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REVIEW ARTICLE

NEUROPROTECTIVE ROLE OF MELATONIN IN OXIDATIVE STRESS VULNERABLE BRAIN

Y. K. GUPTA*, MADHUR GUPTA** AND K. KOHLI**

**Neuropharmacology Laboratory,*
Department of Pharmacology,
All India Institute of Medical Sciences,
New Delhi – 110 029

and

***Department of Pharmacology,*
Lady Hardinge Medical College,
New Delhi – 110 001

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Abstract : The brain is deficient in oxidative defense mechanisms and hence is at greater risk of damage mediated by reactive oxygen species (ROS) resulting in molecular and cellular dysfunction. Emerging evidence suggesting the activation of glutamate gated cation channels, may be another source of oxidative stress, leading to neuronal degeneration. Oxidative stress has been implicated in the development of neurodegenerative diseases like Parkinsonism, Alzheimer's disease, Huntington's disease, amyotrophic lateral sclerosis, epileptic seizures, and stroke. Melatonin, the pineal hormone, acts as a direct free radical scavenger and indirect antioxidant. It is suggested that the increase in neurodegenerative diseases is attributable to a decrease in the levels of melatonin with age. Melatonin has been shown to either stimulate gene expression for the antioxidant enzymes (superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase) or to increase their activity. Additionally, it neutralizes hydoxyl radical, superoxide radical, peroxyl radical, peroxynitrite anion, singlet oxygen, hydrogen peroxide, nitric oxide, and hypochlorous acid. Unlike other antioxidants, melatonin can easily cross all morphophysiological barriers, e.g., the blood brain barrier, and enters cells and subcellular compartments. Though evidence are accumulating to suggest the potential of melatonin in neurodegenerative conditions, much information needs to be generated before the drug can find place in neurology clinics.

Key words : melatonin, epilepsy, neurodegenerative disorders, oxidative stress, antioxidants, free radicals, seizure, reactive oxygen species

*Corresponding Author : E-mail : ykg@hotmail.com, Fax : +91–11–26589803

The term 'oxidative stress' was first coined by Sies to account for the imbalance between oxygen species (ROS) and the antioxidant opposing forces (1). ROS may be oxygen centered radicals possessing unpaired electrons such as superoxide dismutase anion and hydroxyl radical, or covalent molecules such as hydrogen peroxide. The fact that oxygen is ubiquitous in aerobic organisms has led to the concept of the oxygen paradox; namely the fact that this life supporting molecule is also a precursor to the formation of harmful reactive oxygen species (ROS). ROS can damage virtually any biological molecule in its vicinity including DNA, essential proteins, and membrane lipids (2).

Why brain is more vulnerable to oxidative stress?

The central nervous system (CNS) is especially vulnerable to free radical damage because of brain's high oxygen consumption, its abundant lipid content, and the relative paucity of antioxidant enzymes as compared with other tissues (3). Moreover, brain has a high ratio of membrane surface area to cytoplasmic ratio, extended axonal morphology prone to injury, and neuronal cells are non-replicating. ROS can increase the permeability of the blood brain barrier, alter tubulin formation, and inhibit the mitochondrial respiration. If unchecked, it can lead to a geometrically progressing lipid peroxidation. Evidence also indicate that ROS may stimulate extracellular release of excitatory aminoacids (4). Glutamate is the major excitatory aminoacid in the brain. It acts through various types of ionotropic receptors, the most significant being, NMDA receptors. There seems to be a bi-directional relationship between the ROS production and release of excitatory aminoacids (5). Free radicals generated in the brain are also reported to influence gene expression, subsequently effecting apoptosis and neuronal death (6).

