CHAPTER ONE

LITERATURE REVIEW

1.1 AN INTRODUCTION TO NEUROSCIENCE

The transformations that have brought modern human kind to its current dominant intellectual status on earth follow a pattern governed by certain fundamental rules of nature that thread their way through the fabric that constitutes the history of brain and behaviour. Neuroscience is a vast area of biological sciences, and consists of several approaches to the study of the brain. The advent of neuroscience has been a great source of excitement in attempts to understand the biological basis of brain function and abnormal behaviour.

Over the last decade, there has been an unexplained increase in the number of neurodegenerative diseases, especially Alzheimer's dementia, Parkinson's disease, and amyotrophic lateral sclerosis that appears to go beyond the natural aging of the population. Recently, scientists have suspected a complex relationship between environmental toxins and the chronic death of neurons. Humans are likely to be exposed to a complex mixture of chemical agents in their residential and occupational environments. There is increasing concern that chronic low-level exposure of the developing and aging nervous systems to such environmental neurotoxicants such as rotenone may adversely influence the onset and severity of certain neurological disorders. Thus, there exists a dire need to combat the various neurological disorders and as a result the field of neuroprotection, has become a rapidly advancing area of neuroscience. Though substantial therapeutic interventions are in progress, brain research is nonetheless only at its beginning.

1.2 THE BRAIN

The human brain is a fascinating and enigmatic machine. It is a highly sophisticated and complex organ responsible for mood, behaviour, memory, learning and thought processes to name just a few. The basic structural units that comprise the brain are cells called neurons and glia. For the

efficient functioning of the brain, all of the one hundred billion cells in this complex organ must effectively communicate with each other and failure to do so may cause or contribute to brain dysfunction.

The brain and nervous system are especially vulnerable to free-radical damage for a number of reasons. First, it has one of the highest concentrations of polyunsaturated fat contents of any tissue in the body. Polyunsaturated fats are especially prone to lipid peroxidation. Second, the brain has very high oxygen content. It consumes 20% of the blood's oxygen, even though it makes up only 2–4% of the body weight (Hoyt *et al*, 1997). Third, the nervous system, especially the brain and spinal cord, undergoes a progressive accumulation of tissue iron levels with ageing and the brain is poor in endogenous antioxidants too (Dawson and Dawson, 1996).

1.3 MECHANISMS OF NEURODEGENERATION

Neuronal cells of the CNS, with few exceptions, cannot divide or regenerate, and as such pathological processes causing neuronal loss generally have irreversible consequences (Rang *et al*., 1995). Brain degenerative diseases are normally characterised by late onset, gradual progression, and finally death, but the aetiology and mechanisms are still unknown. However, oxidative stress and excitotoxicity are thought to play an important role in eliciting various neurological disorders and of the ageing process.

1.3.1 FREE RADICALS AND OXIDATIVE STRESS

Most atoms and molecules remain reasonably stable when placed in contact with living cells. However, free radicals are group of particles that are considered to be less benign. Free radicals are unstable, highly reactive molecules characterised by the presence of unpaired electrons in their outermost shells (Schipper, 1998). Although there are a variety of free radicals produced by molecules, those that are produced from molecular oxygen have received considerable interest.

Molecular oxygen is required by living organisms and biological systems to survive and is depended upon heavily. Any free radical involving oxygen can be referred to as reactive oxygen species (ROS), which includes the superoxide anion radical (O_2^-), the hydroxyl radical ($OH^{\bullet}$), hydrogen peroxide (H_2O_2), nitric oxide (NO) and peroxynitrite ($ONOO^-$). The production of oxygen free radicals is a natural consequence of aerobic metabolism, with these molecules being constantly generated in the body by normal metabolic processes (Ames *et al*., 1993). They are "the price we pay for breathing, the inescapable by-products of living in a world full of oxygen," to quote New York Times science writer Natalie Anger.

1.3.1.1 Superoxide Radical

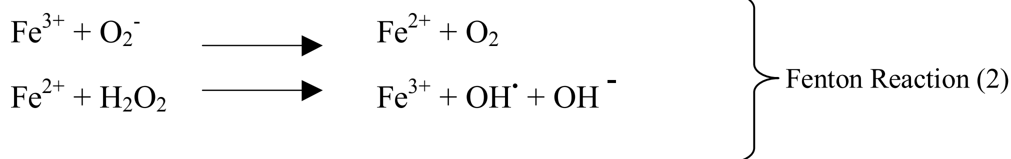
Superoxide (O_2^-) is generated by multiple enzymatic and non-enzymatic pathways and is often at the start of the oxidative stress cascade. A major source is via the cellular electron transport chains, such as those of mitochondria, chloroplasts and the endoplasmic reticulum (Halliwell & Gutteridge, 1990) where some electrons passing through the chain “leak” directly from the intermediate electron carriers onto O_2. Since oxygen accepts one electron at a time, O_2^- is formed (Halliwell, 1992).

Superoxide anions are generated enzymatically by a number of oxidases, such as xanthine oxidase and the oxidase that is found in the plasmalemma of phagocytic cells (Reiter et al., 1995). Activated phagocytic cells (such as monocytes, neutrophils, eosinophils and macrophages including microglia) also produce superoxide, which plays an important part in the mechanism by which bacteria are engulfed and destroyed (Colton and Gilbert, 1987). Thus excessive activation of phagocytic cells (as in chronic inflammation) can lead to free radical damage.

The toxicity of superoxide is seen in its ability to inhibit certain enzymes and thereby attenuate vital metabolic pathways, as well as in its effects on other major classes of biological molecules (McCord, 2000). *E. coli* deficient in superoxide dismutase (SOD) activity show increased rates of mutagenesis (Touati and Farr, 1990), illustrating the role of the radical in DNA damage.

1.3.1.2 Hydroxyl radical

The $OH^{\bullet}$ radical is probably the most reactive of the ROS species (Poeggler *et al*., 1993; Dawson and Dawson, 1996) as it will react with almost all molecules in living cells (Fridovich, 1996). Hydroxyl radicals are short-lived and can be formed from O_2^- and H_2O_2 through the Haber-Weiss reaction or through the interaction of metals such as iron or copper and H_2O_2, through the Fenton reaction (Halliwell & Gutteridge, 1985) as shown in the equations below.



The hydroxyl radical has been implicated in damage to proteins, carbohydrates, DNA, and lipids (Reiter *et al*., 1995; Dawson& Dawson, 1996; Volterra *et al*., 1994). Action on DNA results in strand breakage and chemical alterations of the deoxyribose and of the purine and pyrimidine bases. A main physiological target of free radicals is the polyunsaturated fatty acids of cell membranes and the resultant degradation causes alterations in membrane structure and function (Viani *et al*., 1991).

1.3.1.3 Nitric Oxide

Nitric oxide (NO) is a free radical released by several cell types, especially vascular endothelial cells and phagocytes (Moncada *et al*., 1991). NO is formed by nitric oxide synthase (NOS). The process involves the conversion of L-arginine to L-citrulline (Knowles & Moncada, 1994). Neurons produce NO mainly by a calcium-dependent activation of neuronal NOS (nNOS) (Stewart and Heales, 2003). NO has been suggested to be involved in both the normal

functioning of excitatory amino acids such as glutamate and in the damaging effects produced by their generation in excess (Dawson *et al*., 1991; Forstermann *et al*., 1991).

1.3.1.4 Peroxynitrite

The interaction of NO with superoxide radical leads to formation of peroxynitrite ($ONOO^-$), a reaction that occurs at a threefold faster rate than that of the dismutation of superoxide by SOD (Beckman *et al*., 1993).

$O_2^- + NO^\cdot \longrightarrow ONOO^- + H^+$ (3)

Therefore the formation of peroxynitrite depends on the concentration of superoxide and NO in the cell. At physiological pH, peroxynitrite may be able to diffuse over several cell diameters to produce cell damage by oxidising lipids, proteins and DNA (Beal, 1997).

Figure 1.1 depicts the generation of oxygen free radicals which is an important contributing factor in several chronic human diseases, including atherosclerosis and related vascular diseases, mutagenesis and cancer, neurodegeneration, immunologic disorders, and even the ageing process.

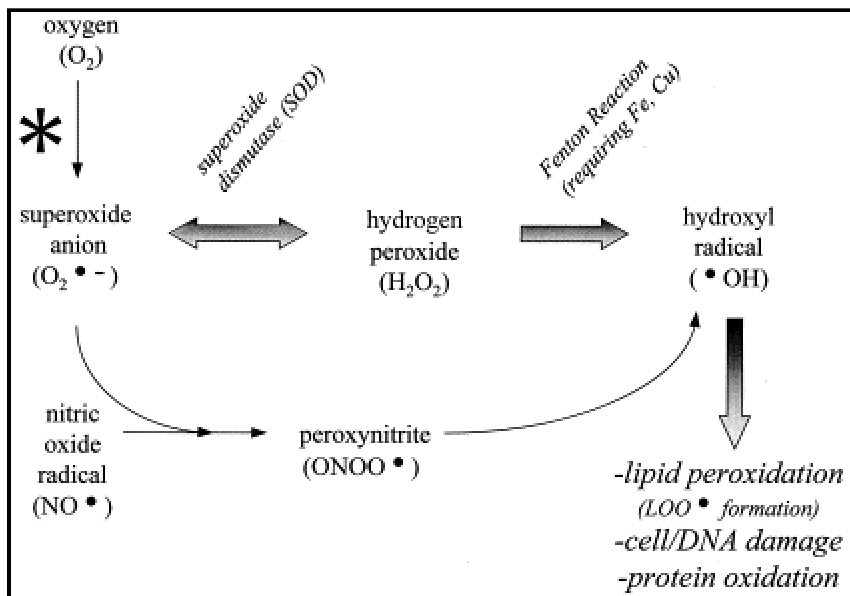


FIG 1.1 *Generation of oxygen free radicals (Beyer, 1998)*

1.3.2 LIPID PEROXIDATION

There are major biochemical changes in the brain affecting the neuronal membrane, which is the "site of action" for many essential functions of the brain. Lipid peroxidation is one of the major outcomes of free radical-mediated injury that directly damages biological membranes and generates a number of secondary products that possess neurotoxic activity (Halliwell and Chirico, 1993). Lipid peroxidation has been defined as the oxidative deterioration of polyunsaturated lipids, i.e. those lipids containing more than two carbon-carbon double covalent bonds (Halliwell, 1992). Of all the major classes of biomolecules, membrane lipids are probably the most susceptible to oxidative attack because of the high levels of polyunsaturated fatty acids (PUFA) present in these macromolecules.

