1. Introduction:

1.1. *Free Radicals:*

1.1.1. *Definition and generation*:

Free radicals are molecules which possess an unpaired electron in their outermost orbits. A paired electron is energetically preferred in bonded systems; thus, most radicals are highly reactive and readily abstract an electron from another molecule to form the paired configuration and, therefore, most of them are also transient (Symons and Gutteridge 1998). In biological systems, the majority of free radicals are generated during oxygen (O_2) metabolism. O_2 is one of the most abundant substances (20.95%) in the atmosphere. Aerobic organisms have adapted to use oxygen as the electron recipient during the process of mitochondrial adenosine triphosphate (ATP) production. O_2 is necessary for most forms of life. Its reduction to water via the respiratory electron transport chain represents a highly efficient source of energy. However, O_2 reduction is a mixed blessing. Not only does it permit a considerably higher yield of bio-available energy, ATP, but its byproducts are also life-threatening. This is because incompletely reduced oxygen species may damage biological molecules (Halliwell 1992). In a properly working electron transport chain, one O_2 molecule is reduced to water with the stepwise acceptance of 4 electrons. Unfortunately, this is not always the case; a single electron transfer to O_2 forms the superoxide anion radical ($O_2^{\cdot -}$). $O_2^{\cdot -}$ receives another electron and forms peroxide, a process prominently associated with dismutation to hydrogen peroxide (H_2O_2) and O_2, in the presence of protons. Transfer of an additional

electron to H_2O_2 generates the hydroxyl radical ($^{\cdot}OH$). The sequential steps of single-electron donation to O_2 are described in a simplified scheme 1.

$$O_2 + e^- \rightarrow O_2^{\cdot -}$$

$$O_2^{\cdot -} + e^- \rightarrow H_2O_2$$

$$H_2O_2 + e^- \rightarrow {}^{\cdot}OH$$

$${}^{\cdot}OH + e^- \rightarrow H_2O \quad (\textit{Scheme 1)}.$$

The intermediate H_2O_2 is not a free radical since it lacks an unpaired electron. However, due to its high reactivity and as a precursor of $^{\cdot}OH$ it is referred to as a reactive oxygen species (ROS), a term also used to describe toxic O_2 derivatives.

In addition to ROS, nitrogen-containing free radicals and their radical-generating intermediates, referred to as reactive nitrogen species (RNS), represent another category of biologically highly important substances. Like the ROS, they are a source of damage to biomolecules (Jenner 1998). Nitric oxide ($NO^{\cdot}$) is the basic form of these reactive species. The principal pathway for $NO^{\cdot}$ generation is enzymatic (Perez-Severiano 1998). Nitric oxide synthase (NOS) isoforms (Marletta 1993), found in endothelial cells, neurons, leukocytes and various other cell types, are responsible for the synthesis of this gaseous metabolite, $NO^{\cdot}$. $NO^{\cdot}$ functions in intercellular communication and intracellular autostimulation processes. Its synthetic pathway is illustrated in Scheme 2.

$$\text{L-arginine} + O_2 \xrightarrow{\text{NOS}} \text{L-citrulline} + NO^{\cdot} + [H]^* \quad (\textit{Scheme 2})$$

(* transferred to redox-active coenzymes)

In addition to this major enzymatic pathway, $NO^{\cdot}$ can also be produced by redox-

sensitive release from multiple forms of storage such as S-, Fe- and N-nitrosocompounds.

$O_2^{\cdot -}$, $^{\cdot}OH$ and $NO^{\cdot}$ are the sources of other radicals. They can be generated enzymatically or non-enzymatically both *in vitro* and *in vivo* (Cohen 1994). $^{\cdot}OH$ can initiate radical reactions in lipid to form the alkyl radical ($L^{\cdot}$) as well as the peroxyl radical ($LOO^{\cdot}$). $O_2^{\cdot -}$ is either dismutated to H_2O_2 and O_2 or couples with $NO^{\cdot}$ to form the peroxynitrite anion ($ONOO^-$), which has much greater cytotoxicity than either of its precursors (Beckman 1994). These multiple intermediates play important roles in the physiology and pathology of organisms and are considered to be biologically significant.

