH, bilirubin), all of which suppress oxidative stress [37].

Similarly, the RNS, $^{\bullet}NO$, is seen to play important roles in the body, where it serves as an intracellular second messenger, stimulating guanylate cyclase and protein kinases [7]. It aids in relaxing the smooth muscles in blood vessels and functions as a cellular redox regulator by regulating enzymatic activity by nitrosylating the proteins [7]. $^{\bullet}NO$ is also crucial for nonspecific host defense and for destroying intracellular pathogens and tumors [6]. It does so by regulating the growth, function, and death of crucial immune cells, including macrophages, T lymphocytes, antigen-presenting cells, mast cells, neutrophils, and natural killer cells [38]. $^{\bullet}NO$ is also believed to have a potential microbicidal effect via the reaction of $^{\bullet}NO$ with iron or thiol groups on proteins forming iron-nitrosyl complexes. These complexes can induce nitrative stress in the microbial cells, which can lead to cell death [38]. In conclusion, ROS and RNS are continuously produced owing to metabolic activities, and they are vital to human health at low or moderate levels.

DETRIMENTAL EFFECTS OF OXIDANT SPECIES

An imbalance between the formation and neutralization of ROS and RNS species, favoring their high levels, leads to ‘oxidative stress.’ Under such conditions, the oxidant species attack biological components such as lipids, nucleic acids, and proteins [6]. The mechanism of the damaging effects of oxidant species on these cellular structures has been discussed below:

Lipids

Polyunsaturated fatty acid (PUFA) residues of phospholipids are most susceptible to oxidation by free radicals [7]. These membrane lipids are subject to lipid peroxidation, which can result in the loss of membrane functioning, for example, decreased fluidity, and the inactivation of membrane-bound enzymes and receptors [7]. Lipid peroxidation is a chain mechanism and involves three events: initiation, propagation, and termination. An initiating free radical, which can be hydroxyl, alkoxyl, $ROO^{\bullet}$, or $OONO^{-}$, can oxidize numerous lipid molecules through sequential, self-propagating chain reactions [39]. Of the mentioned free radicals, the $^{\bullet}OH$ is the most active and is likely to initiate the peroxidation process. The catalytic metal ions, copper

(Cu^{I}) or iron (Fe^{II}) also aid in initiating the chain reaction [39]. Lipid peroxidation is initiated when a free radical attacks hydrogen from a methylene group (CH2) in a fatty acid which results in the formation of a carbon-centered lipid radical (L$^{\bullet}$). This L$^{\bullet}$ then reacts with O_2 to form a lipid peroxyl radical (LOO$^{\bullet}$), which undergoes rearrangement through a cyclization reaction to form endoperoxides. PUFAs such as linoleic acid (LA) (18:2), arachidonic acid (AA) (20:4), eicosapentaenoic acid (EPA) (20:5), and docosahexaenoic acid (DHA), are targets of free radical-initiated lipid peroxidation, yielding a diverse array of products [39]. The rate at which these PUFAs get oxidized is subject to the number of -CH2- centres in the molecule that are flanked by two double bonds (bisallylic methylene) [39].

The primary products of free radical-initiated peroxidation of PUFAs are lipid hydroperoxides (LOOH). Oxidation of linoleates yields hydro(pero)xyoctadienoates (H(P)ODEs) [39]. The decomposition of LOOHs yields the HCORs, acrolein, malondialdehyde (MDA), and 4-hydroxy-2-nonenal (4-HNE). MDA and 4-HNE are toxic products of lipid peroxidation as they can damage the DNA and proteins [7]. These products can further propagate the peroxidation process by extracting hydrogen atoms from the other lipid molecules. MDA and 4-HNE have risen as important biomarkers of lipid peroxidation. The other lipid peroxidation products, 4-Hydroxynonenalmercapturic acid (4-HNE-MA) and 4-oxo-2-nonenal (4-ONE) can also be used as biomarkers. MDA and 4-HNE can undergo nucleophilic reaction of proteins with reactive carbonyl species to yield advanced lipoxidation end products. On such important reaction is their reaction with lysine residue proteins to form carboxymethyl lysine (CML), which has risen as an important marker of oxidative stress [49].

Secondary lipid peroxidation products are generated from the non-enzymatic free radical-catalysed peroxidation of AA and other highly unsaturated PUFAs. These secondary lipid peroxidation products include a series of prostaglandin (PG)-like products termed isoprostanes (IsoPs) [7,39]. They are important targets of lipid peroxidation of AA. The abstraction of a bisallylic hydrogen atom and the addition of a molecule of O_2 to AA yields a ROO$^{\bullet}$. Following this, the ROO$^{\bullet}$ undergoes 5-exo cyclization and a second molecule of O_2 gets added to the backbone of the compound to form PGG2-like compounds. F2-isoprostanes (F2-IsoP) is a subclass of IsoPs. The unstable bicycloendoperoxide PGG2-like intermediates are then reduced to give the four F2-IsoP regioisomers, namely the 5, 8, 12 and 15 regioisomer series of F2-IsoP, depending on the carbon atom to which the allylic hydroxyl is attached [30]. The four F2-IsoP regioisomers, each comprises eight racemic diastereomers and depending of the combination of the isomers, they can generate 64 possible compounds [50]. 8-, 9-, 11-, and 12-peroxy radicals of AA are known to make up the F2-IsoPs class [43]. They are the intermediates generated during the formation of the above-mentioned F2-IsoP regioisomers. The F2-IsoPs class of AA are “gold standard” biomarkers of endogenous lipid peroxidation and oxidative stress [7,39]. Although there are assays for various IsoPs, 8-isoprostaglandin $F_2\alpha$ (8-isoPGF$_2\alpha$, also known as 8-epi-PGF$_2\alpha$ or 8-isoprostane; 15-F2t-IsoP) is commonly assessed as a biomarker of oxidative stress. Additionally, there are other F2-IsoPs products such as 11-β-prostaglandin F2α (11-PGF2α) and 15-prostaglandin F2α (15-PGF2α) as well as the isomer of 8-isoPGF2α, 8-Iso-15(R)-Prostaglandin that are quantified as biomarkers of lipid peroxidation. Table 1 summarizes the quantification techniques for the established lipid peroxidation markers.

Nucleic acids

ROS and RNS can oxidatively damage nucleic acids resulting in base substitution, addition, deletion, and other mutations [51]. The oxidative damage caused to DNA and RNA are discussed below.

Deoxyribonucleic Acid (DNA)

ROS, particularly the $^{\bullet}OH$ radical reacts directly with the various components of DNA including the purine and pyrimidine bases, and the deoxyribose sugar backbone. This results in a number of alternations including single and double-stranded breaks in DNA [7]. When the $^{\bullet}OH$ radical attacks pyrimidine by abstracting hydrogen atoms, it produces different pyrimidine adducts like thymine glycol, uracil glycol, 5-hydroxydeoxy uridine, 5-hydroxy deoxycytidine, and hydantoin among others [7]. Similarly, the attack of $^{\bullet}OH$ radical on purine results in the formation of 8-Hydroxy-2'-deoxyguanosine (8-OHdG), 8-hydroxy deoxy adenosine, and 2,6-diamino-4-hydroxy-5-formamidopyrimidine [7]. More specifically, when guanine gets oxidized by $^{\bullet}OH$ radical, a $^{\bullet}OH$ is added to the eighth position of the purine base leading to the formation of the oxidatively modified product, 8-OHdG [52].

8-OHdG is an important biomarker of oxidative DNA damage as it is one of the predominant forms of free radical-induced lesions of DNA [7,52]. Its formation in the transcription factor binding sites can modify the binding of these factors and thus change the expression of related genes. In DNA, 8-OHdG leads to the GC to TA transversion mutation [53]. Due to this, it is known to be mutagenic [2]. The mitochondrial DNA is more prone to ROS attack than the nuclear DNA, as it is near the ROS generation site. Consequently, 8-OHdG levels are higher in mitochondrial DNA than in nuclear DNA [2].

5-formyl uracil, cytosine glycol, 5,6-dihydrothyronine, 5-hydroxy-6-hydro-cytosine, 5-hydroxy-6-hydro uracil, uracil glycol, and alloxan are also some of the free radical-induced adducts of DNA bases [7]. Glycolic acid, 2-deoxytetrodialdose, erythrose, 2-deoxypentonic acid lactone, 2-deoxypentose-4-ulose are the important adducts of the sugar moiety in DNA. Oxidization of the guanine base with ROS results in the formation of 8-Hydroxyguanine (8-OHG, the base moiety of 8-OHdG) [53]. It is an abundant lesion in genomic, mitochondrial, and telomeric DNA and is an essential marker of oxidative damage in DNA.