Several experimental evidences indicate that extensive lipid peroxidation results in loss of membrane integrity, impairment of the function of membrane-transport proteins and ion channels, disruption of cellular ion homeostasis and eventual rupture leading to release of cell and organelle contents such as lysosomal hydrolytic enzymes (Fong *et al.*, 1973; Mattson, 1998).

1.3.2.1 STEPS IN LIPID PEROXIDATION

Lipid peroxidation is initiated by the attack of any species that has sufficient reactivity to abstract a hydrogen atom from a methylene group ($-CH_2-$).
Table 1.1. illustrates the sequence of events which characterise the lipid peroxidation process. During the initiation step (reaction 1) PUFA are attacked by ROS ($In^{\cdot}$), resulting in the removal of a hydrogen atom from the PUFA (RH) and the formation of a lipid-derived radical ($R^{\cdot}$). The propagation step (reaction 2) normally begins with the rapid addition of molecular oxygen to $R^{\cdot}$, to form the lipid peroxyl radical ($ROO^{\cdot}$). This radical can attack other lipids, to generate a further lipid radical and a lipid hydroperoxide (ROOH). A self-perpetuating autocatalytic reaction then follows with reactions 2 and 3 undergoing a number of cycles (Krinsky, 1992). The final step is a termination step, which results when two $ROO^{\cdot}$ radicals react together to form a non-radical product (Burton and Ingold, 1989). The removal of ROS by various antioxidant systems is therefore essential to limit lipid peroxidation in cells.

Table 1.1. Chain sequence for free radical auto-oxidation.

Initiation	$In^{\cdot} + RH \rightarrow InH^{\cdot} + R^{\cdot}$	Reaction 1
Propagation Reactions	$R^{\cdot} + O_2 \rightarrow ROO^{\cdot}$	Reaction 2
	$ROO^{\cdot} + RH \rightarrow R^{\cdot} + ROOH$	Reaction 3
Termination	$2\ ROO^{\cdot} \rightarrow$ Non-radical products	Reaction 4

1.3.2.2 THE ROLE OF IRON IN LIPID PEROXIDATION

Transition metal ions are remarkably good promoters of free radical reactions. Iron is the most abundant transition metal in the brain and is an important component of molecules that undergo redox reactions in cells. However, this property makes iron potentially toxic, since redox reactions may generate reactive oxygen species (Liochev and Fridovich, 1994). As mentioned earlier in section 1.3.1.2, iron participates in the Fenton reaction, in which the very reactive hydroxyl radical is generated.

Brain tissue has the ability to readily accumulate iron (Dawson & Dawson, 1996) and this observation is important as iron ions can contribute to lipid peroxidation in two ways. Firstly, they catalyze formation of free radical species and secondly, decompose hydroperoxides to peroxyl and alkoxyl radicals (Halliwell & Gutteridge, 1990), which are able to abstract hydrogen and lead to further peroxidation. Abnormally high levels of iron as well as oxidative stress have been demonstrated in a number of neurodegenerative disorders including Alzheimer's disease and those characterised by nigral degeneration such as Parkinson's disease, multiple system atrophy, and progressive supranuclear palsy (Campbell, 2001).

1.3.3 THE ROLE OF MITOCHONDRIA IN OXIDATIVE STRESS AND AGEING

Mitochondria have been described as "the powerhouses of the cell" because they link the energy-releasing activities of electron transport and proton pumping with the energy conserving process of oxidative phosphorylation. Mitochondria are also a major source of oxidants and a target for their damaging effects, and, therefore, mitochondrial oxidative stress appears to be a cause, rather than a consequence of cell aging.

Oxidative damage due to the endogenous production of reactive oxygen species by mitochondria is a common and ubiquitous form of oxidative stress in most mammalian cells. During aerobic metabolism, ROS such as superoxide radical ($O_2^{\cdot -}$) and hydrogen peroxide (H_2O_2) are produced by continuous transfer of electrons from the respiratory chain of mitochondria to molecular oxygen. Two sites in the respiratory chain, complex I and complex III have been suggested to be the major source of ROS (Turrens *et al*., 1980; Barja., 1999).

Once the electron transport system is damaged, cellular energy production, in the form of ATP, begins to fail. Many of the cell's functions are energy-dependent, especially cellular repair mechanisms, many transport functions, reproduction, cellular signaling, and ion transport. A defect in energy metabolism may lead to neuronal depolarization, activation of N-methyl-D-aspartate (NMDA) excitatory amino acid receptors, and increases in intracellular calcium, which are buffered by mitochondria (Beal, 1995).

Electron flow through the mitochondrial respiratory chain can be blocked by site-specific inhibitors. The pesticide rotenone, is a specific inhibitor of mitochondrial complex I enzyme. Defects in electron transport complexes or other perturbations of mitochondria can be responsible for excess production of ROS.

1.3.3.1 Complex I and Neurodegeneration

Complex I, also known as NADH:ubiquinone oxidoreductase is the first electron transport energy-conserving enzyme complex of the mitochondrial respiratory chain. It oxidizes NADH

and transfers the electrons via a flavin mononucleotide cofactor and several iron–sulfur clusters to ubiquinone (Q) (Walker *et al*., 1992). Complex I is an integral membrane protein complex composed of 41 subunits, seven of which (ND1, ND2, ND3, ND4, ND4L, ND5 and ND6) are encoded by mitochondrial DNA (mtDNA) (Walker *et al*., 1992).

It appears to have an "L-shape" structure, which consists of two major parts: a hydrophilic promontory (peripheral) part extruding from the membrane into the matrix, and a hydrophobic part that is embedded into the membrane (Takahiro, 2002). This is shown in figure 1.2. The promontory part of complex I mainly contains the redox cofactors such as one FMN (flavin mononucleotide) and 8-9 Fe-S clusters (iron-sulfur). The electron transfer from NADH to Q takes place in this part. The hydrophobic membrane domain consists of the seven subunits, ND1-6, and 4L. The membrane domain is responsible for Q reduction and ion transport. Rotenone, the classical inhibitor of mitochondrial complex I, acts by binding to the ND1 subunit of complex I (Singer & Ramsay, 1994).

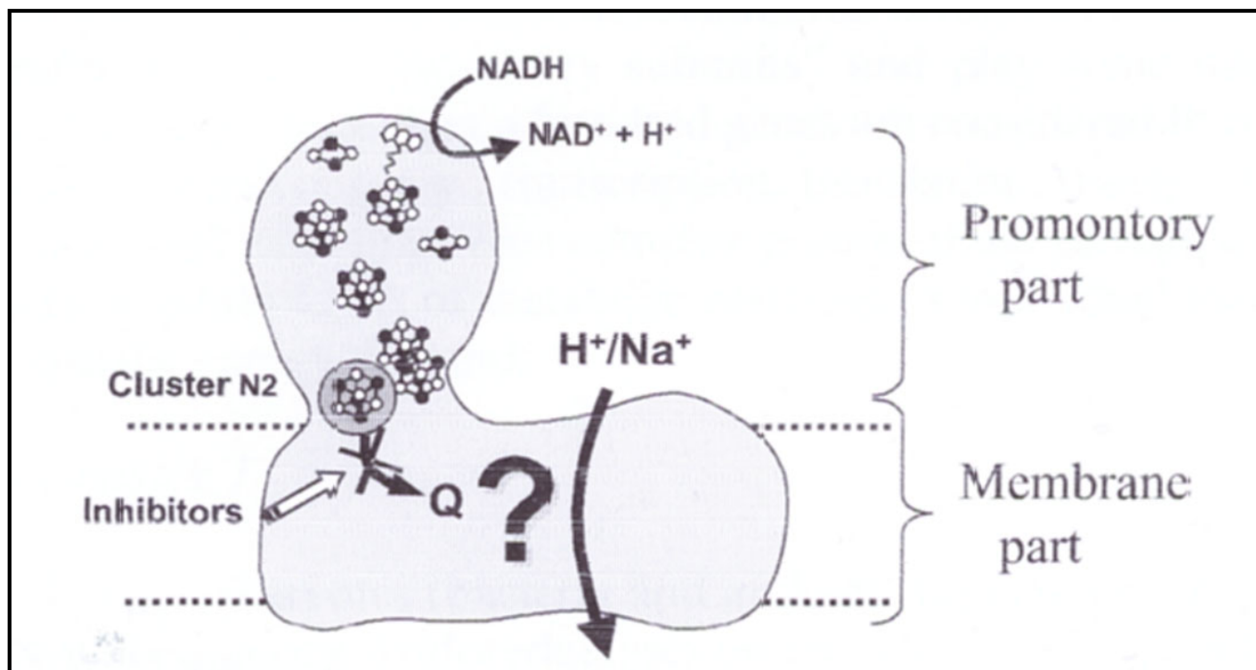


FIG 1.2 *L-shaped appearance of complex I and its cofactor locations (Takahiro, 2002)*

Complex I dysfunction presents the following major problems: (i) impairment of the ability of the respiratory chain to oxidise NADH to NAD, (ii) impairment of the ability of this enzyme to pump protons, which results in a rate of decrease in the ATP synthesis, and (iii) production of superoxide radicals causing mitochondrial DNA mutations, lipid peroxidation and protein denaturation (Boo *et al*., 1998).