Neurological conditions and oxidative stress

Growing data from experimental models and human brain studies suggest that oxidative stress may play an important role in various neurological disorders, such as epilepsy, Alzheimer's disease, Parkinson's disease, stroke, cerebral ischaemia, multiple sclerosis, Huntington's chorea, tardive dyskinesia, and amyotrophic lateral sclerosis etc. (7). The data generated on the neurological conditions is largely based on the experimental models. It is, however, often not well established if an increase in free radicals initiates the disease process or is the sequelae of the pathophysiological changes. That the free radical status is intimately linked to the degenerative processes in most neurological diseases, and its attenuation, so as to retard the vicious cycle of cell degeneration is a matter of great interest to scientists. The different neurological disease processes in which ROS have been implicated have been dealt with below.

Endogenous antioxidant substances in brain

In brain, an array of cellular defense systems exists to counterbalance the ROS. These include enzymatic and nonenzymatic antioxidants that lower the concentration of free radical species and repair oxidative cellular damage. Glutathione functions as a

major antioxidant in tissue defense against free radicals in the brain. Brain is known to synthesize molecules like glutathione and NADPH. But, the concentration of glutathione is relatively in lesser quantities in the brain as compared to the rest organs of the body (3). The natural antioxidant system present in brain can be in form of enzymes like catalase, peroxidase, superoxide dismutase or low molecular weight antioxidants. Low molecular weight antioxidants can be ascorbic and lipoic acids, carotenoids or indirectly acting, like chelating agents (6). Free radical scavengers or antioxidants function as biological bodyguards for essential molecules by either neutralizing reactive species before they multilate a molecule or they repair damage that has been inflicted.

Why melatonin has high therapeutic potential as a neuroprotector?

Recently, a lot of endogenous substances with free radical scavenging properties have been explored for neuroprotection, of which melatonin and adenosine have gained attention. Both melatonin and adenosine are ubiquitously present endogenously in brain. Their concentrations have been found to be raised after seizures and altered in neurological conditions (8, 9). Both melatonin as well as adenosine have a wide safety margin. Both are known to cross the blood brain barrier. The experimental studies have shown the effectiveness of melatonin in Parkinsonism (10), epilepsy (11), stroke (12), Alzheimer's disease (13), movement disorders etc. Similarly, adenosine has been shown to exhibit antiepileptic effect on experimental seizures (14), Parkinson's disease etc. The ultrashort half life is the major limitation of adenosine for using it for chronic conditions. The attempts to develop adenosine analogs with long half life are in experimental stages. In experiments done in our laboratory, an anticonvulsant action of adenosine against PTZ induced seizures in rats has been demonstrated while in chemically kindled seizures adenosinergic agents could offer only incomplete protection. Further, adenosine administration caused significant hypotension and hypothermia. Though, neither hypotension nor hypothermia have been attributed to the antiepileptic effect of adenosine in PTZ induced seizures in rats, these effects can be a serious limitation in their clinical usefulness (15). On the other hand, melatonin's half life of nearly 30–53 minutes (16), no significant side effect except sedation in high doses in experimental studies, make it the most potential endogenous substance to be therapeutically exploited in chronic conditions. The function of melatonin as antioxidant and free radical scavenger is facilitated by the ease with which it crosses morphophysiological barriers like blood brain barrier, intracellular and subcellular barriers (17). Moreover, melatonin even in very high doses has been shown to be extremely safe in humans (18).

How melatonin acts as an antioxidant in brain?

Melatonin, the pineal hormone, was a regular of biological rhythms controlling the phase and amplitude of circadian rhythm by acting both on suprachiasmatic nucleus (SCN), the biological clock that resides in

the hypothalamus as well as on various other cells and tissues of the body (19). Because of this action the hormone has been named as 'chronobiotic' by Armstrong. It was Ianas, who first suggested that melatonin may have a role in scavenging free radicals (20). Melatonin likely works via electron donation to directly detoxify free radicals. In in-vitro and in-vivo experiments, melatonin has been found to protect cells, tissues and organs against oxidative damage induced by a variety of free radical generating agents and processes, including cyanide poisoning, glutathione depletion, ischemia reperfusion, kainic acid induced excitotoxicity, and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (21). Melatonin as an antioxidant is not only effective in protecting nuclear DNA, membrane lipids and possibly cytosolic proteins from oxidative damage but is also reported to alter the activities of enzymes that improve the total antioxidative defense capacity of the organism (22).