The RNS, particularly $OONO^{-}$ interacts with guanine on the DNA to produce a nitrative DNA lesion, 8-nitroguanine (8-NO2-G). The produced 8-NO2-G is unstable and can be spontaneously removed, resulting in the formation of an apurinic site (DNA site missing a base analogue). Additionally, during DNA synthesis, adenine can be paired with 8-NO2-G resulting in G-T transversions. As a result, 8-NO2-G is known to be a mutagenic DNA lesion that can contribute to carcinogenesis [7]. Most of the DNA modifications are implicated in carcinogenesis, aging, neurodegenerative, cardiovascular, and autoimmune diseases [2]. 8-NO2-G has risen as a marker of RNS-induced nitrative DNA damage [46].

Ribonucleic acid (RNA)

RNA is also subjected to free radical damage and happens to be more prone to oxidative damage than DNA. This is owing to its single-stranded nature, lack of an active repair mechanism for oxidized RNA, less protection by proteins than DNA and its being located close to the mitochondria, which is a major ROS generation site [5]. Translation of oxidized mRNA can result in the formation of truncated proteins owing to the translation machinery terminating at the oxidized site, or mutated proteins if the entire mRNA has been translated [61]. As a

result, oxidization of RNA can result in altered protein synthesis which can lead to cell degradation and cell death [61]. This is implicated in various neurological pathologies which will be discussed in the later sections. The attack by RNS on RNA yields the major RNA damage product, 8-hydroxyguanosine or 7,8-dihydro-8-oxo-guanosine (8-oxoG) [62]. It appears to be extremely deleterious due to its high mutagenic potential [62]. Its levels are elevated in various disease conditions Alzheimer's disease (AD), Parkinson's disease (PD), atherosclerosis, hemochromatosis, and myopathies [7]. 8-oxoG is a reliable marker for oxidative damage of RNA [51]. Additionally, oxidation of guanosine on the RNA by a nitro (NO2) group yields 8-nitroguanosine (8-NdG) [63]. 8-NdG is an RNA oxidation marker. The quantification techniques for the established DNA and RNA damage markers are enlisted in Table 2.

Proteins

Oxidant species attack proteins, leading to the formation of protein-protein cross-linkages, which results in the denaturation and loss of protein functionality, loss of enzyme activity, and loss of function of receptors and transport proteins [7]. The free radicals that can attack proteins are $O_2^{\bullet-}$, $^{\bullet}OH$, $ROO^{\bullet}$, alkoxyl, and hydroperoxyl, while the non-radical species are H_2O_2, O_3, HOCl, $^1O_2$, and $OONO^-$ [7]. Following are the various reactions that proteins undergo with oxidant species:

Carbonylation

Oxidative damage to the amino acids, lysine, proline, threonine, and arginine yields carbonyl derivatives via protein carbonylation [7, 32]. This reaction is a stable modification that is induced by ROS via three pathways: direct oxidation of protein-bound amino acids, oxidative cleavage of the protein backbone, and incorporation of carbonyls from glycoxidation or lipoxidation (MDA and 4-HNE reacting with amino groups in proteins) [32]. Aminoadipic acid is formed via the $^{\bullet}OH$ mediated abstraction of the hydrogen in lysine [32]. Glutamic semialdehyde is formed via the abstraction of a proton from arginine or proline, followed by carbon radical oxidization [32]. These are examples of direct oxidation of amino acids, responsible for about 60% of total protein carbonylation in the liver.

During oxidative cleavage of the protein backbone, the cleavage is initiated by $O_2^{\bullet-}$ -mediated alkoxyl radical formation at the α-carbon next to a peptide bond. The fragmentation brought about by the alkoxyl radical takes place either by the diamide pathway (homolytic cleavage of the carbon-carbon bond) or the α-amidation pathway (homolytic cleavage of the carbon-nitrogen bond) [32]. The end products of the former pathway are diamide and isocyanate, while ketoacyl derivatives and amides are products of the latter pathway [32]. Carbonylation from glycoxidation will be explained in the later sections. The presence of carbonyl groups in proteins has been considered a marker of ROS-mediated protein oxidation. Elevated levels have been associated with various pathologies, including protein carbonyls, and are observed in several pathological conditions such as AD, PD, muscular dystrophy, cataractogenesis, rheumatoid arthritis, diabetes, atherosclerosis, respiratory dystrous syndrome, and ageing [7]. Protein carbonyl content is the most used marker of protein oxidation [64]. It is advantageous to quantify protein-bound carbonyl owing to its frequent occurrence in the body. relatively early formation and the relative stability of oxidised protein moieties [64]. They circulate in the body for longer periods as compared to other parameters of oxidative stress, such as

glutathione disulfide (GSSG) or MDA [64]. Lipid peroxidation products are degraded within minutes while cells take hours to days to degrade oxidised proteins [64].

Oxidation of sulfur-containing amino acids

Aminothiol proteins such as cysteine and GSH are highly susceptible to oxidation via alterations of reactive aminothiol residues [65]. Aminothiols can be measured in serum or plasma to assess the oxidant burden [65]. Of these aminothiols, cysteine extracellularly accounts for the major aminothiol pool that reacts readily with oxidants. Under enzymatic or non-enzymatic conditions, the thiol group (-SH) in cysteine's side chain gets oxidized resulting in the formation of a disulfide bond to give cystine [32]. Overoxidation of cystine can lead to the oxidation of cysteine sulfenic acid to cysteine sulfinic and finally sulfonic acid [32]. Several enzymes can control and reverse the formation and cleavage of disulfide bonds. Therefore, the oxidation of cysteine residues is reversible, except for sulfinic and sulfonic acids [66]. Owing to cysteine sulfenic being an intermediate, it is not studied as a marker of oxidative stress. Although sulfenic acids are often unstable and reactive, studying this modification may represent the initial product of two-electron oxidants with the thiolate anion, therefore serving as a marker for oxidant-sensitive cysteine residues [67]. Cysteine and its oxidized form, cystine can give the oxidized potential in the body [32]. However, owing to cysteine's instability and high reactivity to be reduced by other thiols, it does not pose as potentially reliable marker of oxidative stress [67]. As a result, cystine appears to be a better marker of oxidative stress.

Methionine is another sulfur-containing amino acid which is highly susceptible to oxidation by ROS [7]. It can be reversibly oxidized to methionine sulfoxide and irreversibly oxidized to methionine sulfone. Methionine sulfoxide reductases reduce methionine sulfoxide back to methionine. However, they do not target methionine sulfone which is a stable modification [32]. As the major oxidation product of protein-bound methionine is methionine sulfoxide, and methionine sulfone, is produced later to much lesser extent, methionine sulfoxide is considered a marker of protein damage by oxidative stress [68]. Among most thiol oxidized products methionine sulfoxide shows higher stability and is used as an oxidative damage marker.

Oxidation of aromatic moieties

Aromatic moieties in amino acids are favourable targets of protein oxidation [32]. The amino acid, tyrosine is particularly prone to oxidation. Its phenolic side-chain gets easily oxidized, as the intermediary tyrosyl radical is stabilized by mesomeric delocalization of the unpaired electron. The tyrosyl radical can then react with another tyrosyl radical leading to the formation of a protein crosslink, dityrosine [69]. This reaction can be mediated by the oxidative species, $^{\bullet}OH$ and nitrative species, $OONO^{-}$ and nitrosoperoxycarbonate [70]. Therefore, dityrosine is used as a marker indicative of oxidative/nitrative stress [70].

ROS and RNS actively target aromatic amino acid residues leading to the formation of dityrosine-containing crosslinks, called as Advanced Oxidation Protein Products (AOPP). The amount of AOPP in the body has been used as an indicator of oxidative stress [32].3-nitrotyrosine is an irreversible modification product formed by the nitration of tyrosine via the attack of the $^{\bullet}NO_2$ at the ortho-position of the aromatic ring [72]. Thus, nitrotyrosine is a biomarker for endogenous $OONO^{-}$ activity and at large, nitrative stress [73]. The oxidation of phenylalanine residues via $^{\bullet}OH$ yields abnormal isomers ortho- and meta-tyrosine [32]. $^{\bullet}OH$, oxidize tryptophan to hydroxytryptophan, which is then cleaved by O_2 to yield N-formyl

673 kynurenine while a metal catalysed reaction of histidine with $^{\bullet}OH$ leads to the formation of 2-
674 oxohistidine [32]. 2-oxohistidine has been proposed as a marker of protein oxidation, however,
675 the marker still needs to be studied for its sensitivity and specificity in oxidative stress [74].

676 Glycoxidation

677 Glycation is a protein modification process characterized by the formation of intermediate
678 Amadori products and subsequently advanced glycation end products (AGEs) [32]. This
679 reaction is a nucleophilic reaction of amino acid residues with reductive sugars or their reactive
680 degradation products (α-dicarbonyl compounds). Lysine and arginine are readily modified by
681 glycation [32]. It is critical to note that the formation of AGEs usually does not require
682 oxidative conditions and only selected AGEs are generated by oxidation. These AGEs are
683 referred as "glycoxidation products" because they are formed by a combination of glycation
684 and oxidation [32].