Complex I deficiency has been associated with several neurodegenerative disorders and the aging process. Leber's hereditary optic neuropathy (LHON) is a neurodegenerative disease linked to mitochondrial complex I dysfunction (Brown *et al*., 1992). It is the most common cause of blindness in otherwise healthy young men with an incidence of approximately 1 in 50000. Several mitochondrial DNA mutations in the ND1, ND4, and ND6 complex I genes have been identified in LHON (Schapira, 1998). Point mutations in complex I associated with Leber's disease also result in selective damage in the central nervous system. Mutations in the ND1 and ND4 subunits produce optic neuropathy while mutations in the ND6 subunit produce both optic neuropathy and selective basal ganglia degeneration associated with dystonia (Jun *et al.,* 1994).

Parkinson's disease (PD) is another neurodegenerative disease associated with complex I dysfunction. Complex I activity was reported to be defective in multiple tissues from PD patients, including platelets, substantia nigra, nonnigral brain areas, muscle and fibroblasts (Swerdlow et al., 1996). Dopaminergic neurons are especially vulnerable to complex I inhibitors and several groups have reported a generalized and selective defect in complex I of the electron transport chain in PD, raising the possibility that inhibitors of complex I might cause parkinsonism (Schapira, 1990; Betarbet *et al*, 2000).

1.3.4 EXCITOTOXICITY

Excitotoxicity is the paradoxical property exhibited by excitatory amino acids (EAA) such as glutamate, of causing acute neuronal degeneration by excessive stimulation of postsynaptic EAA ionotropic receptors-receptors through which glutamate functions as a transmitter (Olney, 1995). Glutamate, the principal excitatory amino acid in the brain has important roles in several

physiological and pathological events. Excessive activation of glutamate receptors may mediate neuronal injury and death (Lipton and Rosenberg, 1994).

Several disorders such as cardiac arrest, stroke, brain trauma and seizures, can initiate excess glutamate release. This occurs when the oxygen and glucose supply to the brain is dramatically decreased in cases where blood flow ceases such as during a stroke. As a result, neurons cannot generate enough ATP to drive membrane ion pumps and depolarisation of the membrane occurs and consequently calcium leaks into the cell. This calcium triggers the synaptic release of glutamate from vesicles, which further depolarises neurons and raises intracellular calcium, and more glutamate is still released.

There are two principal processes by which EAA induce neurotoxicity, based on the differences in time course and ionic dependence. The first process is termed the acute toxicity and it involves the rapid swelling of neurons caused by excessive depolarisation, ion influx and water entry (Tilson & Mundy, 1995).

The second process, delayed toxicity is one that involves Ca^{2+} influx, which is mediated by the NMDA receptor. The increase in intracellular Ca^{2+} concentration can set off a cascade of pathophysiological events that can lead to excitotoxin-mediated neuronal death (Coyle & Puttfarcken, 1993). Among these processes, is the activation of proteases such as calpain-1. These enzymes can degrade major neuronal structural proteins, and induce cytoskeletal breakdown (Seubert *et al*., 1988). Ca^{2+} can also bind to calmodulin to form Ca^{2+}-calmodulin complexes, which modulate the activity of a large number of enzymes (Ross and Gilman, 1980).

Mitochondria play an important role in the removal of Ca^{2+} from cells after a glutamate insult (White and Reynolds, 1997). Ca^{2+} is sequestered to the mitochondrial matrix, driven by the proton electrochemical gradient generated by the electron transport chain (Ankarcrona *et al*., 1996). The reduction in the electrochemical gradient caused by the Ca^{2+} influx decreases ATP synthesis. Impaired oxidative phosphorylation also enhances vulnerability to excitotoxicity (Beal, 1998). Thus mitochondria are also targets of excitotoxicity such that NMDA receptor activation leads to mitochondrial swelling and the generation of ROS. Substantia nigra (SN)

neurons possess NMDA receptors and there are glutamatergic inputs into the substantia nigra from both the cerebral cortex and the subthalamic nucleus. The activation of NMDA receptors allows calcium influx into the cell, which further results in the neuronal nitric oxide synthase (nNOS) activation and free radical cytotoxicity (Beal, 1995). It is believed that the susceptibility of SN neurons to altered intracellular Ca^{2+} homeostasis is further complicated by an inherent mitochondrial complex I dysfunction (Mizuno *et al.,* 1995). Mitochondrial Ca^{2+} accumulation also leads to the opening of the mitochondrial permeability transition pore which leads to initiation of neuronal death (Murchison and Griffith, 2000).

1.3.5 APOPTOSIS

Many neurological disorders involve cell death, which can occur by either of two distinct mechanisms, necrosis or apoptosis. The term apoptosis was first used by Kerr *et al* in 1972 and is Greek in origin, meaning "dropping off" of petals or leaves from plants or trees (Kerr *et al*, 1972). Apoptosis or "programmed cell death" is a process by which cells undergo physiological cell death in response to diverse stimuli and is essential for normal biological processes such as morphogenesis, tissue homeostasis, and the elimination of damaged or virally infected cells, and may play a role in various pathologic and toxicological process (Thompson, 1995). Apoptosis differs from necrosis in the fact that apoptosis is an active process of cell destruction, characterized by intact plasma membranes, cell shrinkage and the formation of apoptotic bodies whereas necrotic cell death is often characterized by loss of membrane integrity, cell swelling and lysis (Lawrence and Roger, 2000).

The apoptotic process is caused by a cascade of events in which a family of cysteine proteases known as caspases are activated (Thornberry, 1998; Nicholson, 1999). These proteins cleave key cellular substrates that are required for normal cellular function including structural proteins in the cytoskeleton and nuclear proteins such as DNA repair enzymes. The caspases also activate other degradative enzymes such as DNases, which begin to cleave the DNA in the nucleus (Enari *et al*, 1998). The prominent molecular hallmark of apoptosis consists of nuclear fragmentation

from chromatin condensation and internucleosomal DNA breakdown. Figure 1.3 represents the deleterious events occurring in apoptosis.

Mitochondria have the ability to promote apoptosis through the release of proapoptotic factors such as cytochrome c, apoptosis-inducing factor (AIF), caspase-2 and -9 located in the inter-membrane space of the mitochondria or in their matrix. These mediators lead to the activation of a caspase cascade and finally apoptotic death (Lawrence and Roger, 2000). Mitochondria also contribute to apoptosis signaling via the overproduction of free radicals (Sastre *et al*, 2000). These reactive oxygen species can indirectly induce apoptosis by changing cellular redox potentials, depleting reduced glutathione, reducing ATP levels and these changes can accelerate the formation of permeability transition pores, leading to the subsequent release of cytochrome c.

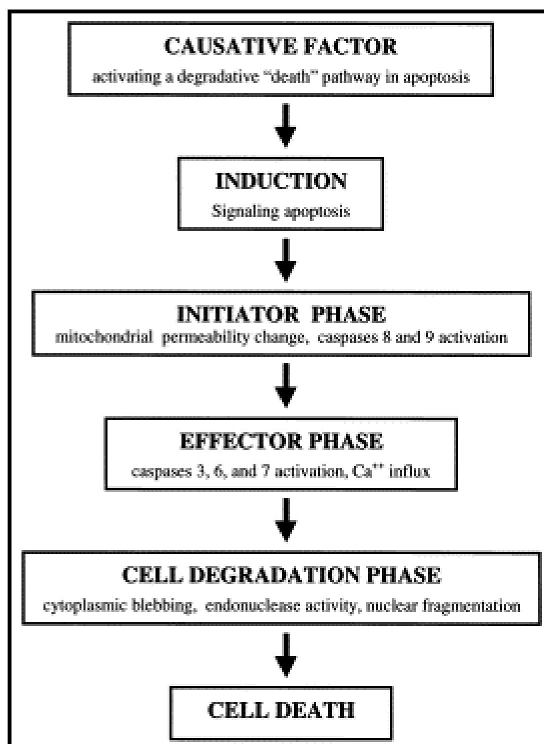


FIG 1.3 *Pathway representing the events in apoptosis (Lawrence and Roger, 2000)*

Many *in vitro* and *in vivo* studies indicate the presence of apoptotic cell death in most of the neurodegenerative disorders such as Alzheimer's, Parkinson's and Huntington's diseases and amyotrophic lateral sclerosis (ALS). Apoptosis appears to be one of the mechanisms leading to

the reduction in neurons in the substantia nigra of Parkinson's patients (Anglade *et al.*, 1997). Dopamine was shown to induce apoptosis in several cell cultures, including chick sympathetic neurons (Zilkha-Falb *et al.*, 1997) and human neuroblastoma cell line SH-SY5Y (Junn and Mouradian, 2001). Selective degeneration of dopaminergic neurons of the SN, as well as DNA fragmentation, was reported in an MPTP-induced PD mouse model (Tatton and Kish, 1997).