Direct scavenging of the reactive oxygen species by melatonin

Definitive evidence that melatonin functioned as a direct scavenger of hydroxyl radicals (˙OH) was provided by Tan et al in 1993 (23). This is widely accepted as the most damaging molecule endogenously produced in aerobic organisms. The ˙OH mutilates any molecule in the vicinity of where it is produced. Melatonin scavenges the ˙OH resulting in the formation of cyclic 3 hydroxymelatonin, a harmless product that is excreted in the urine, which also acts as a free radical scavenger (24). Each molecule of melatonin scavenges two ˙OH, unlike other antioxidants, which lack the ability to quench the hydroxyl radicals. Many other studies have confirmed melatonin's ability to detoxify ˙OH (25, 26). Unlike other well known antioxidants that are exclusively lipid (e.g. vitamin E) or water (e.g. vitamin C) soluble and therefore, exhibit a limited intracellular distribution, melatonin is amphiphilic allowing it to reduce ˙OH mediated damage in both the lipid and aqueous subcellular compartments. Of recent interest has been the discovery that melatonin also directly neutralizes the precussor of ˙OH, namely hydrogen peroxide (H_2O_2) (27). When melatonin scavenges H_2O_2 the product has been identified as N_1 acetyl N_2 formyl 5 methoxykynuramine (AFMK), which in addition is shown to be capable of donating two electrons and, therefore being a direct free radical scavenger in its own right. This has been referred to as the antioxidant cascade where melatonin as well as at least one resulting metabolite are both highly effective scavengers. Indirect evidence suggesting that melatonin neutralized $^1O_2$ was first provided by Cagnoli et al (28).

Lipid peroxidation is a much-studied process in free radical biology where in the peroxyl radical (LOO˙) is generated; oxidizing another adjacent lipid molecule to maintain the chain reaction of lipid peroxidation. Pieri et al claimed that melatonin was a more efficient LOO˙ scavenger as compared to vitamin E (29).

Hypochlorous acid (HOCl), an oxygen or chloride based reactant, is a powerful oxidizing agent that has the capability of damaging a variety of molecules. Melatonin is reported to inactive HOCl leading to the formation of 2-hydroxymelatonin (30).

The nitrogen based peroxynitrite anion ($ONOO^-$) or its metabolites [e.g. peroxynitrous acid (ONOOH)] are almost equivalent to $^{\cdot}OH$ in terms of destructive capacity. Exogenous melatonin administration neutralized this reactant (or its metabolites) reducing thereby, the molecular and physiological damage (31). Furthermore, the steady state levels of $ONOO^-$ are reduced when melatonin scavenges nitric oxide (NO^*) (32). NO normally couples with $O_2^{-\cdot}$ to form $ONOO^-$. Melatonin also reduces the generation of NO^- by inhibiting the activity of its rate limiting enzyme, nitric oxide synthase (NOS). In in-vivo studies where melatonin's efficacy was compared with classical antioxidants in terms of pharmacologically protecting against free radical damage, melatonin was found to be effective at a lower dose than other antioxidants (33).

Action on antioxidant enzymes and gene expression

Melatonin stimulates several important antioxidative enzymes (activity or gene expression) including SOD (34), glutathione peroxidase (35) and glutathione reductase (36). SOD functions as an antioxidant by rapidly removing $O_2^{-\cdot}$ from cells thereby lowering the formation of the highly reactive and damaging $ONOO^-$ (37). Experimental evidence has shown that melatonin also promotes the activity of GRx, thereby helping to maintain high levels of reduced glutathione.