685 The AGE, carboxymethyl lysine (CML), can be formed by oxidative degradation of
686 fructoselysine (Amadori product) [32]. It can also be formed from a reaction between the α-
687 dicarbonyl compound glyoxal and lysine leading to CML formation via an isomerization
688 mechanism. Although the latter mechanism is non-oxidative, glyoxal which is the reactive
689 precursor is mainly formed by oxidative degradation of biological molecules such as
690 carbohydrates, lipids, nucleotides, and serine [32]. Elevated levels of CML can exert stronger
691 oxidizing potential which may lead to oxidative stress [75]. Therefore, CML levels are used as
692 markers of glycoxidation. A crosslink between lysine and arginine residues yields another
693 important glycoxidation product called pentosidine [32]. Although it is found in lower
694 abundance compared to CML, pentosidine is frequently measured glycotoxin in clinical studies
695 and it is important in oxidative stress.

696 Halogenated products

697 The leukocyte-derived enzyme, EPO generally oxidizes the halide, bromide (Br). 3-
698 bromotyrosine on of the products formed by the reaction of free and protein-bound tyrosine
699 residues with either HOBr/OBr-. It can also be formed from the reaction with EPO in the
700 presence of H_2O_2 and plasma levels of halides [12]. Halogenated Br products potentially serve
701 as excellent molecular markers to identify sites where EPO promote oxidative damage because
702 there are no other known pathways in the body that result in covalent incorporation of Br into
703 biomolecules. 3-bromotyrosine has risen as an attractive candidate for molecular markers for
704 eosinophil mediated-oxidative damage of proteins by reactive brominating species [12].

705 Stimulated neutrophils generate $O_2^{\bullet-}$ and H_2O_2 and release MPO. MPO can catalyse the
706 oxidation of chloride by H_2O_2 to give HOCl, which is a strong oxidant that can damage cells
707 [36]. HOCl reacts with tyrosyl residues in proteins to give 3-chlorotyrosine. This is the only
708 physiologic source of chlorotyrosine which makes it a specific marker for oxidant activity of
709 MPO-containing cells, which include neutrophils and monocytes [36].

710 Acrolein

711 Acrolein is another aldehyde product generated from lipid peroxidation. It is a highly reactive
712 molecule [47]. Among most lipid peroxidation products, acrolein is by far the strongest
713 electrophile showing high reactivity with nucleophiles, such as the sulfhydryl group of
714 cysteine, imidazole group of histidine, and amino group of lysine [79]. Studies state that

acrolein was seen to modify lysine and histidine residues of human serum albumin [80]. The acrolein-lysine adduct has been observed to be the major product of acrolein's reaction with amino groups [79]. The excretion of acrolein-lysine adduct has risen as a biomarker of oxidative status; indicative of damage done to the amino acid [79].

Allantoin

Allantoin is the major product of non-enzymatic free-radical oxidation of the antioxidant, UA [47]. It has emerged as a biomarker for monitoring oxidative status. It is important to note that a variation of UA levels do not correlate with variation in allantoin. This implies that formation of allantoin is independent of UA levels. Hence, allantoin can serve as an effective biomarker of systemic oxidative status [47].

The quantification techniques for the protein damage markers have been summarised in Table 3.

ANTIOXIDANTS

The human body is equipped with an antioxidant system that helps combat the effects of oxidants in the body. These antioxidants break radical chain reactions, thereby preventing oxidative stress-related damage. They have heterozygous chemical structures, as their roles require them to work in both hydrophilic and hydrophobic cellular environments [83]. Antioxidants are generally categorized as enzymatic and non-enzymatic antioxidants. However, from a nutritional point of view, they can also be categorized as endogenous and exogenous antioxidants. Technically, all enzymatic antioxidants are endogenous, as well as some non-enzymatic ones such as thiol antioxidants and coenzyme Q10 (CoQ10) [83]. On the other hand, exogenous antioxidants are the ones that need to be obtained from the diet since they are not synthesized in eukaryotic cells [83]. Here, we comprehensively discuss the various enzymatic and non-enzymatic antioxidants.

Enzymatic Antioxidants

In the body, free radicals are quenched by various enzymes. A few of them act directly in scavenging ROS and they are called "primary enzymes," whereas "secondary enzymes" are the ones that indirectly help in reducing oxidative stress by supporting other endogenous antioxidants [83]. They have been discussed in detail:

Primary Enzymes

Primary antioxidant enzymes are the ones that act directly on the main ROS arising from $O_2^{\bullet-}$ and H_2O_2 [83].

Superoxide dismutase (SOD)

SOD the metalloenzyme, primarily catalyses $O_2^{\bullet-}$ dismutation to H_2O_2 and O_2 (Eq.10) (Figure 2) [83]. In turn, the less harmful H_2O_2 can be removed by the other enzymatic antioxidant systems. There are 3 forms of SOD: cytoplasmic Cu/ZnSOD (SOD1), the mitochondrial MnSOD (SOD2), and the extracellular Cu/ZnSOD (SOD3). All 3 forms require catalytic metal (Cu or Mn) for their activation [84]. The SOD system also competes with $^{\bullet}NO$ for $O_2^{\bullet-}$. Consequently, SOD also indirectly reduces the formation of another deleterious ROS, $OONO^-$ (Eq.11), and increases the $^{\bullet}NO$ biological availability which an essential modulator for

endothelial function [83]. Measurement of the primary antioxidant, SOD is integral in assessing the body's antioxidant capability.

$2\ O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 + O_2$… (Equation 10)

$2\ O_2^{\bullet-} + {}^{\bullet}NO \rightarrow OONO^-$… (Equation 11)

Catalase (CAT)

H_2O_2 produced by SODs or from the action of oxidases, such as xanthine oxidase, is reduced to H_2O by CAT and GPx (Figure 2). CAT is primarily located in the peroxisomes. It is seen to have the highest activity in liver and red blood cells [83]. The enzyme exists as a tetramer composed of 4 identical monomers, each containing a heme group at the active site. CAT neutralizes and maintains an optimum level of H_2O_2 in the cell. It breaks down H_2O_2 into one molecule of O_2 and two molecules of H_2O in a two-step reaction [87]. A peroxidase-like compound I intermediate, CpdI is formed at the end of the first step. CpdI is converted back to CAT after a reaction with the second H_2O_2 molecule (Eq. 12&13). Recent studies have been indicating the CAT might also help in scavenge $OONO^-$ [88]. Assessing CAT levels can be indicative of the antioxidant status of the body.

$CAT\ (Fe^{3+}) + H_2O_2 \rightarrow CpdI^+\ (Fe^{4+}= 0) + H_2O$… (Equation 12)

$CpdI^+\ (Fe^{4+}= 0) + H_2O_2 \rightarrow CAT\ (Fe^{3+}) + H_2O + O_2$… (Equation 13)

Glutathione peroxidase (GPx)

The GPx enzyme is a selenium-dependent oxidoreductase which is responsible for the reduction of H_2O_2 and LOOHs [2, 83]. It uses H_2O_2 or organic $ROO^{\bullet}$ as the oxidant, and the tripeptide GSH as the electron donor in a general class I peroxidase catalytic cycle (Eq.14&15) (Figure 2). The enzyme activity depends on the micronutrient cofactor, selenium. For this reason, GPx is often referred to as a selenocysteine peroxidase [91]. The GPx family comprises eight isoenzymes (GPx1-8). GPx1 to 4 incorporate selenocysteine which is a non-standard amino acid, where the sulfur atom of cysteine is replaced by selenium. GPx6 contains selenium only in humans, which is not the case with rodents. GPx5, 7, and 8 do not have selenium and instead have a "normal" cysteine [92].

$$H_2O_2 + 2GSH \xrightarrow{\text{GPx}} 2H_2O + GS - SG\ldots \text{ (Equation 14)}$$

$$ROO^{\bullet} + 2GSH \xrightarrow{\text{GPx}} ROH + H_2O + GS - SG\ldots \text{ (Equation 15)}$$

Among all isoforms, GPx1 is the most abundant and is present in virtually all cells. GPx2 is found in the gastrointestinal tract, predominantly in the intestine, while GPx3 is primarily found in the kidney followed by its presence in extracellular fluids as a glycoprotein [91]. Although most forms of GPx are tetrameric, GPx4 is a monomer and regarded as is phospholipid hydroperoxide. This is because GPx4 is the only GPx enzyme that breaks down phospholipid hydroperoxides [91]. GPx5 is limited to the epididymis of the male reproductive tract in mammals and is regulated by androgens while GPx6 is restricted to embryos and adult olfactory epithelium [93]. GPx7 and GPx8 are present in the endoplasmic reticulum [93]. Quantification of GPx levels can indicate the body's antioxidant capacity.