1.4 NEURODEGENERATIVE DISORDERS

The etiology of neurodegenerative diseases remains enigmatic; however, evidence for defects in energy metabolism, excitotoxicity, and for oxidative damage is increasingly compelling.

1.4.1 AGEING

Ageing is a universal process and yet it is a remarkably difficult phenomenon to define. One widely accepted definition of ageing is “the process(es) that occur during life which culminate in changes that decrease an individual’s ability to handle biological challenges.” Although the process cannot be stopped there are many physiological factors that can either advance or slow the ageing process.

One of the most prevalent theories in aging research is the “free radical theory of aging”, which was first proposed in the 1950s by Denham Harman. The basis of this theory is that oxidative damage accumulates in cells and tissues over time and contributes to the decline in physiologic function with age (Harman, 1956).

Moreover, the aged brain has been shown to be more susceptible to ROS damage since many of the neuroprotective pathways are no longer as effective as they are in younger animals. As vertebrates age, the pineal gland gradually loses its ability to synthesise the neurohormone, melatonin (Reiter, 1992). This is probably because of the reduction in the number of β-adrenergic receptors (responsible for mediation of night time increase in melatonin production) with age (Saarela and Reiter, 1993). Another reason maybe that the neuronal message to the pineal gland in older animals may be weakened by the gradual deterioration in the cells of the

hypothalamic suprachiasmatic nuclei due to the action of EAA on the neurons at this level (Reiter *et a*l., 1996).

1.4.2 ALZHEIMER'S DISEASE (AD)

Alzheimer's disease (AD) is one of the most common and severe progressive neurodegenerative diseases of aging, accounting for a significant number of cases of senile dementia. It is characterised clinically by progressive loss of memory and other cognitive functions, resulting in severe dementia (Heston and White, 1991).

Alzheimer's disease involves selective and progressive degeneration of lower motor neurons in the spinal cord, and the upper motor neurons in the cerebral cortex**.** The main pathological features of Alzheimer's disease comprises of β– amyloid plaques, neurofibrillary tangles and loss of neurons, especially cholinergic neurons of the basal forebrain (Siegel *et al*., 1989).

Substantial evidence for increased oxidative damage in AD exists from studies of postmortem brain tissue. There are consistent increases in lipid peroxidation as assessed by increases in malondialdehyde concentrations (Beal, 1997). Amyloid β-protein which is derived from the amyloid precursor protein (APP) accumulates in plaques and has been implicated in neurotoxicity, where it is thought to induce the production of free radicals (Behl *et al.*, 1994, Goodman & Mattson, 1996) and disrupt calcium homeostasis (Mattson *et al.*, 1992).

Melatonin appears as if it could be a neuroprotective agent against Alzheimer's disease. Pappolla *et a*l reported that melatonin prevents death of neuroblastoma cells exposed to amyloid β-protein and amyloid peptide fragment (Pappolla *et al*., 1997a; Pappolla *et al*., 1997b). It has been shown that patients with AD appear to suffer from diminished amplitude in the melatonin acrophase (Bevier *et al.*, 1992). Rodent experimentation has demonstrated that pharmacological treatment with melatonin prevents premature ageing and delays the onset of various neurodegenerative diseases (Poeggeler *et al.*, 1993).

1.4.3 PARKINSON'S DISEASE (PD)

In 1817, physician and geologist James Parkinson gave the first clear description of what is now known as Parkinson's disease in his *An essay on the shaking palsy* (Parkinson, 1817). He established the condition as a clinical entity and his essay continues to be recognised throughout the world as the classical description of the condition. Parkinson's disease (PD), the second most common neurodegenerative disease after Alzheimer's disease is a progressive disorder of movement, occurring most commonly in the elderly. It is believed to affect approximately 1% of the population over 55 years of age (Linert and Jameson, 2000).

PD is a neurodegenerative disorder characterized by the progressive depletion of dopamine in the caudate/putamen (striatum) resulting from the progressive loss of neurons in the substantia nigra pars compacta (SNpc) (Calne, 1992). Figure 1.4 depicts the nigrostriatal dopaminergic pathway, progressive degeneration of which leads to the major symptoms of PD.

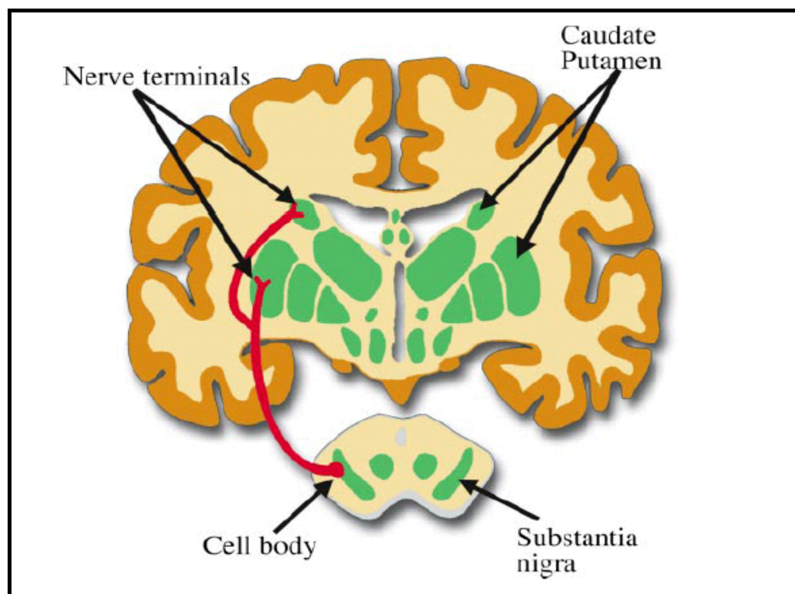


FIG 1.4 *Schematic diagram showing the nigrostriatal dopaminergic pathway. A cross-section of the human brain shows the caudate and putamen and a section through the midbrain shows the substantia nigra. Dopaminergic neurons are indicated in red (Betarbet et al., 2002)*

The clinical symptoms of PD are resting tremor, rigidity, bradykinesia and postural instability. These motor deficits are attributed mainly to a profound reduction of dopamine in the striatum due to a dramatic loss of dopaminergic neurons in the SNpc (Crossman, 1989; DeLong, 1990). Pathologically, PD is characterized by loss of pigmented neurons and gliosis, most prominently in the substantia nigra pars compacta and locus ceruleus (LC) and by the presence of fibrillar cytoplasmic inclusions, known as Lewy bodies (Fahn and Przedborski, 2000). These Lewy bodies (LB) are concentric eosinophilic cytoplamic intraneuronal inclusions with peripheral halos and dense cores, and their presence is essential for the pathological confirmation of PD.

Though the etiology of PD is not well understood, it is believed that it appears to result from several key factors including exposure to unknown environmental toxicants, toxic endogenous compounds and genetic alterations. Postmortem studies strongly implicate that these environmental and endogenous factors cause PD by producing mitochondrial oxidative stress (e.g. complex I inhibition) and damage in the substantia nigra, leading to cell death (Schapira *et al*, 1990; Ebadi *et al*, 1996). Epidemiological studies indicate that rural living, farming, herbicide use, exposure to pesticides, and well-water consumption all increase the risk for developing PD.

As stated earlier, mitochondrial dysfunction and oxidative stress are key factors in the pathogenesis of PD. Brains of PD patients show evidence of elevated oxidative damage to DNA, proteins and lipids, decreased levels of reduced glutathione (GSH) and increased monoamineoxidase (MAO) activity. The findings of decreased activities of glutathione peroxidase and catalase in the SN of PD brains indicate reduced antioxidant defence mechanisms (Fahn and Cohen, 1992). Auto-oxidation of DA also results in the formation of ROS (Lotharius and O'Malley, 2000).

Clinical, epidemiological and experimental studies support the potential role of many different environmental toxicants in the development of PD such as pesticides and herbicides (rotenone, paraquat, heptachlor, dieldrin), metals (manganese, iron, copper), synthetic drug products (MPTP) and plant-related food and natural products (cycads, beta-carboline alkaloids (Betarbet

et al, 2000; Di Monte, 2001; Collins, 2002). Apart from environmental factors, genetic mutations have also been identified as risk factors in PD. Mutations in three different genes such as ∝-synuclein, parkin, and ubiquitin carboxyterminal hydrolase L1 have been associated with familial PD (Polymeropoulos *et al*, 1997; Leroy *et al*, 1998).