Although membrane receptors for melatonin have been identified in many cells, nuclear binding sites for melatonin have also been documented. Either, or both, of these may be involved in the mechanisms by which melatonin promotes the activity of SOD, GPx and GRx (38). Melatonin maintains the optimal fluidity of cellular membranes. It indirectly reduces membrane rigidity by positioning itself within cellular membranes to restrict damage to PUFA by toxic reactants, which is particularly important for the brain (39). At a molecular level, melatonin has been reported to increase tissue levels of mRNA for both manganese SOD as well as copper SOD. It was also documented in the same study that mRNA levels for GSH-Px were also augmented after melatonin administration (40). Another significant observation is that of Pierrefiche and Laborit, who demonstrated that melatonin stimulates the activity of glucose-6-phosphate dehydrogenase in both the liver and the brain (41).

The amphiphilic property of melatonin: advantage for the brain

The importance of melatonin as an antioxidant depends on several characteristics: its lipophilic and hydrophilic nature, its ability to cross all barriers with ease, and its availability to all tissues and cells. It distributes in all cellular compartments, being especially high in the nucleus and mitochondria. Tissues except pineal gland producing melatonin for local use include the retina, cells of the immune system, bone marrow, human ovary, lens and testes (42). Levels of melatonin are two to three orders of magnitude higher than maximal blood melatonin concentrations in cerebrospinal fluid (CSF) (43).

Melatonin in epilepsy

Classically, research into pathophysiology of epileptic seizures has primarily been focused on factors responsible for seizure initiation, but seizures arrest spontaneously and abruptly, and brain remains seizure free for some time thereafter. Thus, the possibility that some endogenous anticonvulsant substances is/are involved was suggested. Numerous animal studies have suggested an anticonvulsant role for the pineal hormone melatonin against various convulsive stimuli (44, 45, 46, 47). In gerbils, pinealectomy causes seizures, an effect which is reversed with exogenous melatonin (48). In rats, melatonin has been reported to inhibit amygala kindled seizures (49) and antibodies to melatonin induced seizures (50). Pinealectomy, which results in absence of melatonin secretion, induces seizures in certain animals within a few hours (51). Melatonin also exerted an anticonvulsant activity against seizures induced by several chemoconvulsants e.g.

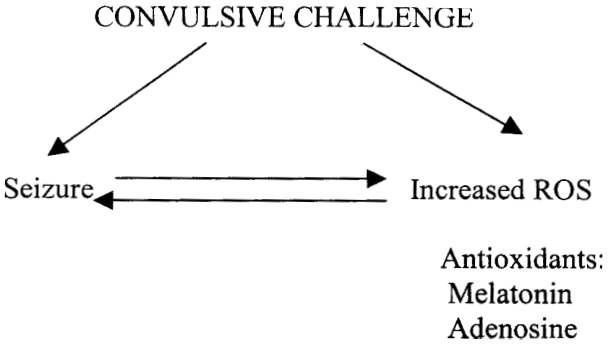

Fig. 1: Involvement of ROS in epilepsy.

quinolinate, kainate, glutamate, NMDA and pentylenetetrazol in rodents (52). Melatonin afforded protection against L-cysteine induced seizures and lipid peroxidation in mice brain (53) (Fig. 1).

In experiments done in our laboratory, a superior protective effect of melatonin over phenytoin in an intracortical $FeCl_3$ model of posttraumatic epilepsy was reported (54). It was also observed that on combining melatonin, 100% protection was achieved with phenytoin and carbamazepine in doses, as low as, ED50 and ED33 respectively. There was no pharmacokinetic interaction, when melatonin was used in combination with either phenytoin/carbamazepine (communicated). In clinical situation, in a study by Molina-Carballo et al in 1997, it was suggested that melatonin may be a useful addition to other anti-epileptic therapy. Add-on melatonin proved to be useful in a female child of myoclonic epilepsy leading to clinical control of severe infantile myoclonic epilepsy in large doses (100 mg daily) for two years. The authors suggested a neuroprotective role of melatonin more likely due to its inhibitory action on glutamate receptors and its potentiation of GABA-benzodiazepine receptors (55). Similar neuroprotection and anticonvulsant action of melatonin was also reported by Sanchez-Forte et al (56). It has been proposed that CSF melatonin is a natural anticonvulsant (57). Melatonin is known to depress brain excitability regulating Na+ K+ ATPase (58) and GABA benzodiazepine receptor complex activities (59). Melatonin also potentiates the capacity of corticotrophic and opiodergic peptides to increase brain benzodiazepine receptors (60). Besides potentiating brain inhibitory neurotransmission melatonin blocks

