CHAPTER I

1. INTRODUCTION

A free radical is a molecule or an atom having a single or more unpaired electron in its outer orbital, making them very unstable and reactive. The word free radical was first described by Moses Gomberg in 1900 (1). In 1950, free radicals were found in the biological systems (2). The free radicals have low stability and high reactivity and also have inference chemical properties by conferring reactivity to different biological targets like lipids, proteins, DNA.

In 1894, Henry J.H Fenton, a British chemist proposed the chemical mechanism catalyzed by iron ions towards formation of free radicals by generation of hydroxyl radicals from hydrogen peroxide and thereby causing toxicity, named as Fenton reaction (3). In 1934, German chemist Fritz Haber, Nobel prize winner along with an Australian chemist Joseph Weiss discovered that superoxide could be converted to hydrogen peroxide and then to hydroxyl radical, named as Haber-Weiss reaction (3). In 1991, Helmut Sies first proposed that the imbalance of pro-oxidant to antioxidant equilibrium by favoring the pro-oxidant results in the oxidative stress and thereby forming the basis for antioxidant therapy for treating various diseases associated with oxidative stress (4).

In 1935, Nikolai Semenov, Nobel prize winner and Russian chemist discovered the biological function of reactive nitrogen species. He described the free radical reactions as initiation, propagation, branching and termination (3).

Most of the living organisms consume 90% of the oxygen by cytochrome oxidase in the electron transport chain by forming water and the electron transport chain is linked by oxidative phosphorylation thereby generating ATP as an energy source (1). The remaining 10% or less molecular oxygen undergoes reduction through one electron successive pathways to superoxide anion radical which in turn convert to hydrogen peroxide by one electron reduction and two proton acceptance (1). The formed hydrogen peroxide (H_2O_2) in turn accepts one electron leads to hydroxyl radical (•OH) along with hydroxyl anion (OH^-) formation. The hydroxyl radical in turn interacts with one more electron and proton, resulting in the formation of water.

1.1 TYPES OF REACTIVE SPECIES

The free radicals are chemically reactive molecules having nitrogen or oxygen named as reactive nitrogen species (5) or reactive oxygen species (ROS). ROS includes hydroxyl radical,

superoxide and hydrogen peroxide like non radical species (3). RNS include peroxynitrite and nitrogen dioxide (3).

Table 1.1 Reactive Oxygen Species

Free radical
Hydroxyl: OH^-
Alkoxyl: RO-
Peroxyl: RO_2-
Superoxide : $O_2^{\cdot -}$
Hydroperoxyl: HO_2
Non free radical
Singlet Oxygen: (O)
Hypochlorous acid: HOCL
Ozone: O_3
Hydrogen peroxide: H_2O_2
Hypobromous acid: HOBR

Table 1.2 Reactive Nitrogen Species

Free radical
Nitrous acid: NHO_2
Nitric Oxide: NO
Non free radical
Peroxynitrite: ONOO
Nitrogen dioxide: NO_2

1.2 SOURCE FOR ROS AND RNS GENERATION

ROS, a natural byproduct is formed in the biological process of the cell. The increase in ROS levels results in cellular damage. ROS formation and decomposition occur by both endogenous and exogenous from different sources.

1.2.1 Endogenous ROS and RNS

In the biological process, oxidation is important for energy production in catalytic reactions pertaining to oxygen. In the cell, ROS is produced in mitochondria in which the oxidative

phosphorylation occurs resulting in the superoxide radical ($\bullet O^{2-}$) formation as a byproduct. These superoxide radicals are highly reactive to nitric oxide thereby producing peroxynitrite and nitrogen dioxide like reactive nitrogen species by activating the enzymes like NADPH oxidase. In biological systems, ROS are produced during cytochrome P450 metabolism by oxidoreductase enzymes. Phagocytic cells are also responsible for ROS production during the host cell defense mechanism. Some of the endogenous factors responsible for oxidant formation are electron transport chain occurring in mitochondria, plasma membrane and endoplasmic reticulum, peroxidases, lipoxygenases, nitric oxide synthase, cytochrome P450, haemoglobin, myoglobin, xanthine oxidase, prostaglandin synthases and autooxidation of catecholamines, metal ions and glucose etc. (6).