1.4.3.1 MODELS OF TOXIN-INDUCED PARKINSONISM

There are various neurotoxin-based models that have been important in elucidating the molecular cascade of cell death in dopaminergic neurons. These include the following:

1.4.3.1.1 The MPTP Model of PD

In 1982, a number of young Californian drug-addicts developed a severe parkinson-like syndrome after injecting a potent pethidine derivative contaminated with 1-methyl-4-phenyl-1, 2,3,6-tetrahydropyridine (MPTP) (Langston *et al.*, 1983). Patients exhibited symptoms very similar to PD with bradykinesia, rigidity, postural instability and resting tremors. After administration, MPTP crosses the blood-brain barrier and is metabolised to its active metabolite 1-methyl-4-phenyl-2, 3-dihydropyridinium ion (MPP^+), by monoamine oxidase type B. MPP^+ is selectively taken up into dopaminergic neurons via its affinity for the dopamine neurotransporter. MPP^+ toxicity is believed to result from inhibition of complex I of the mitochondrial ETC leading to oxidative stress (Nicklas *et al*, 1985). Exposure to MPTP results in nigrostriatal dopaminergic degeneration in a number of species including mice, cats and primates with 50-93% cell loss in the SNpc and more than 99% loss of dopamine in the striatum (Hantraye *et al*, 1993), thus rendering it as a valuable model in studying the mechanisms of PD pathogenesis.

1.4.3.1.2 Rotenone Model of PD

The role of environmental toxins and complex I dysfunction in PD is further supported by a novel model based on chronic systemic exposure of the pesticide, rotenone to rats (Betarbet *et al*,

2000). This *in vivo* model suggested that chronic systemic inhibition of complex I by rotenone exposure induces Parkinsonian features in rats such as selective nigrostriatal dopaminergic degeneration, selective striatal oxidative damage, and formation of ubiquitin- and ∝-synuclein-positive inclusions. This will be discussed further in a later section.

1.4.3.2 IRON AND PARKINSON'S DISEASE

Iron is thought to play an important role in the development of PD and other neurodegenerative diseases. In Parkinson's disease patients, the iron content, particularly the ferric iron is increased in brain tissue (Sofic *et al*, 1988). The selective damage to melanin-containing substantia nigra neurons in Parkinson's disease is possibly initiated by the interaction between iron and neuromelanin, resulting in the accumulation of ferric iron. SN neurons might be at a special risk for damage by free radicals because of their dopamine metabolism. Dopamine auto-oxidation leads to the formation of hydrogen peroxide as a by-product and, if not effectively detoxified by glutathione, hydrogen peroxide might potentially induce the generation of highly reactive hydroxyl radicals in the presence of excess iron.

1.5 ROTENONE

1.5.1 INTRODUCTION

Rotenone (ROT) is a commonly used organic pesticide that has a variety of known and speculated biological effects. It is the most potent member of the rotenoids, a family of isoflavonoids extracted from Leguminosae plants. Rotenone was first isolated in 1929 from the roots of a plant, locally known as 'barbasco' or cube belonging to the Lonchocarpus species. Two species of this genus, *L. utilis* and *L. urucu*, contain relatively high concentration (5-12%) of rotenone and captured attention as an insecticide (Jeremy, Internet). It is also derived from the root of various plants of the Derris (Jewel vine) or Téphrosia (Hoary pea) species from Southeast Asia, Central and South America, and can be found in at least 68 species of Legumes. Rotenone is present mainly in the roots of the plants. The roots are dried, ground, and the active principle extracted with solvents such as chloroform, and benzene (Gosselin, 1984; Hayes, 1982). It is a

selective, non-specific botanical insecticide with some acaricidal and piscicidal (fish-killing) properties. Rotenone is used in home gardens for insect control, for lice and tick control on pets, and for fish eradication as part of water body management.

Rotenone has been used for centuries in South America to paralyze fish. It acts as a fish poison because rotenone is taken up rapidly across the gills, thus damaging the fish respiration and thereby resulting in an inability of fish to use oxygen in the release of energy during normal body processes.

This molecule was studied in Japan by Roten and hence the name 'rotenone'. Trade names for products containing rotenone include Chem-Fish, Cuberol, FishTox, Noxfire, Rotacide, Sinid and Tox-R. It is also marketed as Curex Flea Duster, Derrin, Cenol Garden Dust, Chem-Mite, Cibe Extract and Green Cross Warble Powder. The compound may be used in formulations with other pesticides such as carbaryl, lindane, thiram, piperonyl butoxide, pyrethrins and quassia.
Formulations include crystalline preparations (approximately 95% pure), emulsified solutions (approximately 50% pure), and dusts (approximately 0.75 to 5% pure).

1.5.2 CHEMISTRY OF ROT

Chemical name of Rotenone: 1, 2, 12a-tetrahydro-8, 9-dimethoxy-2 (1-menthylethenyl-(1) benzopyrano (2, 4-b) furo (2,3-h) (1) benzophyran-6 (6H)-ONE.

Chemical Formula: $C_{23}H_{22}O_6$
Composition:
Carbon...............70,40%
Hydrogen............5,62%
Oxygen..............24,34%

1.5.2.1 STRUCTURE OF ROT

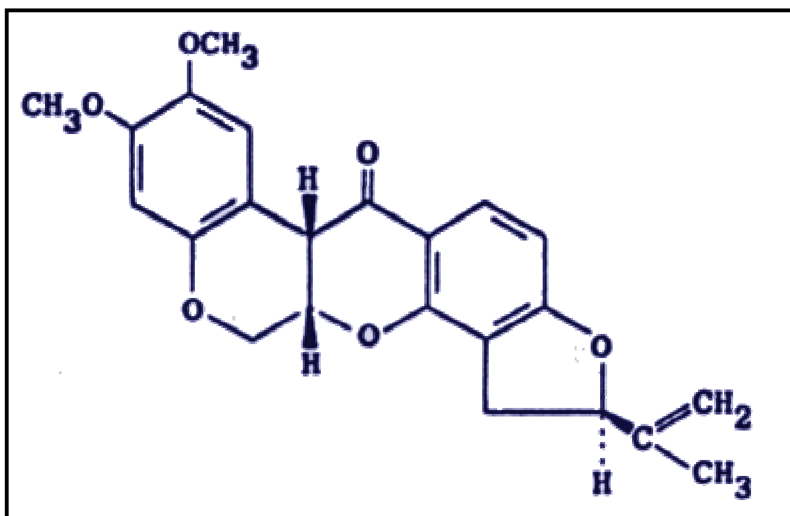


FIG. 1.5 *Chemical Structure of Rotenone*

The structure-activity study, reported by Hirosuke is based on the premise that rotenone bears some structural resemblance to the ubiquinone respiratory coenzymes (Hirosuke, 1992)

1.5.2.2 Physical and Chemical Properties of ROT

It is a "steroid shaped" molecule having a molecular weight of 394.43.

Appearance: Crystal white powder

Water Solubility: Slightly soluble in water, 15mg/L at 100°C.

Solubility in other solvents: soluble in alcohol, acetone, carbon disulfide, chloroform, ether and several organic solutions.

Melting Point: 163°C.

Rotenone is unstable in the presence of air and light.

1.5.3 MODE OF ACTION OF ROT

Rotenone is a cell respiratory enzyme inhibitor. It acts by blocking the NADH:ubiquinone oxidoreductase (complex I) enzyme in the mitochondrial respiratory chain, which in turn results in failure of the respiratory functions (Ernster *et al*., 1963). Thus rotenone acts by moderately depleting the cellular energy levels and causing acute cell death.

1.5.4 PHARMACOKINETICS

1.5.4.1 Absorption

Gastrointestinal absorption of rotenone is low and incomplete. In animals, rotenone is hundreds of times more toxic intravenously than orally. Fats and oils increase absorption (Gosselin, 1984).

1.5.4.2 Distribution

Rotenone, being extremely lipophilic crosses biological membranes easily and is independent of dopamine transporters and thus gets into brain very rapidly (Heikkila *et al*, 1985).

1.5.4.3 Metabolism

Rotenone undergoes metabolism by NADP-linked hepatic microsomal enzymes. Several metabolites have been identified as rotenoids (rotenolone I and II, hydroxy and dihydroxyrotenones, etc.) with toxicity similar to rotenone. One mechanism of metabolism is demethylation (Hayes, 1982).

1.5.4.4 Elimination

In the mouse and rat, approximately 20% of a dose of rotenone is recovered in urine within 24 hours of oral administration (Hayes, 1982).

1.5.5 TOXICITY

Rotenone is classified by the World Health Organisation (WHO) as a moderately hazardous, Class II pesticide (WHO, 2000-2001). The cytotoxicity of rotenone is attributed to its irreversible binding and inactivation of NADH dehydrogenase complex of the mitochondrial electron transport chain (Heikkila *et al*., 1985). Rotenone is believed to be moderately toxic to humans with an oral lethal dose estimated from 300 to 500 mg/kg.

1.5.5.1 Acute Toxicity

The acute oral toxicity of rotenone is moderate for mammals, but there is a wide variation between species. Local effects on the body include conjunctivitis, dermatitis, sore throat, congestion, and vomiting. Inhalation of high doses can cause increased respiration followed by depression and convulsions. Hypoglycaemia may also occur. Cardiopulmonary arrest was reported as the cause of death in one case (De Wilde, 1986).

1.5.5.2 Chronic Toxicity

Growth retardation and vomiting result from chronic exposure of rotenone to rats and dogs. Agricultural workers and pesticide applicators are occupationally exposed. Occupational chronic exposure produces immediate dermatitis characterized by a red-violet colour, slight oedema, and itching (IPCS, 1992).

1.5.5.3 Clinical Effects of Rotenone

Although the main target organs of ROT toxicity are the central nervous system, skin and mucous membrane, other organs are also affected.