1.1.2 *Functions and oxidative stress*:

Free radicals, especially the oxygen-based and nitrogen-based free radicals, exhibit dual roles; they can be either beneficial or harmful to the development and survival of organisms. Free radicals can serve as second messengers in signal transduction pathways regulating various cellular functions including gene expression, immune responsiveness, inflammatory reactions, vasomotor functions, cell proliferation, and apoptosis (Wolin and Mohazzab-H 1997). Moreover, $NO^{\cdot}$, at physiological levels, can protect against heart injury induced by ischemia/reperfusion (Bolli et al. 2001) and also serves as a non-synaptic neuromodulator implicated in the facilitation of learning and consolidation of memory in vertebrates (Qiang et al. 1997; Hawkins et al. 1998). However, excessive free radical production contributes to the etiology of many disorders. The damage caused by free radicals is referred to as the oxidative stress (Sies and Cadenas 1985). Up to 4% of the consumed oxygen is transformed to ROS

due to its partial reduction (Halliwell 1994). When ROS escape detoxification by the antioxidant defense system, they attack macromolecules including DNA, lipids, carbohydrates and proteins to induce oxidative tissue damage.

It was estimated that some 10,000 oxidative DNA lesions are produced per human genome per cell per day (Ames 1989). $^{\cdot}OH$, a principal player in oxidative DNA damage, can modify purine and pyrimidine bases and deoxyribose, and it can also cleave the phosphodiester DNA backbone. If these DNA lesions are not properly healed by enzymatic DNA repair mechanisms, a biological consequence can be malign transformation or cell death.

Proteins are also the targets of free radical attack. Free radicals modify both the structure and function of proteins. For example, free radicals can abstract the hydrogen from the cysteine sulfhydryl group (SH), thereby leading to the formation of a disulfide bond, a process associated with loss of function in some enzymes. While oxidation of proteins is less well characterized, however, several classes of damage have been documented, including oxidation of sulfhydryl groups mentioned above or reduction of disulfides, oxidative adduction of amino acid residues near to metal-binding sites via metal-catalyzed oxidation, reactions with aldehydes, protein-protein cross-linking, and peptide fragmentation (Starke-Reed and Oliver 1989; Stadtman and Oliver 1991). Of the various products formed during the oxidation of proteins, carbonyl derivatives of several amino acid residues have been identified (Stadtman and Oliver 1991). It has been shown that protein carbonyl products increase with free radical generation. Significantly, these increases correlate well with measured losses in the activities of the

oxidation-sensitive enzyme glucose-6-phosphate dehydrogenase (G6PD) (Sohal et al. 1995).

Lipid peroxidation is a radical chain reaction (Gutteridge and Halliwell 1990). A free radical, especially $^{\cdot}OH$, initiates this reaction by abstracting a hydrogen from the unsaturated lipid to form an alkyl radical; thereafter, the alkyl radical combines with O_2 to generate a lipid hydroperoxyl radical. The lipid hydroperoxyl radical has the ability to abstract a hydrogen atom from the double bond of a neighboring unsaturated lipid molecule to form an alkyl radical, thereby propagating the cycle. This self-propagating radical chain reaction of lipid peroxidation is illustrated in Scheme 3.

$$LH + R^{\cdot} \rightarrow RH + L^{\cdot}$$

$$L^{\cdot} + O_2 \rightarrow LOO^{\cdot}$$

$$LOO^{\cdot} + LH \rightarrow LOOH + L^{\cdot} \quad (\textit{Scheme 3})$$

Here LH represents a lipid; $R^{\cdot}$, radical; $L^{\cdot}$, alkyl radical; $LOO^{\cdot}$, lipid hydroperoxyl radical; LOOH, lipid peroxide, respectively.

Ultimately during lipid breakdown, intramolecular reactions and decomposition yield cyclic endoperoxides and aldehydes. A primary effect of lipid peroxidation is the decreasing membrane fluidity, which alters membrane properties and results in cellular damage.

Free radicals were first hypothesized to be involved in the biological processes such as aging by Harman (1956). This free radical theory of aging is based on the accumulated oxidative tissue damage throughout life. The accumulated oxidative injury in somatic cells is predicted to be the causative factor of aging. For the last decade, this theory has gained popularity. Many studies have also indicated that a variety of

diseases including Alzheimer's, Parkinson's disease, heart disease, diabetes and arthritis are at least partially related to oxidative stress (Lubec 1996).