GPx's function is also coupled with the action of the enzyme, glutathione reductase (GR). GPx neutralizes H_2O_2 using GSH as a reducing agent. This results in the oxidation of GSH to GSSG. The flavoprotein enzyme, GR, regenerates GSH from its oxidized form, with NADPH as a source of reducing power (Figure 2). Therefore, the action of GR is crucial for enabling GPx's antioxidant function. Quantification of GR levels is clinically significant as it indicates the level of GR present which helps maintaining the antioxidant pool [96].

Secondary Enzymes

In addition to the primary enzymes discussed earlier, the degradation of H_2O_2 is facilitated by a group of thiol-containing enzymes, which include the thioredoxin system comprising thioredoxins (TRX) and thioredoxin reductases (TRR), thioredoxin peroxidases (PRX), and glutaredoxins (GRX).

Thioredoxin system

The thioredoxin system comprises TRX, TRR, and NADPH. It is a major disulfide reductase system which are critical for defense against oxidative stress [100]. The small proteins, TRXs that are thiol antioxidants interact directly with reactive species like H_2O_2, $^{\bullet}OH$, and $OONO^-$, and effectively convert them into less harmful molecules. Within cells, there are two primary forms of thioredoxin: one is the cytosolic and nuclear variant called thioredoxin-1 (TRX1), and the other is the mitochondrial isoform known as thioredoxin-2 (TRX2) [101]. TRXs undergo oxidation while scavenging for oxidants but are subsequently restored to their active, reduced state by TRRs. TRRs are enzymes that utilize NADPH as a cofactor to transfer electrons to the oxidized thioredoxin, converting it back to its reduced and active form, which can then continue its role in maintaining the redox balance within the cell [102]. It has been stated that mammalian TRR has three different isoenzymes, cytosolic TRXR1, mitochondrial TRXR2 and TRXR3 [103]. The TRX system is present in various cellular compartments, allowing it to maintain redox balance and shield the cell against oxidative stress [100]. The TRX protein can be used as a marker, with its increased levels indicative of oxidative stress. The upregulation of TRX is a protective response to counteract the damaging effects of oxidative stress [104, 105].

Thioredoxin peroxidases (PRX)

PRX, comprise a large family of thiol-dependent peroxidases that catalyse the reduction of H_2O_2, alkyl hydroperoxides, and $OONO^-$ [106]. PRX is among the most abundant proteins in erythrocytes. They catalyse the reduction of H_2O_2 or other peroxides, using electrons provided by thioredoxins. In this process, the PRX themselves undergo oxidation and become a disulfide, which is later reduced back to their active form by TRRs. Six PRX isoforms are present in humans – PRDX1, PRDX2, PRDX3, PRDX4, PRDX5, PRDX6 [107]. Unlike the other PRDX isoforms that are present in various cellular compartments such as the cytoplasm, mitochondria, and endoplasmic reticulum, PRDX5 is specifically localized in the peroxisomes. Accumulation of oxidized PRX indicates disrupted cellular redox homeostasis, with intermolecular disulfide and hyperoxidized forms accumulating under increased oxidative stress, serving as markers of cellular damage caused by ROS, and compromised redox balance [108].

c. Glutaredoxins (GRX)

GRX are a family of small redox-regulating proteins that facilitate the reduction of disulfide bonds in target proteins, like thioredoxins. They use GSH as a cofactor in their redox reactions. GRX play a crucial role in cellular defense against oxidative stress and in the repair of damaged proteins. The two most studied human GRXs are the dithiol isoforms GRX1, which mainly exists in the cytosol, and GRX2, which is located in the mitochondria, cytosol or nucleus depending on gene splicing [111]. GRX can be a useful marker for assessing the degree of oxidative stress.

Together, these secondary enzymes comprising thioredoxin-based systems and GRXs contribute to the effective degradation of H_2O_2 as well as other oxidative species and help maintain cellular redox homeostasis, thus protecting cells from oxidative damage [111].

Non-enzymatic Antioxidants

Endogenous Non-enzymatic Antioxidants

Glutathione (GSH)

GSH is a tripeptide composed of three amino acids: cysteine, glutamic acid, and glycine. It is the most abundant thiol antioxidant and is present in cytosol, nuclei, and mitochondria. It serves as the major soluble antioxidant in these cell compartments, playing crucial protective roles against oxidative/nitrative stress. It possesses the ability to directly scavenge $^{\bullet}OH$ and $^1O_2$, bolstering its effectiveness as an antioxidant [113]. In the body, GSH exists in two isoforms: the reduced form known as GSH and the oxidized form known as GSSG. GSSG is produced when GSH reacts with oxidizing agents such as H_2O_2 or free radicals. The antioxidant capacity of thiol compounds, like GSH, is attributed to the presence of a sulfur atom, which readily accommodates the loss of a single electron during free radical neutralization [57]. Monitoring the levels of total GSH (GSH + 2 GSSG + protein-bound GSH) and the GSH:GSSG ratio serves as reliable indicators of oxidative stress [114]. A depletion in these levels and a decreased GSH:GSSG ratio highlight the presence of oxidative stress, signifying the importance of GSH's role in maintaining cellular redox balance [103].

Uric acid (UA)

UA is a weak organic acid and the end-product of purine nucleotides degradation. It is an integral part of the body's antioxidant system. In the extracellular fluid, at a physiological pH of 7.4, UA mainly exists in the ionized form of urate, while in the urine, which is usually acidic, the un-ionized UA form predominates. UA contributes to over half of the blood plasma's antioxidant capacity [117]. It acts as an effective antioxidant, scavenging $OONO^-$ and other ROS. Additionally, UA may assist in the removal of $O_2^{\bullet-}$ by inhibiting the degradation of SOD. The removal of $O_2^{\bullet-}$ helps prevent its reaction with $^{\bullet}NO$, thereby blocking the formation of $OONO^-$. In this manner, UA aids in reducing oxidative stress and its elevated levels serve as a biomarker of [118].

Albumin

Albumin is the most abundant circulating protein in mammals including humans. It is an antioxidant that is capable of scavenging $^{\bullet}OH$. It exists in three isoforms named as

mercaptalbumin (reduced albumin), non-mercaptalbumin-1 and -2 (oxidized albumin), respectively [121]. Oxidization of albumin results in the loss of its antioxidant properties to give, oxidized albumin which further contributes to oxidative stress. Increased levels of oxidized albumin can be indicative of oxidative burden in the body [122]. *In vivo* studies suggest that albumin's redox state shifts to a more oxidized state in response to the severity of the pathological condition in various diseases such as liver diseases and renal failures [123].

Bilirubin

Bilirubin is a yellowish-orange pigment and a byproduct of the breakdown of heme, which is found in haemoglobin, myoglobin, and other heme-containing proteins in red blood cells. Bilirubin exists in various isoforms, with bilirubin IXα being the primary isoform in vivo (approximately 99%), while isoforms IIα and XIIIα are present in lower proportions [124]. Bilirubin has been identified as a potent antioxidant, shielding lipids from oxidation by effectively scavenging $ROO^{\bullet}$, and $^1O_2$. Its presence in serum significantly contributes to the overall antioxidant capacity in blood plasma [125]. This is achieved via its actions on quenching newly formed free radicals, preventing chain reactions that lead to lipid peroxidation. Elevated levels of bilirubin in the bloodstream indicate enhanced antioxidant actions making it a valuable marker for assessing oxidative stress [125].

Coenzyme Q10 (CoQ10)

CoQ10 is a powerful antioxidant naturally found in mitochondria. It is an important component of the electron transport chain where it shuttles electrons between various enzyme complexes as well as accepts free radicals that have escaped and which could form free radicals [128]. It combats oxidative stress by inhibiting lipid peroxidation caused by H_2O_2 [129]. It has also shown to protect DNA against H_2O_2-induced oxidation [130]. In biological systems, CoQ10 exists in two redox states: the reduced form (ubiquinol, $CoQ10H_2$) and the oxidized form (ubiquinone, CoQ10) [131]. CoQ10's antioxidant function is mainly attributed to its reduced ubiquinol form ($CoQ10H_2$), which is essential for neutralizing free radicals. The $CoQ10H_2$ acts as an electron donor in the cellular environment. When exposed to H_2O_2 radicals, $CoQ10H_2$ donates electrons to neutralize them, effectively transforming H_2O_2 into harmless H_2O and O_2 molecules. However, this reduced form needs to be continually regenerated from its oxidized form, ubiquinone (CoQ10). Owing to its antioxidant abilities, CoQ10 levels are used as biomarkers to assess oxidative stress [132].