glutamatergic dependent brain excitability thus acting as an antiexcitotoxic compound (61). Several authors have proposed that excitatory aminoacids are involved in the triggering and maintenance of seizures in human convulsive disorders (62). The fact that some metabolites of the kyneurinine pathology are ligands for excitatory aminoacid receptors raises the possibility that tryptophan metabolites may be involved in the pathogenesis of seizure disorders (63).

We have recently conducted a double blind randomized placebo controlled trial on epileptic children, and studied the effects of add-on melatonin therapy. It was found that melatonin led to an induction of the antioxidant enzymes in the melatonin combination groups. Add-on melatonin administration also improved the sleep behaviour and quality of life of patients markedly. Further it was observed that there was no pharmacokinetic interaction between melatonin and valproate/ carbamazepine in the treatment groups (unpublished work).

Melatonin in Alzheimer's disease

In recent years considerable data has been generated indicating that the brain in AD is under oxidative stress, and this may have a role in the pathogenesis of neuron degeneration and cell death in this disorder. Direct evidence supporting increased oxidative stress in AD are increased brain iron, aluminium, and magnesium. In brain, these are capable of stimulating free radical generation, lipid peroxidation and PUFA, protein and DNA oxidation, diminishing energy metabolism, advanced glycation end products (AGE), MDA, SOD-1 in senile plaques. Studies have shown that amyloid beta peptide is capable of generating free radicals (Fig. 2) The neuroprotective role of melatonin in the presence of beta amyloid protein has been reported (64). Melatonin has also been reported to inhibit the formation of β amyloid protein from its precursor and reduce aluminium ion induced peroxidation (65). Recently, some authors reported that melatonin and pinoline reduced, in a concentration-dependent manner, lipid peroxidation due to aluminum, $FeCl_3$ and ascorbic acid in the synaptosomal membranes (66). In experiments done in our laboratory, the effectiveness of melatonin in preventing the cognitive deficits as well as the oxidative stress caused by intracerebroventricular streptozotocin in rats was demonstrated and its potential in age-related neurodegenerative disorders where oxidative stress and cognitive impairment are involved was suggested (67).

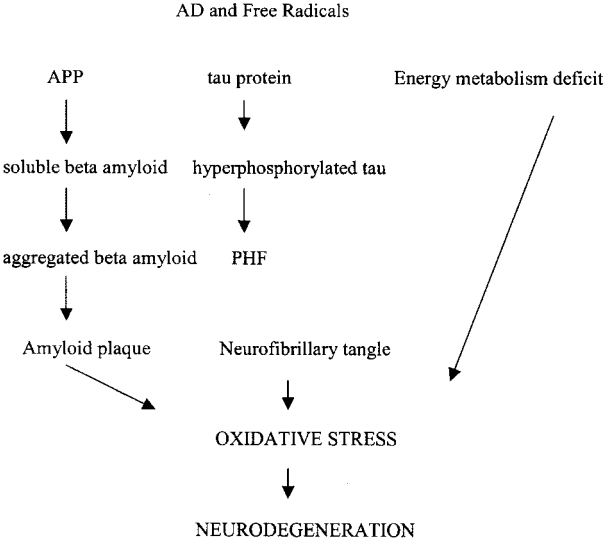

Fig. 2: Involvement of ROS in Alzeimer's disease.