1.5.5.3.1 Cardiovascular (CVS) Effects

Acute CVS effects include tachycardia, hypotension, impaired myocardial contractility.

1.5.5.3.2 Respiratory Effects

These include tachypnoea, followed by respiratory depression, cyanosis and hypoxemia. Asphyxia occurs due to respiratory arrest. Chronic respiratory effects include pharyngitis and bronchitis (IPCS, 1992).

1.5.5.3.3 Central Nervous System Effects (CNS)

Central nervous manifestations are muscle tremors, inco-ordination, clonic convulsions and lethargy.

1.5.5.3.4 Gastrointestinal Effects (GIT)

Acute effects include nausea, vomiting and abdominal cramps whereas GIT lesions and bleeding are observed with chronic administration.

1.5.5.3.5 Hepatic and Renal Effects

Chronic poisoning may produce fatty changes in liver and in the kidneys.

1.5.6 Overdose Management

Vitamin K3 (Menadione) antagonises the inhibition by rotenone of mitochondrial respiration. Intravenous administration of Vitamin K3 and glucose is recommended (Gosselin, 1984). Alkaline solutions taken orally also decompose the molecule. Additional management involves maintaining airway and respiration, administering oxygen if cyanosis is present. Gastric lavage maybe indicated if a large amount of rotenone was ingested.

1.5.7 ROTENONE AND PARKINSON'S DISEASE

The link between rotenone and PD has been suspected for several years. Rotenone parallels MPP^+ and 6-hydroxydopamine in damaging rat striatal DA terminals after stereotaxic injection into the median forebrain bundle (Heikkila *et al*., 1985) and is neurotoxic to neuroblastoma cell cultures (Sherer *et al*., 2001). As mentioned earlier (section 1.4.3.1.2), chronic infusion of rotenone via a cannula in the jugular vein for 1–5 weeks reproduced many of the PD symptoms

in rats including dopaminergic neurodegeneration, formation of Lewy bodies and behavioural changes characterised by hypokinesia, hunched posture, and severe rigidity (Betarbet *et al.*, 2000). The rotenone model shows that the features of PD can be produced by systemic complex I inhibition. Unlike MPTP, rotenone does not depend on dopamine transporter and can easily cross the blood–brain barrier.

Rotenone caused accumulation and aggregation of ∝-synuclein, progressive oxidative damage, and caspase-dependent death in an *in vitro* cell system thus linking with a number of events implicated in PD pathogenesis (Sherer *et al.*, 2002). Günter *et al* reported that rotenone and MPP^+ increased free radicals and reduced proteasomal activity via adenosine triphosphate (ATP) depletion and these deficits have been described in the substantia nigra in Parkinson's disease (Günter *et al.*, 2003).

Rotenone has been reported to have a selective toxicity on dopaminergic cells *in vitro* (Semper *et al.*, 1995) and *in vivo*. The herbicide, paraquat (PQ) has also emerged as a risk factor for PD and has reported to cause nigral degeneration by promoting oxidative stress (Sun and Li., 2001). Despite the fact that dopaminergic neurons are susceptible to oxidative injury, environmental toxins such as rotenone and PQ can cause mitochondrial dysfunction and oxidative damage, both of which are associated with PD. This is clearly depicted in figure 1.6.

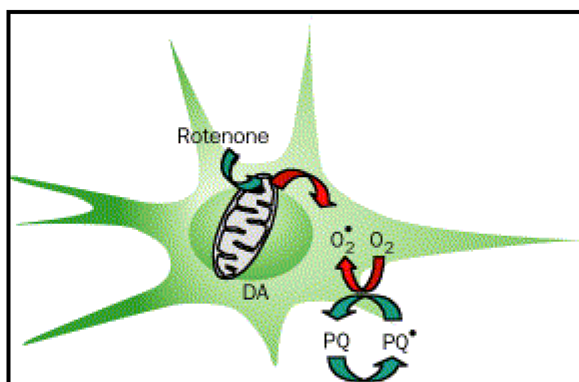


FIG. 1.6 *Toxin-induced oxidative stress in dopaminergic neurons. Both rotenone and paraquat (PQ) generate superoxide anions ($O_2^{\bullet-}$) by blocking mitochondrial electron flow and redox cycling (Di Monte, 2003)*

1.6 NEUROPROTECTIVE STRATEGIES

Numerous cellular defence mechanisms exist to prevent the build up of ROS, and collectively help to protect living organisms against oxidative damage. It is only when there is either an increase in oxyradical production, or a decrease in cellular ROS defence systems, that neurotoxicity occurs. These defence systems include superoxide dismutase and catalase [which collectively remove superoxide ($O_2.^-$) and hydrogen peroxide (H_2O_2) from the cytoplasm], glutathione peroxidase and glutathione reductase, and vitamins E and C.

Due to the continuous generation of superoxide anions in the mitochondria, the mitochondria possess an efficient antioxidant system composed of superoxide dismutase, glutathione peroxidase, glutathione reductase, glutathione, NAD(P) transhydrogenase, NADPH, vitamins E, C (Halliwell & Gutteridge, 1989) and mitochondrial respiration itself (Guidot *et al*., 1995).

Antioxidants obtained from dietary source such as ascorbic acid (vitamin C), α–tocopherol (vitamin E) and carotenoids, can scavenge damaging free radicals. Antioxidants inhibit the chain reaction of lipid peroxidation by scavenging intermediate peroxyl radicals. Vitamin E is believed to be the most important chain breaking antioxidant inhibitor of lipid peroxidation in humans (Burton & Ingold, 1989).

Melatonin, the principal neurohormone secreted by the pineal gland is an endogenous free radical scavenger and it is widely believed that melatonin may be of therapeutic use for protection against neurodegeneration.

1.6.1 THE PINEAL GLAND

1.6.1.1 History of the Pineal Gland

The famous anatomist, Herophilos (325-280 B.C.), at the University of Alexander in Egypt, first discovered the pineal organ in man. Later, René Descartes (1596-1650) described the pineal as

the "seat of the soul". He named it based on the fact that the pineal was the only unpaired part of the brain, and thus the soul could exercise its function more particularly from the pineal.

In the late 19th century and the beginning of the 20th century, there was an explosion in pineal organ research. Bernard (1813-1878) and Brown-Sequard (1817-1894) discovered the endocrine glands and hormone producing organs, and this led to greater interest of physiologists in the pineal gland. At the end of the 19th century, the mammalian pineal gland was seriously considered as a candidate for hormonal production. In 1898, Heubner described a boy suffering from a pinealoma, and showing signs of precocious puberty. Later, in 1930, Marburg also thought that development of the primary and secondary sex organs was caused by hypopinealism, due to pineal degeneration (Pevét, 1984).

In the last 50 years, scientists have confirmed that the pineal gland is an endocrine organ, and determined the neuronal connections between the pineal gland and the hypothalamus. The discovery of melatonin, the chief hormone by the pineal gland, has stimulated worldwide research in this field.

1.6.1.2 Pineal location and anatomy

The pineal gland is located within the brain. It develops from an evagination of the neural tube, which becomes the diencephalon (Ariëns Kappers, 1979). It varies in size, shape and location in different species. In humans, the gland is located on the dorsal surface of the hypothalamus, occupying a central position between the two cerebral hemispheres (Yu and Reiter, 1993). It is a highly vascularized tissue and consists of two types of cells: pinealocytes and neuroglia. In humans, pinealocytes predominate and produce both melatonin and peptides e.g. arginine-vasotocin (Brzezinski, 1997).

In the rat, the pineal is located in the superior part of the *sulcus transversus cerebri* at the surface of the brain. It lies between the cerebral hemispheres, anteriorly, and the cerebellum posteriorly. The pineal gland is attached to the brain by the pineal stalk, and it consists of pinealocytes, pinealoblasts and fibrocytes.

1.6.1.3 Pineal Indole Metabolism

Indole metabolism in the pineal gland occurs in the pinealocytes. The different stages are outlined in figure 1.7. It commences with the uptake of tryptophan from the blood stream. Some of the tryptophan is utilized in the synthesis of pineal proteins, but the majority is converted to 5-hydroxytryptophan by the enzyme tryptophan hydroxylase (Lovenberg *et al*, 1967). This step occurs in the presence of oxygen, ferrous iron, and a reduced pteridine cofactor (Snyder and Axelrod, 1964). In the synthesis of serotonin from tryptophan, this appears to be the rate-limiting step.

5-hydroxytryptophan is converted via L-amino acid decarboxylase to 5-hydroxytryptamine (5HT), also known as serotonin. Serotonin can then be metabolised in three different ways (same figure):

(1) A fraction of the serotonin undergoes methoxylation by hydroxyindole-O-methyltransferase (HIOMT) to form 5-methoxytryptamine. The methyl group is donated by S-adenosylmethionine.

(2) It may undergo deamination and oxidation reactions, where serotonin is oxidized by the enzyme monoamine oxidase to 5-hydroxyindole acetaldehyde. 5-hydroxyindole acetaldehyde is an unstable intermediate and undergoes further metabolism (Axelrod *et al*, 1969). The acetaldehyde is converted to 5-hydroxyindoleacetic acid by aldehyde dehydrogenase (Wurtman and Larin, 1968). A proportion of the 5-hydroxyindole acetaldehyde is converted to 5-hydroxytryptophol by alcohol dehydrogenase (McIsaac and Page, 1959) and is then methoxylated by HIOMT to form 5-methoxytryptophol (Wurtman *et al*., 1968).