Melatonin

Melatonin is an endogenous hormone derived from tryptophan. It is mainly released from the pineal gland in the dark. Along with regulating functions such as sleep, circadian rhythm, immunity, and reproduction, it is also seen to act as an effective antioxidant [134]. Melatonin can easily cross the blood-brain barrier and can enter circulation where it protects biomolecules against damage caused by free radicals by acting as a direct scavenger to detoxify ROS and RNS [134]. It neutralizes $^{\bullet}OH$ and the $OONO^-$ generated within the cells. It also scavenges $^1O_2$,

$O_2^{\bullet-}$, H_2O_2, $^{\bullet}NO$, and HOCl [134]. Moreover, melatonin and its metabolites can also indirectly reduce oxidative stress by enhancing the activities of antioxidative defense systems via stimulating the expression and function of antioxidant enzymes, as well as GSH [134]. It can also inhibit the activity of NOS, which produces $^{\bullet}NO$. Therefore, melatonin is seen to play an integral role in the body's antioxidant defenses [134].

Alpha-Lipoic acid (ALA)

ALA, synthesized in the mitochondria, is a caprylic acid-derived antioxidant. It plays an important role in bioenergetic reactions such as the Krebs cycle. It also plays a crucial role in nutrient breakdown. ALA is a sulfur-containing antioxidant. Unlike most antioxidants, which are active only in the lipid or aqueous phase, ALA is active in both phases. It is a very potent endogenous antioxidant as it acts as a chelating agent for metal ions, a quenching agent for ROS ($O_2^{\bullet-}$, $^{\bullet}OH$, and HOCl,), and a reducing agent for the oxidized form of GSH and vitamins C and E. The presence of heavy metals in the bloodstream are responsible for oxidative stress. However, ALA being an eminent antioxidant, removes metals from the bloodstream via chelation and prevents oxidative stress. Studies have shown that oxidants can lead to cell death via lysosomal breakage caused due to the involvement of intralysosomal iron which catalyses Fenton reactions. This results in peroxidative damage to lysosomal membranes. ALA protects lysosomes against such oxidative insults by chelating intralysosomal iron and consequently, preventing intralysosomal Fenton reactions. On digestion, ALA is converted to dihydrolipoic acid (DHLA). Like ALA, DHLA is also a strong antioxidant that quenches free radicals in both aqueous and lipid phases [135,136].

Exogenous Non-enzymatic Antioxidants

Vitamin A

Vitamin A encompasses a group of vital fat-soluble compounds known as retinoids and provitamin A carotenoids, with β-carotene being one of the most prominent examples. These compounds play a crucial role as dietary antioxidants, as they possess the remarkable ability to scavenge and neutralize free radicals directly [137]. Specifically, β-carotene, when metabolized in vivo, acts as a primary antioxidant by scavenging $^1O_2$. By preventing the formation of LOOHs through its reaction with $^1O_2$, β-carotene effectively curtails lipid peroxidation, thus safeguarding cellular structures from oxidative damage. Therefore, vitamin A is an important biomarker with its low levels being indicative of oxidative stress [137].

Vitamin C

Vitamin C, or ascorbic acid, is a water-soluble essential nutrient obtained through the diet. It exists in various forms, including ascorbic acid and its oxidized form, dehydroascorbic acid. Vitamin C is a potent reducing agent and an important scavenger of oxidants such as $^{\bullet}OH$, H_2O_2, and $^1O_2$ [140]. While neutralizing oxidant species, vitamin C is rapidly oxidized to DHA and removed from the blood. However, vitamin C can also act as a pro-oxidant, especially in the presence of transition metal ions like iron or copper. This dual function is vital for maintaining cellular redox balance. Monitoring changes in vitamin C levels in the blood can provide insights into the body's oxidative stress status [140].

Vitamin E

Vitamin E, a fat-soluble antioxidant, comprises eight different types: α-, β-, γ-, and δ-tocopherol, and α-, β-, γ-, and δ-tocotrienol. Among these, α-tocopherol demonstrates the highest antioxidant activity, effectively transferring hydrogen to various ROS like $O_2^{\bullet-}$ and ROO$^{\bullet}$. Its oxidized form can be restored to its active reduced state with the help of ascorbic acid, which donates electrons to the tocopheroxyl radical, converting it back to its antioxidant form, alpha-tocopherol [143]. A decrease in vitamin E levels in urine can serve as an indicator of reduced antioxidant status, indicating a compromised ability to combat oxidative stress and maintain cellular health, given its vital role as a primary fat-soluble antioxidant [144].

Selenium

Selenium is an essential trace element classified as a micronutrient and plays a vital role in various biological processes. It is a part of the group of antioxidant enzymes known as selenoproteins. Selenium acts as a powerful antioxidant, helping to combat oxidative stress by neutralizing harmful free radicals, thereby protecting cells from damage. It specifically helps in preventing lipid peroxidation of H_2O_2. Its incorporation into selenoproteins, such as GPxs and thioredoxin reductases, enables these enzymes to detoxify ROS and maintain redox homeostasis. Its levels are often quantified to assess the body's antioxidant capacity [146].

Zinc

Zinc is a trace element in the human body. Of its many functions, it plays a crucial role in reducing oxidative stress. As an ion, it helps inhibiting the production ROS and RNS via its structural role in antioxidant proteins and its influence on metallothionein induction (proteins rich in thiol groups that are induced to bind and store zinc). By binding to thiol groups of antioxidant enzymes, zinc shields them from oxidation, demonstrating its direct antioxidant activity [148]. Additionally, zinc functions as a cofactor for the important primary antioxidant, SOD1. Its deficiency can suppress SOD1 activity, making zinc levels an indirect marker of oxidative stress. Decreased zinc levels in cells are often associated with increased oxidative damage [148]. Monitoring zinc levels may provide insights into the body's antioxidant defense system and overall oxidative balance [148]. However, it's worth noting that more studies need to be conducted in humans to further understand the full extent of zinc's role as a biomarker of oxidative stress.

Polyphenols

Polyphenols are natural compounds present in plants that exhibit antioxidant activities. They are ingested via the consumption of fruits, vegetables, cereals, and beverages containing polyphenols. Fruits such as grapes, apples, pear, cherries, and berries, and beverages such as red wine, tea, or coffee, contain polyphenols. Herbs, spices, chocolates, cereals, and dry legumes are also rich in polyphenols [149]. 8000 phenolic compounds have been identified in the plants. Polyphenols can include flavonoids such as flavanols, flavones, isoflavones, anthocyanidins, resveratrol, curcumin, tannins, lignans, and phenolic acids [149]. The phenolic compounds and flavonoids are known to interact with ROS/RNS and can terminate their reaction. Polyphenols can react with NOS and may modulate the $^{\bullet}$NO production. Flavonoids

such as quercetin, silibin, and luteolin can inhibit the enzyme xanthine oxidase, which produces free radicals [149]. Regular intake of polyphenols can boost the body's antioxidant capacity.

The quantification techniques for the established endogenous and exogenous antioxidant markers are summarized in tables, 4 – 7. An imbalance between the body's antioxidant system and oxidants, favoring the generation of oxidants leads to oxidative stress. The Supplementary Table 1 summarizes the antioxidant capacity of the body to quench and manage the concentrations of various oxidant species in the body.

OXIDATIVE STRESS IN AGING

Aging is defined as an intrinsic, universal, multifactorial, and progressive process characterized by tissue degeneration and progressive loss of organ function, ultimately leading to increased mortality [150, 151]. It is a multifactorial process. Of the many theories, the 'free radical theory of aging,' also known as the 'oxidative stress theory of aging' has been of great interest [151]. The theory hypothesizes that aging is associated with structural damage caused due to the accumulation of oxidative damage to crucial macromolecules (lipids, DNA, RNA, and proteins) brought about ROS and RNS [150]. The increase in oxidative stress could be brought about by the failure of several defensive mechanisms to respond to the ROS-induced damage, particularly in the mitochondria [151].

Aging is associated with structural and functional changes in the mitochondria [152], which is accompanied by the alterations of biophysical properties of the membrane including alteration in the electron transport chain complexes activities, decreased fluidity, and energy imbalance and mitochondrial failure [151]. Reduced oxidative phosphorylation results in increased ROS production [153]. This gives rise to impaired cellular homeostasis and mitochondrial function leading to the increased vulnerability to oxidative stress [151]. Increased ROS can activate the pro-apoptotic protein, p66Shc which further contributes to the production of ROS. This, in turn, promotes the accelerated damage of the mitochondria, leading to apoptosis and finally resulting in the process of aging [153]. Therefore, p66Shc which is responsible for ROS generation and apoptosis induction is regarded as a link between ROS and aging [153].