1.2.2 Exogenous ROS and RNS

Cellular events such as toxicant exposure, radiation damage, smoking and environmental pollution lead to exogenous ROS production thereby causing oxidative stress. During radiolysis, the hydroxyl radical (•OH) formed is also responsible for the oxidative stress. Hydrogen peroxide binds to macromolecules like deoxyribonucleic acid (DNA) thereby causing cellular damage. Some of the exogenous factors responsible for oxidant formation are UV radiation, ultrasound radiation, nitrogen oxides, sulfur oxides, oxidized food stuffs, drug metabolism, smoking like combustion process, metal ions like Fe, Cu overload, etc. (6).

Exogenous anti-oxidants like Ascorbic acid, Vitamin E, Phenolic acids (benzoic acid, cinnamic acid etc), Flavonoids (flavones, flavanone, etc) are available in fruits and vegetables counteract with the endogenous defense system thereby decreasing the disease incidence (4).

1.3 PHYSIOLOGICAL ROLES OF ROS AND RNS

In the healthy tissues, ROS and RNS are well regulated by maintaining the homeostasis at the cellular level. ROS and RNS acts as second messengers to signaling molecules. In mitochondria, ATP is formed from ADP by oxidative phosphorylation and is used as free energy in the biological process. Some of the important roles are: in target protein, hydrogen peroxide interacts with cysteine thiolate anions and oxidize it to sulfenic form thereby altering its structure as well as function. ROS plays a vital role in cell proliferation of cancer cells by maintaining ROS in higher level than normal cells by enhancing pathways in redox signaling like NF-κB (nuclear factor kappa B). ROS and RNS play a physiological role in vascular functions, platelet activation, blood pressure regulation and also in regulation of smooth muscle (7).

1.4 CELLULAR DEFENSE AGAINST ROS

For the survival of aerobic life, reactive oxygen species detoxification is predominant there by generating a balance between the ROS production along with its removal and an imbalance leads to the cause of oxidative stress. Cells normally mitigate the harmful effects of ROS through defense mechanisms which include antioxidants like vitamin E, ascorbic acid and glutathione having low molecular weight along with antioxidant enzymes like thioredoxins, superoxide dismutase, catalase, lactoperoxidases (7).Water soluble molecule like ascorbic acid has the capacity to reduce ROS, where as lipid soluble molecule like vitamin E also known as α-tocopherol also plays a similar role in membranes thereby reducing ROS.

In 1968, Irvin Fridovich discovered superoxide dismutase (8) in which one molecule of hydrogen peroxide (H_2O_2) with oxygen (O_2) results from the conversion of two superoxide anions by catalyzing process (3).

$$2\ O^{2-} + 2H^{+} \rightarrow H_2O_2 + O_2$$

Hydrogen peroxide converts to water and oxygen by catalase thereby completing the detoxification proposed by SOD enzyme.

$$2\ H_2O_2 \rightarrow 2\ H_2O + O_2$$

Table 1.3 Different types of free radicals and their scavenging system

Types of free radicals/oxidants	**Defense system**
Hydroxyl radical	Mn-SOD, Cu, Zn-SOD
Superoxide anion	Superoxide dismutase (8)
Peroxy radicals ($ROO^{\cdot}$)	Tocopherol, Ubiquinone
Hydroperoxides (ROO)	Catalase, Glutathione reductase, Selinium
Hydrogen peroxide (H_2O_2)	Catalase, Selinium Glutathione Peroxidase
Transition metal ions	Chelators (Ferritin, Transferrin)

Some of the antioxidants which are non-enzymatic small molecules also play a significant role in ROS detoxification. One such important molecule against intra-cellular defence is glutathione, a tripeptide having glutamyl-cysteinyl-glycine residues having sulphhydryl group, responsible for oxidizing glutathione via NADPH-dependent reductase by generating the reduced form. Selenium containing enzymes like glutathione peroxidase catalyze organic peroxides, hydrogen

peroxide to alcohols. Oxidative stress of an organism is calculated based on the ratio of oxidized form of glutathione (GSSG) to its reduced form (9) which serves as an dynamic indicator (10).

1.5 CELLULAR TARGETS OF ROS

In normal functioning of cells, ROS are formed as a product and its heightened levels leads to deleterious effects. The harmful effects of ROS may occur from significant alteration of intracellular targets such as amino acids oxidation in proteins, polyunsaturated fatty acids (PUFA) oxidation in lipids, DNA damage (7).