Melatonin in movement disorders

Parkinson's disease is characterized by the loss of dopaminergic neurons in the subtantia nigra pars compacta. These neurons are particularly vulnerable to oxidative stress because of the ROS, which are normally generated in the dopamine metabolism. Iron could increase oxidative stress by promoting the formation of O_2 from H_2O_2 via the Fenton reaction. Reductions in glutathione levels have also been reported. Evidence is now accumulating to suggest that NO generation could contribute to neuronal cell death in Parkinson's disease. Melatonin is known to detoxity NO as well NOS, therefore can be of potential use in Parkinson's disease as a free radical scavenger. Recently, synergistic effects of melatonin and deprenyl against MPTP-induced mitochondrial damage and DA depletion was reported (68).

Another group of workers used MPTP to induce oxidative changes in the brain of mice, and reported a complete reversal in the lipid peroxidation products by the use of melatonin (69). In our laboratory, the ferric chloride induced model of experimental Parkinson's disease has been standardized. The role of adenosine and its analogues has been studied and a negative interaction between dopaminergic and adenosinergic system in striatum has been substantiated (70). Recently, a case report described development of Parkinsonism caused by lipopolysaccharide contamination of the brain due to an open wound, wherein, the inflammation of the CNS was observed in Positron Emission Tomography, which was reversed by melatonin administration. This is the first case report describing toxic parkinsonism in consequence of lipopolysaccharides in the CNS and an effective treatment with melatonin, aminoacid DL phenylalanine, and DHEA (71). Tardive dyskinesia has been and continues to be a significant problem associated with long-term antipsychotic use (72). Evidence has been accumulating reporting increased oxidative damage from antipsychotic medications. Based on this, the use of antioxidants, such as melatonin, may be considered as a preventive measure for tardive dyskinesia. Melatonin recently has been investigated in experimental models of tardive dyskinesia. Vacuous chewing movements (VCM's) in rats are widely accepted as an animal model of tardive dyskinesia. Rats chronically treated with haloperidol developed vacuous chewing movements, which were dose dependently reversed by melatonin (73). The study suggested a potential role of melatonin for the prevention and treatment of neuroleptic induced orofacial dyskinesia.

Melatonin in cerebral ischemia

Acute ischemic stroke is the third largest cause of mortality and is the single largest cause of adult disability. The present therapeutic approaches in stroke are primarily vascular (reperfusion) or neuronal (neuroprotection). The perplexing problem with the reperfusion is the massive generation of free radicals, which starts the cascade of events leading to neuronal death. Realizing this, the role of antioxidants in stroke is being widely researched. Free radical generation during cerebral ischemia may underlie delayed neuronal death. It has been proposed that during ischemia, ROS and excitatory amino acids may cooperate in neuronal damage (Fig. 3). Excitatory events may stimulate ROS, but there is also

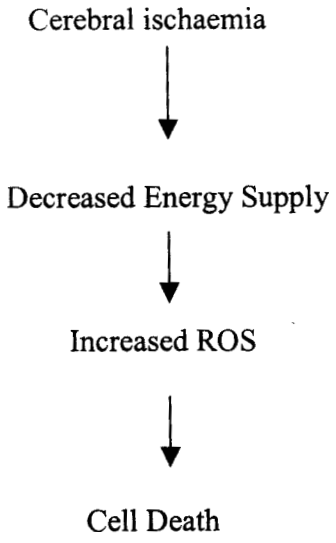

Fig. 3 : Relationship between cerebral ischemia and cell death.