Not only the increased production of ROS and RNS but also the decline in the efficiency of endogenous antioxidant systems with age leads to oxidative stress [150]. A study conducted by Reddy et al., 1998, assessed the levels of lymphocyte free radical generation ($O_2^{\bullet-}$ & H_2O_2), DNA damage, and antioxidant enzyme levels (GST, SOD, and CAT) in healthy individuals between 20-80 years [154]. They found that $O_2^{\bullet-}$ & H_2O_2 progressively increased while the antioxidant enzyme levels showed a gradual decrease from younger to older age [154]. The age-dependent decline in antioxidants has been attributed to various reasons such age-associated nutrition and hormonal changes. Malnutrition in older individuals resulting from poor nutritional habits, loss of appetite, or intestinal malabsorption may lead to deficiencies in trace elements such as Zn^{2+} ions, essential for SOD1 activity or selenium, essential for the synthesis of selenoenzyme GPx, thus weakening the body's antioxidant system [155]. The age-associated reduction in the secretion of the pineal hormone, melatonin which regulates both, the expression of genes coding for antioxidant enzymes such as SOD, GPx, and GR and directly influences their activities can also be the cause of declining antioxidant capabilities with age [154].

Studies have shown that oxidative stress can induce cellular senescence which is another factor that leads to aging. It is a physiological mechanism that stops cellular proliferation in response to damages that occur during replication [150]. Oxidative stress can promote cellular senescence as it causes DNA lesions, accelerates telomere shortening, and activates molecular pathways leading to growth arrest [153]. Senescent cells acquire an irreversible senescence-associated secretory phenotype (SASP). SASP involves the secretion of soluble factors (interleukins, chemokines, and growth factors), degradative enzymes like matrix metalloproteases (MMPs), and insoluble proteins/extracellular matrix components. ROS and RNS can induce cellular senescence by exerting effects on various SASP components [150].

Oxidative stress leading to cellular senescence by affecting SASP components is the pathogenesis of various conditions including cardiovascular diseases, acute and chronic kidney disease, neurodegenerative diseases, macular degeneration, biliary diseases, and cancer [150]. Vascular calcification which is a pathophysiological consequence of atherosclerosis can be caused due to SASP-driven osteoblastic trans differentiation of senescent smooth muscle cells [150]. In the neurodegenerative condition, AD, brain tissue biopsies were shown to have increased levels of p16, MMP, and IL-6 [150]. Oxidative stress is fundamental in age-associated conditions, thereby affecting lifespan and longevity. Increased inflammation is a pervasive feature of aging [156]. Given the close relationship between oxidative stress, inflammation, and aging, the oxidation-inflammatory theory of aging or 'oxi-inflamm-aging' has been hypothesized. The theory believes that aging is the resultant of the loss of homeostasis due to a chronic oxidative stress that affects the regulatory systems, including the nervous, endocrine, and immune systems. This may result in the consequent activation of the immune system giving rise to an inflammatory state. In this manner, chronic oxidative stress and inflammation feed each other forming a continuous vicious cycle, and consequently, increases the age-related morbidity and mortality [150].

CONCLUSION

Oxidative stress is a phenomenon in which excessive oxidant species attack cellular macromolecules such as lipids, nucleic acids, and proteins. Studies have indicated that oxidative stress is an important factor driving the process of aging and it can also be associated with age-related pathologies. This warrants the need to assess and effectively understand mechanisms of oxidative stress in the body along with its reliable quantification. As directly quantifying oxidative stress is not feasible, indirect quantification of oxidative stress by measuring oxidative damage markers (lipid peroxidation, nucleic acid and protein damage markers) and antioxidants (enzymatic and non-enzymatic) can indicate the degree of oxidative stress in the body. Oxidative stress is involved in the mechanism of aging. Managing oxidative stress could delay the expression of SASP factors that leads to cellular senescence, therefore delaying aging.

ABBREVIATIONS

ROS - Reactive oxygen species
RNS - Reactive nitrogen species
$O_2^{\bullet -}$ - Superoxide anion radical
H_2O_2 - Hydrogen peroxide
$^{\bullet}OH$ - Hydroxyl radicals
$^1O_2$ - Singlet oxygen

SOD - Superoxide dismutase
CAT - Catalase
GPx - Glutathione peroxidase
UA - Uric acid
GSH – Glutathione
$ROO^{\bullet}$ - Peroxyl radical
$^{\bullet}NO$ - Nitric oxide
$^{\bullet}NO_2$ - Nitrogen dioxide
HOCl - Hypochlorous acid
HOBr - Hypobromous acid
O_3 - Ozone
HNO_2 - Nitrous acid
NO^+ - nitrosyl cation
NO^- - Nitroxyl anion
N_2O_3 - Dinitrogen trioxide
N_2O_4 - Dinitrogen tetraoxide
NO_2^+ - Nitronium (nitryl) cation
ROOH - Hydroperoxides
HCOR - Aldehydes
$OONO^-$ - Peroxynitrite
O_2 - Molecular oxygen
HO_2 - Hydroperoxyl radical
$HOO^{\bullet}$ - Per hydroxyl radical
NOS - Nitric oxide synthases
nNOS - Neuronal NOS
eNOS - Endothelial NOS
iNOS - Inducible NOS
NO_2^- - Nitrite
NO_3^- - Nitrate
CO_2 – Carbon dioxide
$ONOOCO_2^-$ - Peroxo carboxylate
ONOOH - Peroxynitrous acid
$NO_2^{\cdot}+$ - Nitrosonium cation
HOCl - Hypochlorous acid
HOBr - Hypobromous acid
MPO – Myeloperoxidase
EPO - Eosinophil peroxidase
$^{\cdot}Q^-$ - Semiquinone anion
NOX - NADPH oxidase
UVA - Ultraviolet A
8-oxoGua - 8-Oxo-7,8- dihydroguanine
PFAS - Per- and polyfluoroalkyl substances
GST - Glutathione-S-transferases
GR – Glutathione reductase
BPA - Bisphenol A
BPAG - Bisphenol A glucuronide

BPAS - Bisphenol A sulfate
Dox – Doxorubicin
GSTP1 - Glutathione S-transferase pi 1
DCFDA - 5-(and -6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate
DCFH - 2',7'-dichlorodihydrofluorescein
DCF - 2′,7′-dichlorofluorescein
DHE – Dihydroethidium
MitoSOX - Mitochondria-targeted dihydroethidium
cGMP - cyclic guanosine monophosphate
MAPK - Mitogen-activated protein kinase,
PI3Ks - Phosphoinositide 3-kinases
PTEN - Phosphatase and TENsin homolog
Nrf2 - Nuclear factor erythroid 2-related factor 2
Keap1 - Kelch-like ECH-associated protein 1
NQO1 - NAD(P)H quinone oxidoreductase 1
Srx1 - Sulfiredoxin 1
PUFA - Polyunsaturated fatty acids
CH_2 – Methylene group
$L^{\bullet}$ - lipid radical
$LOO^{\bullet}$ - Lipid peroxyl radical
LA - Linoleic acid
AA - Arachidonic acid
EPA - Eicosapentaenoic acid
DHA - Docosahexaenoic acid
LOOH - Lipid hydroperoxides
H(P)ODEs - Hydro(pero)xyoctadienoates
MDA – Malondialdehyde
4-HNE - 4-Hydroxy-2-nonenal
4-HNE-MA - 4-Hydroxynonenalmercapturic acid
4-ONE - 4-Oxo-2-nonenal
CML - Carboxymethyl lysine
PG - Prostaglandin
IsoPs - Isoprostanes
F2-IsoP - F2-isoprostanes
8-iso$PGF_2\alpha$ - 8-isoprostaglandin $F_2\alpha$
11-PGF2α - 11-β-prostaglandin F2α
15-PGF2α - 15-prostaglandin F2α
8-OHdG - 8-Hydroxy-2’-deoxyguanosine
8-OHG - 8-Hydroxyguanine
8-NO2-G - 8-nitroguanine
8-oxoG - 8-hydroxyguanosine or 7,8-dihydro-8-oxo-guanosine
AD - Alzheimer’s disease
PD - Parkinson’s disease
8-NdG - 8-nitroguanosine
GSSG - Glutathione disulfide
AOPP - Advanced Oxidation Protein Products

AGEs - Advanced glycation end products
CoQ10 - Coenzyme Q10
TRX - Thioredoxins
TRR - Thioredoxin reductases
PRX - Thioredoxin peroxidases
GRX – Glutaredoxins
TRX1 - Thioredoxin-1
TRX2 - Thioredoxin-2
$CoQ10H_2$ – Ubiquinol
ALA - Alpha-Lipoic acid
DHLA - Dihydrolipoic acid
MMPs – Metalloproteases
SASP - Senescence-associated secretory phenotype

DECLARATIONS

Disclosure

The data and materials in this manuscript have not been published elsewhere and are not under consideration by another journal.