1.5.1 DNA damage: During oxidation of DNA, because of low oxidation potential Guanine is more susceptible and the most common are 2,6-diamino-4hydroxy-5-formamidopyridine and 8-oxo-7,8-dihydroguanine because of the addition of hydroxyl radical at C_8 position of Guanine. 8-oxo-7, 8-dihydroguanine serves as oxidative stress biomarker because of its abundance, stability and well studied. Similarly, for adenine also 4, 6-diamino-5formamidopyrimidine and 8-oxo-7, 8-dihydroadenine are generated (7, 11).The addition of hydroxyl radical to 5 or 6 position of pyrimidine ring leads to formation of thymine glycol, cytosine glycol, uracil glycol thereby causing premutagenic lesions. Some of the other premutagenic lesions are xanthine, hypoxanthine (7).

1.5.2 Lipid damage: In oxidative stress, the unsaturated lipids get to convert to polar lipid hydroperoxides thereby leads to the generation of epoxides, aldehydes which are highly reactive. The measurement of malondialdehyde is an indirect assessment of oxidative stress. There was an increase in the membrane fluidity because of the lipid peroxidation, leading to the efflux of cytosolic solutes (7).

1.5.3 Protein damage: The oxidation of proteins effect cell structure and its metabolic process as well as cell signaling due to oxidation sensitive proteins like phosphatases, kinases, etc., (12).

1.6 ROLE OF FREE RADICALS IN VARIOUS DISEASES

In the cellular process, the effect of ROS serves as a function of the strength and duration, in context to the exposure. As a result of prominent alteration of intracellular targets, ROS became familiar to be involved in a variety of disorders by pathogenesis there by flourishing diseases like diabetes mellitus, Parkinson's disease, cancer, cardiovascular diseases, cataracts, AIDS, atherosclerosis, chronic inflammation, hypertension (7). Oxidative DNA damage results in mutagenesis and carcinogenesis.

1.7 OXIDATIVE STRESS AND ORGAN TOXICITY

Oxidative Stress

Oxidative stress infers to the imbalance between the free radicals production and antioxidants present in the body resulting in cell damage. In 1936, the basic principle of stress and its responses were originated whereas the concept of oxidative stress was formulated in 1985 as a concept of redox biology (13).

Clinical and epidemiological studies show that the environmental factors like lifestyle (e.g., smoking, pesticides, metals, exposure to ionizing radiation, and a few evident pharmacological drugs etc.,) and diet lead to various diseases such as organ toxicity and cancer.

In humans, oxidative stress is predominantly responsible in some disease pathogenesis like cancer (2), diabetes (3), Alzheimer's disease (2), acute lung injury (13), cataracts (6), atherosclerosis (6), diabetes mellitus (6), AIDS (6), chronic inflammation (6), hypertension (6), Parkinson's disease (3).

The major mechanisms for oxidative stress induced organ toxicity include

a) Drug-induced organ toxicity
b) Chemical induced organ toxicity
c) Metal induced organ toxicity

1.7.1 Drug-induced organ toxicity: Oxidative stress induced by drugs is involved as a means of toxicity in various tissues, organ systems such as kidney, liver, eyes, nervous, cardiovascular systems. Very well-distinguished drugs are linked with oxidative stress due to their adverse events such as Doxorubicin (Cardiomyopathy, nephropathy), Azidothymidine (Skeletal myopathy, cardiac toxicity), Cisplatin (Nephrotoxicity, ototoxicity), Aminoglycoside (nephrotoxicity), Diclofenac (Nephrotoxicity, hepatotoxicity), and Paracetamol (Hepatotoxicity) (4).

Cisplatin is used for the treatment of lung, testicular, gastrointestinal, bladder cancers etc. Clinical use of cisplatin is plagued by ototoxicity, neurotoxicity (neuropathy) and nephrotoxicity (14-16). About one-third of cisplatin treated patients shown irreversible renal damage after taking prophylactic measures also.

Gentamicin is an antibiotic having aminoglycoside used for treating life threatening gram-negative infections. The exact mechanism by which it induces nephrotoxicity is because of the involvement of reactive oxygen species (17).

Cyclosporine A is used widely as a first line immunosuppressive drug in solid organ transplantation. Even though it has the success of clinical transplantation, backlogged by nephrotoxicity caused by the oxidative stress made it under weighs its therapeutic values (18).

Doxorubicin is a widely used anthracycline drug which leads to generation of free radicals thereby causing the oxidative stress by production of ROS and RNS during its intracellular metabolism and thereby producing superoxide anion correlating with the cellular injury (19). Autooxidation of Adrenalin, Epinephrine, xenobiotics etc are also responsible for generation of ROS (1).