1.1.3. *Antioxidative defense:*

To resist these notoriously cytotoxic free radicals and the associated oxidative damage to macromolecules, organisms have evolved an antioxidant defense system over the course of evolution. Cells are equipped with an impressive repertoire of endogenous enzymatic mechanisms to metabolize free radicals or repair the oxidative damage and also utilize many low molecular weight antioxidants (LMWAs) of either endogenous origin or derived from dietary intake of fruits and vegetables (Yu 1994).

a) *Antioxidative enzymes*: The most important antioxidant enzymes include superoxide dismutase (SOD) which accelerates the dismutation of $O_2^{\cdot -}$ to H_2O_2 and O_2, catalase and glutathione peroxidase (GPx) both of which decompose H_2O_2 to water, glutathione reductase (GRd) which recycles oxidized glutathione (GSSG) to its reduced form (GSH), and G6PD which catalyzes the formation of a most basic cellular reductant, β-nicotinamide adenine dinucleotide phosphate (reduced form) (NADPH). These enzymes cooperate in directly or indirectly metabolizing ROS. For example, H_2O_2 formed from $O_2^{\cdot -}$ by SOD is subsequently reduced to water by catalase or GPx. In the latter case, GSH, which serves as a hydrogen donor, is oxidized to its disulfide, GSSG. The recycling of GSSG back to GSH is the function of GRd. A co-factor in this reaction, i.e., NADPH, is supplied by the action of G6PD. The links among the antioxidant enzymes are essential and mutually supportive and the final objective is the

detoxification of free radicals. The antioxidant enzymes and the LMWA provide the first line of defense against oxidative insults.

b) *LMWAs*: LMWAs can be generated endogenously or obtained from the diet. These include hydrophilic antioxidants such as ascorbate, urate and glutathione, lipophilic antioxidants such as tocopherols (vitamin E), flavonoids, carotenoids and ubiquinol, and the iron chelator curcumin. Via electron donation, LMWAs directly quench and scavenge free radicals or chelate transition metals, thereby inhibiting their participation in prooxidative processes. The LMWAs are very important in protecting against oxidative stress since there are no enzymatic mechanisms to detoxify the most highly reactive radical species, $^{\cdot}OH$. The $^{\cdot}OH$ will damage any enzyme, which it encounters. Thus, direct free radical scavengers are the only means of neutralizing it.

c) *Repair mechanisms*: These include enzymes which can identify and repair oxidized molecules, such as glycosylase which specifically recognizes and excises oxidized bases from double-stranded DNA (Bohr and Anson 1995), phospholipase A_2 which cleaves lipid peroxides from phospholipids (Pacifici and Davies 1991), and proteases that act specifically on oxidized proteins (Rivett 1990). In addition to enzymes, some LMWAs such as β-nicotinamide adenine dinucleotide (reduced form) (NADH) and NADPH also participate the repair mechanisms. For example, NADH can donate an electron to the oxidized guanosine radical in DNA to regenerate the guanosine (Kirsch and Groot 2001).

In addition to the above-mentioned substances, a tryptophan derivative, melatonin, was found to be a potent free radical scavenger and a broad spectrum antioxidant roughly a decade ago (Hardeland et al. 1993; Tan et al. 1993a; Reiter 1998). Melatonin

has some unique antioxidant properties which distinguish it from those of the classic antioxidants.

1.2. *Melatonin*:

1.2.1. *Structure and properties*:

Melatonin {other chemical names: N-acetyl-5-methoxytryptamine, 3-(N-acetyl-2-aminoethyl)-5-methoxyindole} is a derivative of an essential amino acid, tryptophan. Melatonin was first isolated and identified by Lerner et al (1958). The chemical formula of melatonin is C(13)H(16)N(2)O(2) with a molecular mass of 232.28. The melting point of melatonin is 116-118 ^{o}C. Its structure is illustrated in Fig. 1.

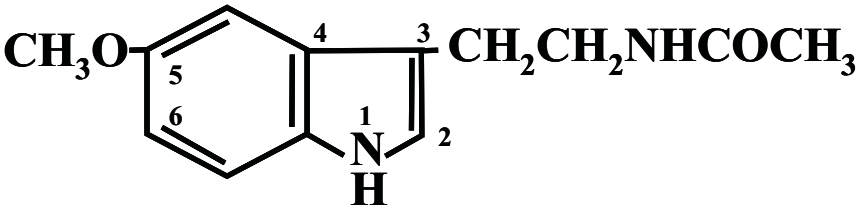

Fig. 1. Chemical structure of melatonin

The melatonin molecule consists of an indole moiety with two side chains, i.e., a methoxy and an N-acetyl-aminoethyl group. The methoxy group connects to carbon 5 and the aminoethyl side group chain connects with carbon 3 of the indole ring. The main C-C distance in the benzene ring is 1.402 Å, while those of C-C and C-N distances in the pyrrole ring are 1.405 Å and 1.394 Å, respectively. The indole portion of melatonin is planar, the average deviation of the atoms from the plane is 0.011Å and the maximum deviation is 0.022 Å for N^2. The acetyl group and the N^1 atom are almost strictly in a plane. This plane forms a dihedral angle of 12^{o} with that of the indole ring [this may be