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TABLES

Table 1

Lipid Peroxidation markers			
Marker	**Sample**	**Technique**	**Reference**
Lipid hydroperoxides (LOOH)	Tissue, plasma, serum lipoproteins	Chemiluminescence-Based HPLC Detection, Iodometric Assay, Ferrous Oxidation of Xylenol	[41,42]
Malondialdehyde (MDA)	Serum, plasma, urine, CSF, erythrocytes, saliva	TBARS assay*, HPLC, GC-MS	[32]
4-hydroxy-2-nonenal (4-HNE)	Serum, plasma, urine, CSF, tissue	GC-MS*, HPLC–MS/MS, ELISA, IHC	[45,46 32]
4-Hydroxynonenalmercapturic acid (4-HNE-MA)	Urine	HPLC–MS/MS	[46].
4-oxo-2-nonenal (4-ONE)	Urine	Isotope-dilution mass spectrometry	[48]
8-isoprostaglandin F2α	Urine	GC-MS* ELISA HPLC–MS/MS	[43,46]
11-β-prostaglandin F2α (11-PGF2α),	Urine	HPLC–MS/MS	[46]
15-prostaglandin F2α (15-PGF2α)	Urine	HPLC–MS/MS	[83]
8-Iso-15(R)-Prostaglandin F2α	Urine	HPLC–MS/MS	[83].

Table 1. Markers of lipid peroxidation

* *Indicates the 'gold standard technique' for a given marker*

Abbreviations: HPLC - High-Performance Liquid Chromatography; TBARS - Thiobarbituric acid reactive substances; GC-MS - Gas Chromatography-Mass Spectrometry; HPLC–MS/MS - High-Performance Liquid Chromatography with Tandem Mass Spectrometry; ELISA - Enzyme-Linked Immunosorbent Assay; IHC – Immunohistochemistry;

Table 2

Nucleic acid damage markers			
Marker	**Sample**	**Technique**	**Reference**
DNA Damage			
8-Hydroxy-2'-deoxyguanosine (8-OHdG)	Saliva, serum, plasma, tissue, urine	LC-MS, ELISA, HPLC	[89, 90]
8-Hydroxyguanine (8-OHG)	Serum, urine, saliva	HPLC, HPLC with an ECD	[83,92, 93]
8-Nitroguanine (8-NO2-G)	Peripheral lymphocytes, Urine	HPLC with an ECD, HPLC–MS/MS	[83,94]
RNA Damage			
8-hydroxyguanosine (8-oxoG)	Urine	HPLC–MS/MS	[83]
8-Nitroguanosine (8-NdG)	Urine	HPLC–MS/MS	[83]

Table 2. Nucleic acid damage markers

Abbreviations: LC-MS - Liquid chromatography mass spectrometry; ELISA - Enzyme-Linked Immunosorbent Assay; HPLC - High-Performance Liquid Chromatography; ECD - electrochemical detector; HPLC–MS/MS - High-Performance Liquid Chromatography with Tandem Mass Spectrometry

Table 3

Protein damage markers			
Marker	**Sample**	**Technique**	**Reference**
Carbonylation			
Protein carbonylation content	Plasma, serum, tissue, aqueous humor, saliva	Spectrophotometric DNPH assay coupled to protein fractionation by HPLC*, ELISA, immunoblot, IHC, cytochemistry	[32,64]
Oxidation of sulfur-containing aromatic amino acids			
Cystine	Serum, plasma	HPLC	[32,65]
Methionine sulfoxide	Serum, plasma	Western blotting, LC-MS techniques	
Oxidation of aromatic amino acids			
Dityrosine	Serum, plasma, urine	LC-MS, spectrophotometric assay, spectrofluorimetric assays, HPLC–MS/MS	[32, 46]
Advanced oxidation protein products (AOPP)	Serum, plasma, and saliva	Spectrophotometry*	[32, 71]
Nitration			
Nitrotyrosine	Serum, plasma, urine	Mass spectroscopy*, IHC, ELISA, HPLC, LC-MS	[32,46]
Glycoxidation			
Carboxymethyl lysine (CML)	Serum, plasma, tissue	ELISA, Spectrophotometry, IHC, immunoblot, HPLC–MS/MS	[32,46]
Pentosidine	Serum, plasma, tissue	ELISA, Spectrophotometry, IHC, immunoblot, HPLC–MS/MS	[32, 75]
Halogenated products			
3-bromotyrosine	Urine	LC/MS/MS, HPLC–MS/MS	[46, 77]
3-Chlorotyrosine	Plasma, serum, whole blood, urine	HPLC–MS/MS	[46, 78]
Acrolein	Urine, tissue	ELISA IHC	[81,82]
Allantoin	Urine	Rimini–Schryver reaction-colorimetric assay*, LC-MS/MS, HPLC–MS/MS	[46,47]

Table 3. Protein damage markers

* *Indicates the 'gold standard technique' for a given marker*

Abbreviations: DNPH - 2,4- dinitrophenylhydrazine; HPLC - High-Performance Liquid Chromatography; ELISA - Enzyme-Linked Immunosorbent Assay; IHC – Immunohistochemistry; LC-MS- Liquid chromatography mass spectrometry; HPLC–MS/MS - High-Performance Liquid Chromatography with Tandem Mass Spectrometry; LC-MS/MS - Liquid chromatography electrospray ionization tandem mass spectrometry

Table 4

Endogenous primary enzymatic antioxidant markers			
Marker	**Sample**	**Technique**	**Reference**
Superoxide dismutase (SOD)	Serum, plasma, erythrocytes, tissues, urine	Phenyltetrazol chloride assay INT assay 4-methoxy-6-nitro assay XTT assay NBT assay	[85-89]
Catalase (CAT)	Erythrocytes, Serum, Plasma, Tissues	UV spectrophotometry Iodometry Chemiluminescence Polarimetry Titration	[89,90]
Glutathione peroxidase (GPx)	Erythrocytes, whole blood, plasma, tissue	Spectrophotometry Ellaman's reagent CUPRAC reagent O-phthalaldehyde reagent Polarographic GSH analysis ELISA	[94,95]
Glutathione reductase (GR)	Serum, plasma, saliva	ELISA, Goldberg and Spooner enzymatic reaction	[97-99]

Table 4. Endogenous primary enzymatic antioxidant markers

Abbreviations: INT - 2-(4-idophenyl) 3-(4-nitrophenol)-5-phenyltetrazolium; XTT - 3-{1-[(phenylamino)-carbonyl]-3,4-tetrazolium}-bis (4-methoxy-6-nitro) benzenesulfonic acid; NBT - nitro blue tetrazolium; ELISA - Enzyme-Linked Immunosorbent Assay

Table 5

Endogenous secondary enzymatic antioxidant markers			
Marker	**Sample**	**Technique**	**Reference**
Thioredoxins (TRX)	Serum, urine	ELISA	[104, 105]
Thioredoxin peroxidases (PRX)	Erythrocyte	Western blotting, Reverse Phase HPLC	[109, 110]
Glutaredoxins (GRX)	Serum	Fluorescent GRX activity assay	[112]

Table 5. Endogenous secondary enzymatic antioxidant markers

Abbreviations: ELISA - Enzyme-Linked Immunosorbent Assay; HPLC - High-Performance Liquid Chromatography

Table 6

Endogenous non-enzymatic antioxidant markers			
Marker	**Sample**	**Technique**	**Reference**
Glutathione (GSH)	Whole blood, Plasma, Serum, Tissues, Urine	Ellman's reagent assay LC-MS/MS Colorimetry Fluorometry HPLC Spectrophotometry	[115, 116]
Uric acid (UA)	Blood, Urine, Serum	Colorimetry LC-MS-TOF, HPLC	[119, 120]
Bilirubin	Plasma, serum, urine, feces	Diazo transfer reaction* HPLC Direct spectrophotometry Transcutaneous methods. Chemiluminescence Polarography Fluorometry	[126, 127]
Coenzyme Q10 (CoQ10)	Plasma, Tissues, Platelets	HPLC-ECD UV-detector HPLC-MS LC-MS/MS	[133]

Table 6. Endogenous non-enzymatic antioxidant markers

* *Indicates the 'gold standard technique' for a given marker*

Abbreviations: LC-MS/MS - Liquid chromatography electrospray ionization tandem mass spectrometry; HPLC - High-Performance Liquid Chromatography; LC-MS-TOF - Liquid chromatography time-of-flight mass spectrometry; ECD- electrochemical detector; UV – Ultraviolet; HPLC-MS - High-performance liquid chromatography coupled to mass detection

Table 7

Exogenous non-enzymatic antioxidants markers			
Marker	**Sample**	**Technique**	**Reference**
Vitamin A	Serum, Plasma, Tissues	APCI/LC-MS Reversed phase HPLC	[138, 139]
Vitamin C	Blood, Tissues, Urine	Dinitrophenylhydrazine method EC-HPLC UV-HPLC Reversed phase HPLC	[141, 142]
Vitamin E	Whole blood, Plasma, Serum, Urine	LC-MS/MS GC-MS Reversed phase HPLC Fluorimetry	[145]
Selenium	Plasma, Serum, Blood, Urine	Graphite-furnace atomic-absorption spectrometry HGAAS MFS ICP-MS	[147]