1.7.2 Chemical induced organ toxicity: Chemical induced oxidative stress is well characterized for chemicals such as ethanol, carbon tetrachloride, tert-butyl hydroperoxide (Hepatotoxicity), potassium dichromate (nephrotoxicity). Some other metals like arsenic, lead, mercury etc., induce oxidative stress by the thiol binding ability thereby able to produce free radicals. Carbon tetrachloride is metabolized in the endoplasmic reticulum of liver by cytochrome P450 leading to the free radicals generation, which in turn attacks the lipids causing hepatotoxicity (20).

1.7.3 Metal induced organ toxicity

For the biological system, the metals like iron, copper are essential for health which may be toxic due to metal homeostasis (12). In environment, the toxic metals like lead, mercury, cadmium, arsenic are widely found and humans get exposure to these metals via numerous sources like contaminated soil, air, food and water. The transition metals act as catalysts in oxidation of biological macromolecules causing toxicity via oxidative tissue damage.

These metals are of two types based on their redox function namely redox-active metals like chromium, copper, iron which undergoes redox cycling and redox in-active metals like cadmium, lead, mercury etc., deplete the cells antioxidant enzymes as well as thiol- containing antioxidants resulting in raise in ROS production via oxidative stress, leading to tissue toxicity (12).

Cadmium is one of the heavy metal pollutants and toxic. It induces oxidative stress in cells there by responsible for the toxic effects such as cardiotoxicity, nephrotoxicity (21). On chronic environmental exposure, cadmium induces nephrotoxicity by thickening of glomerular basement membrane, tubule interstitial fibrosis and also by reducing the glomerular filtration rate. It is also responsible for causing genotoxicity (12).

Lead (Pb) and Mercury (Hg) are harmful heavy metals. For both humans and animals upon acute and chronic exposure to these metals leads to affect organs such as blood, brain, liver, bone, kidneys and reproductive system causing rigorous health damage, highly nephrotoxic. Iron, copper also cause oxidative stress, homeostasis, neurodegenerative diseases and also responsible for causing cancer (12).

1.8 NATURAL PRODUCTS AS ANTI-OXIDANTS

The molecules that are responsible for inhibiting the free radical reactions thereby resulting in inhibition of cellular damage are called antioxidants. In all the living organisms, there is an occurrence of antioxidant defence mechanism which varies from species to species (22). The consumption of natural antioxidants from plants, particularly polyphenols and flavonoids, inhibit the extent of free radical reactions, thereby protecting the human body from diseases, by attributing to potential health benefits (23). The use of herbal remedies was documented from China, India, Egypt, Greek, etc., since ancient days. As most of the developing countries are depending on the traditional medicine for their health care is for the sake of the occurrence of flavonoids, phenols, glycosides, tannins etc., which exhibit best sources of antioxidant, antimicrobial, anticancer activities towards the cure of many diseases. Synthetic antioxidants like gallic acid esters, butylated hydroxytoluene etc., are used against rancidity in food preservation (4).

Depending upon their solubility, the antioxidants are of lipid soluble like carotenoids, Vitamin E and water soluble like ascorbic acid etc. The herbal remedies are being preferred in recent ethnopharmacological surveys as modality to complementary and alternative medicine (24). The rationale use of herbal products has been increased tremendously to about 80% of the population over the past two decades for treatment of various health problems (25). The dietary supplements of anti-oxidants like Vitamin A, lutin, resveratol, vitamin E, ascorbic acid etc., are responsible for the reduction of oxidative stress above the normal which cannot be withdrawn by the endogenous defence mechanism (4).

Phytomedicine is a complex mixture of phytoconstituents originated from plant sources and used as a medicine or drug. The plant extract consists of phytoconstituents which are generally believed to work synergistically (26). Currently, most of the physicians use the pharmaceuticals having ancient history of usage as herbal remedies such as aspirin, opium, quinine, taxols (27). Several antioxidant polyherbal formulations are available in market to treat various disorders

and organ toxicity such as Arjunarishta for heart (28), Liv-52, Livolin, Hepatomed, Tefroli, Jigrine and Livergen for hepatoprotective activity (29), Cystone, Nephrol for nephrotoxicity (29), Tulasi, Curcumin for cancer (30, 31) and Karela, Fenugreek for diabetis (32, 33).

Most of the drug investigation is turned towards the phytomedicine which is in usage from several years back by various civilizations in the form of herbal formulations. The most important reason for seeking phytomedicine is the belief that it promotes healthier living.