evidence that ROS can lead to excitatory aminoacids and thus a bi-directional relationship is an attractive hypothesis (6). Transient ischaemia elevates cerebral levels of both excitatory aminoacids and rates of hydroxyl radical formation. Melatonin treatment has been shown to be highly effective in different *in vivo* and *in vitro* models of excitotoxicity or ischemia/ reperfusion in multiple animal species. Recently, in a study, melatonin at 5 mg/kg given as a single injection or multiple injections protected against focal cerebral ischemia when commenced within 2 hours of onset (74). In our laboratory, middle cerebral artery occlusion model of acute cerebral ischemia has been standardized. Recently, enhanced protection with the use of a combination of melatonin plus meloxicam in the middle cerebral artery occlusion model of acute ischemic stroke in rats has been shown (75). Another study from our laboratory had exhibited neuroprotective effect of melatonin on ischemia reperfusion injury induced by middle cerebral artery occlusion in rats (76).

Melatonin in Huntington's chorea

Quinolinic acid, an active metabolite of the tryptophan-kynurenine pathway has been implicated in the pathophysiology of neurodegenerable disorders. As the neuronal damage by quinolinic acid is very similar to that observed in Huntington's chorea, this model is useful for studying the neuropathology of Huntington's chorea. It has been reported that melatonin administration before and after quinolinic acid administration reduced the lipid peroxidation induced by quinolinic acid (77). As there is a paucity of studies using melatonin in Huntington's chorea, its potential in the pharmacotherapy of this neurodegenerative disease is to be established.

Melatonin in amyotrophic lateral sclerosis

There is an overload of reactive oxygen species and peroxynitrite, a derivative of nitric oxide, in sporadic amyotrophic lateral sclerosis. Jacob et al have recently reported a neuroprotective role of melatonin in ALS patients, who tolerated high doses of melatonin (78). The role of melatonin in this disease needs to be explored further conducting well-designed clinical trials.

Melatonin : The dilemma

Since melatonin is endogenously produced, the organisms have evolved mechanisms to remove excessive amounts

from the body. Virtually all exogenously administered antioxidants have a dose at which they become toxic. Melatonin, even when given in massive amounts (300 mg daily) for prolonged periods (up to 5 years) to humans has not produced untoward side effects. Furthermore, most antioxidants also exhibit devastating pro-oxidant actions under some conditions (18). Melatonin is obviously a phylogenetically old compound that has survived evolution without any chemical structural modification whatsoever. But, the dose and the duration for which it can be used chronically is yet not conclusive. It has been used in doses of 3 mg to 300 mg in clinical trials. The bulk of the studies that have tested the antioxidant capacity of melatonin have used pharmacological doses. This does not mean, however, that melatonin is not relevant as an antioxidant at physiological concentrations. Quite the contrary, a number of studies have shown that surgical removal of the pineal gland leads to exaggerated free radical damage. For example, when compared to intact rats, pinealectomized animals exhibited much greater free radical based neural damage induced by ischemia-reperfusion (79). Furthermore in rats as well as in humans, blood levels of melatonin positively correlate with the ability of this fluid to detoxity free radicals (80). There is a need for controlled clinical trials using melatonin as an add-on therapy in patients of different neurodegenerative disorders. Moreover, there may be different dose requirements for different neurodegenerative conditions, and needs to be established. It has been reported that melatonin exerts its neuroprotective action in various neurodegenerative disorders through its antioxidant and free radical scavenging property. However, it remains to be ascertained whether MT_1 or MT_2 melatonin receptors play any role in neuroprotection, and that the neuroprotection is attributable to the free radical scavenging property of melatonin, and not through its receptors needs to be studied. The role of melatonin in Parkinsonism is complex. On one hand, melatonin may exacerbate symptoms because of its putative interference with dopamine release, and on the other hand, protect against neurodegeneration by virtue of its antioxidant properties and its effect on mitochondrial activity (81). This interaction of melatonin needs to be explored in further studies.

The pineal gland, once considered a part of the body without any significant function gradually gained importance when melatonin was found to be the factor regulating sleep behavior. Now the antioxidant property has made it an important molecule in research, understanding the etiopathology and pharmacotherapy of neurodegenerative disorders.

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