Table 7. Exogenous non-enzymatic antioxidants markers

Abbreviations: APCI - Atmospheric pressure chemical ionization; LC-MS- Liquid chromatography mass spectrometry; HPLC - High-Performance Liquid Chromatography; EC- Electrochemical Detection; UV- Ultraviolet; LC-MS/MS - Liquid chromatography electrospray ionization tandem mass spectrometry; GC-MS - Gas Chromatography-Mass Spectrometry; HGAAS - Hydride-generation atomic absorption spectrometry; MFS- molecular fluorescence spectrometry; ICP-MS - HPLC coupled to inductively coupled plasma-mass spectrometry

FIGURES

Figure 1

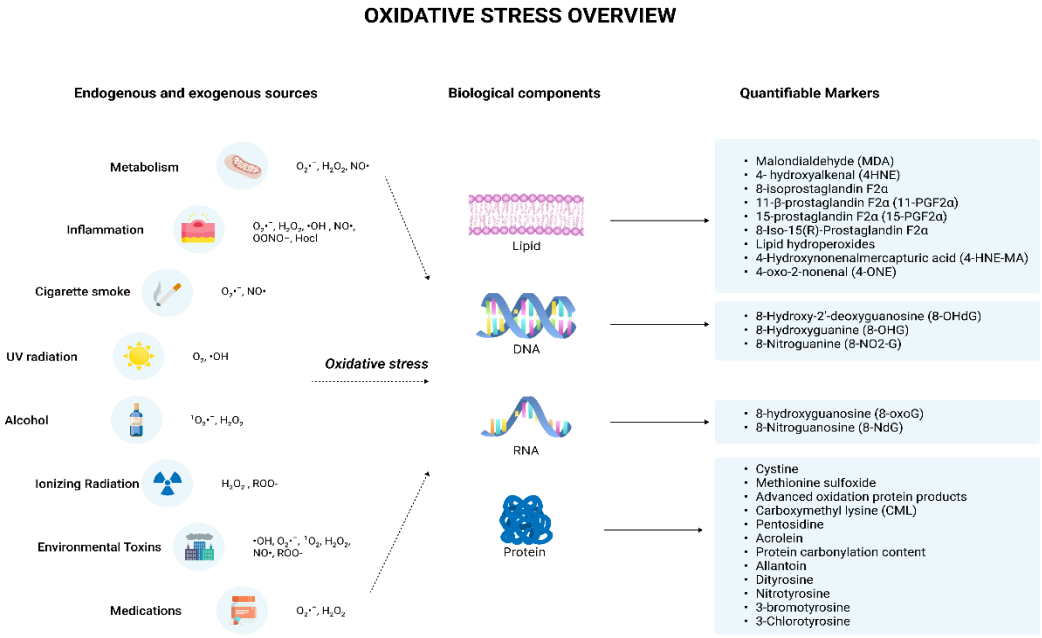

Figure 1. Oxidative stress overview

Abbreviations

$O_2^{\bullet-}$ - Superoxide anion radical
H_2O_2 - Hydrogen peroxide
•OH - Hydroxyl radical
NO• - Nitric oxide
OONO− - Peroxynitrite
Hocl - Hypochlorous acid
$^1O_2$ - Singlet molecular oxygen
ROO- - Hydroperoxides
DNA - Deoxyribonucleic acid
RNA - Ribonucleic acid

Figure 1. Oxidative stress overview. The illustration indicates the various sources that can trigger the production of reactive oxygen species and reactive nitrogen species. The endogenous sources include metabolism and inflammation while the exogenous sources include cigarette smoke, UV radiation, alcohol, ionizing radiation, environmental toxins, and medications. These sources lead to the production of various free radicals such as $O_2^{\bullet-}$, H_2O_2, •OH, NO•, OONO−, Hocl, $^1O_2$, and ROO- which can give rise to oxidative stress. This results in the damage of various cellular components including lipids, DNA, RNA, and protein leading to the formation of damaged products. The damaged products act as good markers of oxidative stress indicating the degree of oxidative stress-mediated damage for each component.

Figure 2

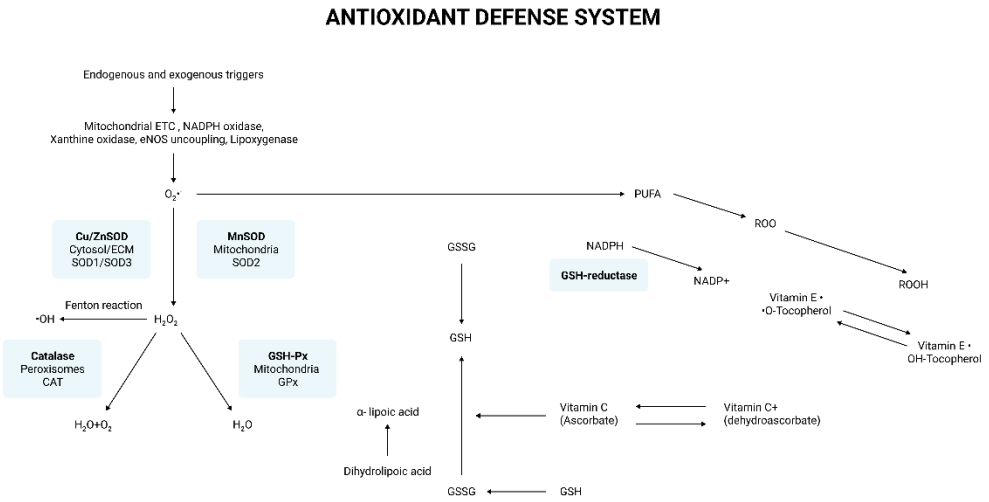

Figure 2. Antioxidant defense system.

Abbreviations

ETC - Electron transport chain
eNOS - Endothelial nitric oxide synthase
O_2•$^-$ - Superoxide anion radical
SOD – Superoxide dismutase
ECM - Extracellular matrix
H_2O_2 - Hydrogen peroxide
•OH - Hydroxyl radical
GSH-Px - Glutathione peroxidase
H_2O – Water
O_2 - Oxygen
GSSG - Glutathione disulfide (oxidized form)
GSH – Glutathione (reduced form)
PUFA - Polyunsaturated fatty acids
ROO• - Lipid peroxyl radical
ROS- Reactive oxygen species

Figure 2. Antioxidant defense system. The illustration indicates the various antioxidants that help combat oxidative stress in the body. Endogenous and exogenous triggers lead to the production of ROS. Mitochondrial ETC, NADPH oxidase, xanthine oxidase, eNOS uncoupling, and lipoxygenase produce O_2•$^-$. The SOD system comprises cytoplasmic Cu/ZnSOD (SOD1), the mitochondrial MnSOD (SOD2), and the extracellular Cu/ZnSOD (SOD3). SOD is the first enzyme to catalyze O_2•$^-$ to H_2O_2, which, in turn, is reduced to water by catalase and GSH-Px. SOD-derived H_2O_2 may contribute to oxidative stress by the production of the •OH radical via the Fenton type reaction. GSH-Px neutralizes oxidant species using GSH as a reducing agent, which results in the formation of the oxidized, GSSG. GSSG is recycled back to GSH through the action of another enzyme called GSH reductase, which uses NADPH as a cofactor.

$O_2^{\bullet -}$ can initiate lipid peroxidation by the oxidative degradation of lipids such as PUFAs in cell membranes. This results in the formation of ROO•. Vitamin E is an important antioxidant scavenging ROO• and on doing so vitamin E (OH-Tocopherol) gets converted into tocopherol radical (•O-Tocopherol). GSH can help regenerate tocopherol by donating electrons to the tocopherol radical, converting it back to its antioxidant form. The important antioxidant, vitamin C (ascorbic acid) can also help regenerate the antioxidant form of OH-Tocopherol from its radical form. Ascorbic acid can be oxidized in the extracellular environment in the presence of metal ions to its less active form, dehydroascorbic acid. Dehydroascorbate can be recycled back to ascorbic acid through various cellular mechanisms facilitated by enzymes and the donation of electrons. The reduced form of OH-Tocopherol indirectly contributes to this recycling process by donating electrons. Additionally, the conversion of GSH to GSSG can also facilitate the regeneration of ascorbic acid from dehydroascorbate.

α-Lipoic acid is a very potent antioxidant as it active in both, lipid and aqueous phases. It directly scavenges and neutralizes ROS by donating electrons, thereby reducing oxidative damage. During this process, α-lipoic acid is oxidized to dihydrolipoic acid. Dihydrolipoic itself is a powerful antioxidant and has the ability to recycle and regenerate the other antioxidants, vitamins C and E, back to their active forms. Now, dihydrolipoic can be regenerated to α-lipoic acid by GSH. This regenerative capacity is crucial for maintaining a pool of active antioxidants in the cell and maintaining the cellular defense against oxidative stress.