(3) The major fraction of serotonin is converted to melatonin. This is a two-step process. First, N-acetyltransferase (NAT) transfers an acetyl group from acetyl coenzyme A to the amino group of serotonin to form N-acetylserotonin. N-acetylserotonin is the precursor of

melatonin (Klein *et al.*, 1971). N-acetylserotonin is then O-methylated by HIOMT to form melatonin. Melatonin is the principal pineal hormone and has been the focus of the larger part of pineal research.

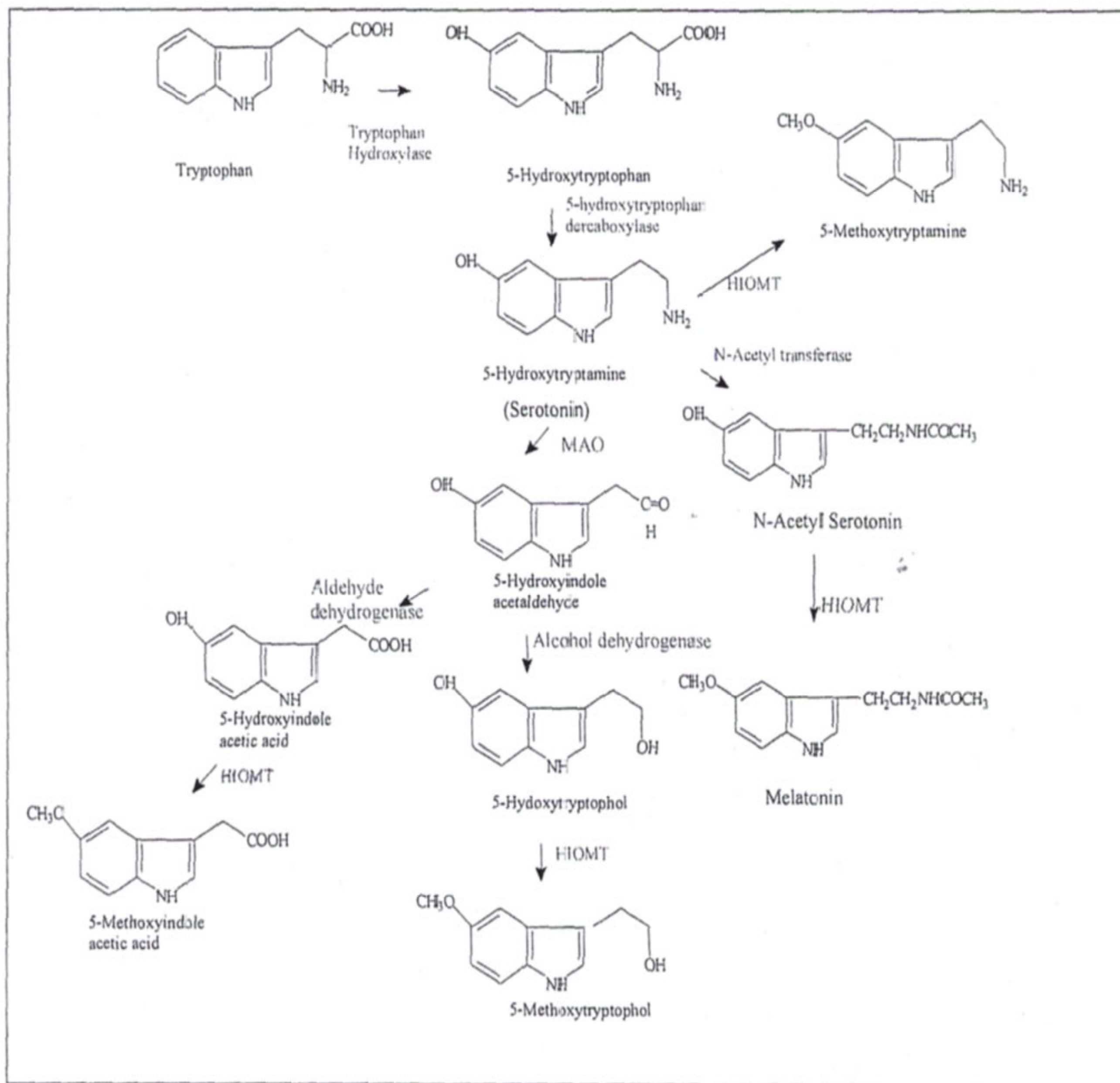


FIG 1.7 *Schematic representation of pineal indole metabolism (modified from Young and Silman, 1982)*

1.6.2 MELATONIN (MEL)

1.6.2.1 History

Melatonin, 5-methoxy-N-acetyltryptamine, is evolutionally speaking, a highly conserved molecule that exists in organisms as different as algae and humans (Poeggeler *et al.*, 1993; Saarela & Reiter, 1993). In 1917, McCord and Allen reported that amphibians fed with bovine pineal extracts exhibited skin lightening. Later, Lerner and co-workers (1958) isolated the compound responsible for this action from bovine pineals. Since it caused aggregation of melanin granules in melanophores, Lerner and colleagues named the compound melatonin.

1.6.2.2 Structure of Melatonin

The chemical structure of melatonin is shown in figure1.8:

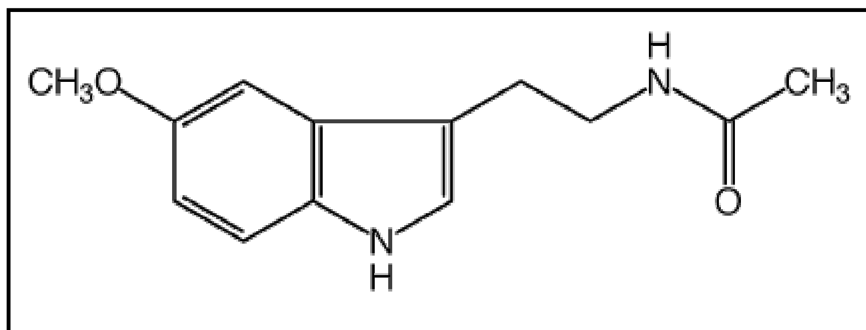


FIG. 1.8 *Chemical structure of melatonin*

1.6.2.3 Synthesis

Melatonin is the product of tryptophan metabolism by the pineal gland. It is synthesized from serotonin via acetylation reactions catalysed by NAT and HIOMT (As shown in the figure 1.7 above).

The biosynthetic pathway of melatonin is regulated by a visual signal that originates in the retina. During the hours of darkness, the retina initiates the production of melatonin, by passing a signal along unmyelinated fibres to the suprachiasmatic nucleus (SCN) of the hypothalamus (Illnerovà *et al*, 1993). The SCN is the body's internal clock which controls circadian rhythmicity of biological events and behaviour (Saarela & Reiter, 1993; Delagrange & Guardiola-Lemaitre, 1993). From the SCN, a projection descends to the intermediolateral cell column in the upper thoracic region of the spinal cord, where fibres project to the superior cervical ganglion. From here postganglionic sympathetic fibres terminate at the pineal gland (Haines, 1997). When stimulated during darkness, the postganglionic sympathetic fibres release noradrenaline which binds to specific β–adrenergic receptors on the pinealocyte cell membrane (Saarela & Reiter, 1993), thus activating melatonin production in the pineal gland.

Melatonin synthesis is not confined only to the pineal gland. It is also synthesised in the retina, the Harderian gland, the intra-orbital lacrimal glands, hypothalamus, the gut, the inner ears, peripheral mononuclear cells, and platelets (Menendez-Pelaez and Reiter, 1993). Pineal synthesised melatonin is predominately responsible for the circulating melatonin in the blood. Reiter (1989) has proposed that extrapineal synthesised melatonin sites compensate for melatonin production in pinealectomized animals.

1.6.2.4 Circadian variation in melatonin synthesis

In vertebrates, melatonin production in the pineal gland exhibits a circadian rhythm, with the highest levels of melatonin being produced during the night. The rhythm of melatonin synthesis depends primarily on the activity of the N-acetyltransferase enzyme (NAT). This enzyme is considered to be the rate-limiting enzyme in the synthesis of melatonin (Reiter, 1994). Noradrenaline released by the postganglionic synaptic fibres activates the adenylate cyclase via a stimulatory guanine nucleotide-binding regulatory protein, G_S (Reiter, 1991). Adenylate cyclase catalyses the conversion of ATP to cAMP (Strada *et al*., 1972).

The increase in intracellular levels of cyclic AMP results in elevated NAT activity. It increases the transcription of mRNA and increases NAT (Reiter, 1991; Brezezinski, 1997). NAT then converts serotonin to N-acetylserotonin which is converted to melatonin (Figure 1.7). NAT

activity has been shown to be lower during the day (Reiter, 1997), resulting in lower melatonin production during the day. At night, blood melatonin levels reach values of 150pg/ml. In humans, melatonin secretion peaks at night between 2:00 am and 4:00 am, and gradually decreases thereafter throughout the proceeding 24-hour period (Waldhauser *et al*., 1984). As a result of this circadian rhythm, the physiological levels of melatonin in body fluids and tissues vary according to the light/dark cycle.

1.6.2.5 Secretion and Distribution of Melatonin

Melatonin is secreted from the pineal gland directly into the bloodstream via the pineal capillaries, which drain into the surrounding venous sinuses. The majority of circulating melatonin in the bloodstream is plasma bound (60-70%), while melatonin is present in its free form in the cerebrospinal fluid (Feuer, 1990). Melatonin is both highly lipophilic, as well as, relatively water-soluble (Shida *et al*., 1994). Thus, it can easily traverse the blood-brain barrier as well as other morphophysiological barriers, and is believed to have ready access to every cell in the organism (Menendez-Pelaez and Reiter, 1993).

1.6.2.6 Metabolism of Melatonin

The half-life of melatonin in rats is only about 20 minutes (Gibbs and Vriend, 1981). Approximately 75% of the melatonin taken up by the liver is converted by oxidation to 6-hydroxymelatonin by cytochrome-P-450-dependent microsomal mixed-function oxidase enzymes (Kopin *et al*., 1961). Most of the 6-hydroxymelatonin is further conjugated to sulphate, rendering 6-sulphatoxymelatonin, and to a lesser extent to glucuronic acid. In the brain, melatonin is converted to N-acetyl-N-formyl-5-methoxykynurenamine by the enzyme indoleamine-2,3-dioxygenase. This is then converted to N-acetyl-5-methoxykynurenamine (Reiter, 1991b).

1.6.2.7 Melatonin Receptors

ML1 and ML2 are the two pharmacologically distinct families of melatonin receptors. These receptors are membrane-bound. ML1 receptors are believed to be involved in renal function,

sleep induction, circadian rhythms, reproduction, and the contractility of cerebral arteries (Feuer, 1990). ML2 receptor functions are unknown at present.

Brezezinski reported that melatonin diffuses into cells and activates the intracellular sites through binding to cytosolic calmodulin (Brezezinski, 1997). Once melatonin has bound to calmodulin, it can influence calcium signalling through interactions with downstream effector enzymes e.g. adenylyl cyclase and phosphodiesterases. Melatonin may also interact with a nuclear family of orphan receptors called retinoid-Z receptors. This implies that once melatonin is in the nucleus, it may regulate gene expression and also function as a free radical scavenger (Brezezinski, 1997).

1.6.2.8 Functions of Melatonin

Melatonin has many and varied functions in mammalian system.

i. Free radical scavenger: melatonin is the most potent hydroxyl radical scavenger (Reiter *et al*., 1994). It has also been shown that melatonin scavenges the peroxyl radical ($ROO^{\cdot}$) (Melchiorri *et al*., 1995). This is discussed in section 1.6.3.

ii. Reproduction: melatonin causes a marked decrease in sperm motility and at high concentrations, completely abolishes its movement (Osthuizen *et al*., 1986).

iii. Immunocompetence: it has been shown that melatonin administration enhances the immune response (Pierpaoli and Maestroni., 1987). Melatonin administration restores the immune dysfunction from soft-tissue trauma and haemorrhagic shock (Wichman *et al*., 1996).

iv. Jet-lag: melatonin is the main synchronizer of the sleep/wake cycle (Bersani and Garavini, 2000). It exerts a therapeutic effect by normalising the sleep/wake cycle in several sleep phase disorders (e.g. jet-lag, advanced sleep onset and delayed sleep phase syndrome) (Zisapel, 1999).

v. Ageing: relative melatonin deficiencies with persistent serotonin have been reported to promote ageing (Rozencwaig *et al*., 1987).

vi. Metal binding: Limson *et al.* (1998) reported that melatonin binds/complexes with metals. This has important consequences in the possible role of melatonin in metal toxicity e.g. In Alzheimer’s disease, where an accumulation of aluminium is

suggested to be a contributing factor in the etiology of the disease and in iron toxicity.

1.6.3 FREE RADICAL SCAVENGING AND ANTIOXIDANT ACTIONS OF MELATONIN

Melatonin possesses an electron-rich aromatic indole ring and acts as an electron donor, thereby reducing and repairing electrophilic radicals. Ring opening of melatonin through interactions with free radicals could explain the antioxidant properties displayed by this compound (Hardeland *et al*., 1993).

1.6.3.1 *In vitro* evidence

Ianas *et al* (1991) was the first to report melatonin's antioxidant actions. However, these authors also reported that melatonin had prooxidant actions. Later, Tan *et al* (1993) reported the free radical scavenging actions of melatonin. Here, $OH^{\bullet}$ was generated by exposing H_2O_2 to ultraviolet light (UV) and a spin trap employing 5,5-dimethylpyrroline-N-oxide was used to determine $OH^{\bullet}$ concentrations. The scavenging properties of melatonin were compared to glutathione and mannitol, (both known scavengers). The results showed that melatonin is a more effective free radical scavenger, and no prooxidant actions of melatonin were uncovered.

Tan *et al* further showed that melatonin is a more effective $OH^{\bullet}$ scavenger, when compared to serotonin, N-acetylserotonin or 5-methoxytryptamine (all chemically related to melatonin). These structure-activity studies showed that the 5-methoxy group on melatonin's indole nucleus and the N-acetyl group on the side chain, are both necessary for the efficient scavenging activity of melatonin.

Various studies soon followed with Poeggeler *et al*, (1994) showing that melatonin could remove $OH^{\bullet}$ from a reaction mixture consisting of $FeSO_4$ and H_2O_2.

1.6.3.2 *In vivo* evidence

Tan *et al* (1993) conducted a series of experiments using safrole, a highly toxic carcinogen that generates free radicals which damage nuclear DNA. The DNA adducts quantified in the liver of rats that were treated melatonin and safrole showed significantly reduced damage in comparison to those animals treated with safrole alone.

Lipopolysaccharide induces extensive tissue damage and multiple organ failure in humans (Ghezzi *et al*, 1986). LPS is believed to damage polyunsaturated fatty acids in membranes through the generation of toxic oxygen radicals. Sewerynek *et al* (1995) treated animals with LPS and the resulting damage to lipids was readily counteracted by systemic melatonin administration.

Paraquat, a highly toxic herbicide, is often used experimentally to investigate the processes of lipid peroxidation in the lung. Melchiorri *et al* (1995) administered paraquat to rats either alone or in combination with melatonin and the indole was able to confer protection against the oxidative toxicity induced by the herbicide.

1.6.3.3 Effects of melatonin on the antioxidant defence system

Melatonin has been shown to affect a number of enzymes related to the antioxidative defence system. Barlow-Walden *et al* found that exogenously administered melatonin stimulated the activity of the antioxidant enzyme, glutathione peroxidase in the brain (Barlow-Walden *et al* 1995). Glutathione peroxidase is involved in the metabolism of H_2O_2 and is therefore an essential antioxidant enzyme.

Melatonin can also stimulate glutathione production by stimulating hepatic and cerebral glucose-6-phosphate dehydrogenase. This causes an increase in NADPH levels, which promotes the production of glutathione by glutathione reductase (Pierrefiche and Laborit, 1995). Glutathione is a necessary cofactor for glutathione peroxidase.

Pozo *et al* demonstrated that physiological concentrations of melatonin are able to inhibit NOS activity (Pozo *et al*., 1997). The inhibition was dose dependent and occurred because of melatonin binding to calmodulin. This prevents calmodulin binding to the NOS peptide, thus inhibiting the initiation of NOS activity.

1.7 OBJECTIVES OF THIS STUDY

There is a compelling need to develop effective animal models for exploring the mechanisms and treatment of neurodegenerative diseases. This study was undertaken to determine whether melatonin could act as a neuroprotective agent in a model of rotenone-induced neurotoxicity. This aim was of interest since rotenone, initially thought to be a safe pesticide, has shown to influence the onset and progression of neurological disorders such as Parkinson's disease. There is a growing concern about the untoward health effects of environmental toxins such as rotenone. The pineal hormone, melatonin, is a well-established free radical scavenger and antioxidant and has been reported to be beneficial in curtailing the severity of various neurodegenerative diseases. Thus melatonin could be an ideal candidate to afford protection against rotenone-induced neuronal damage.

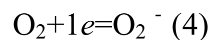
The objectives of this study were to investigate the possible neuroprotective effects of melatonin on rotenone-induced oxidative stress and resultant neuronal damage, through the employment of various biological assays and inorganic studies.

CHAPTER TWO

SUPEROXIDE RADICAL GENERATION

2.1 INTRODUCTION

Partial reduction of molecular oxygen generates reactive oxygen species (ROS), which comprehend both free radicals and neutral molecular species. This molecular oxygen is capable of accepting an additional electron to create a more reactive form of oxygen known as superoxide radical. (Cheeseman *et al*., 1993):



$$O_2 + 1e = O_2^{\,-} \quad (4)$$

The superoxide anion appears to be the first oxygen reaction product generated under physiological and pathological conditions. Mitochondria continuously generate small amounts of superoxide radical anions through monoelectronic reduction of oxygen at intermediate steps of the electron transport chain (Alicia, 1999). It has been understood that the major sites of superoxide formation in the mitochondrial electron transport chain are within respiratory complex I, the NADH-ubiquinone oxidoreductase, and complex III, the ubiquinol-cytochrome *c* oxidoreductase and are diagrammatically represented in figure 2.1 (Barja, 1999). What makes these two sites candidates for superoxide anion production is that the flavin mononucleotide (FMN) of complex I and ubiquinone in complex III can exist in a semiquinone anion form, which contains an unpaired electron that can be donated to molecular oxygen to form superoxide anion (Bailey *et al*., 2002). The semiquinones generated within complex I have been identified as responsible for generating damaging superoxides (Brian, 1998).

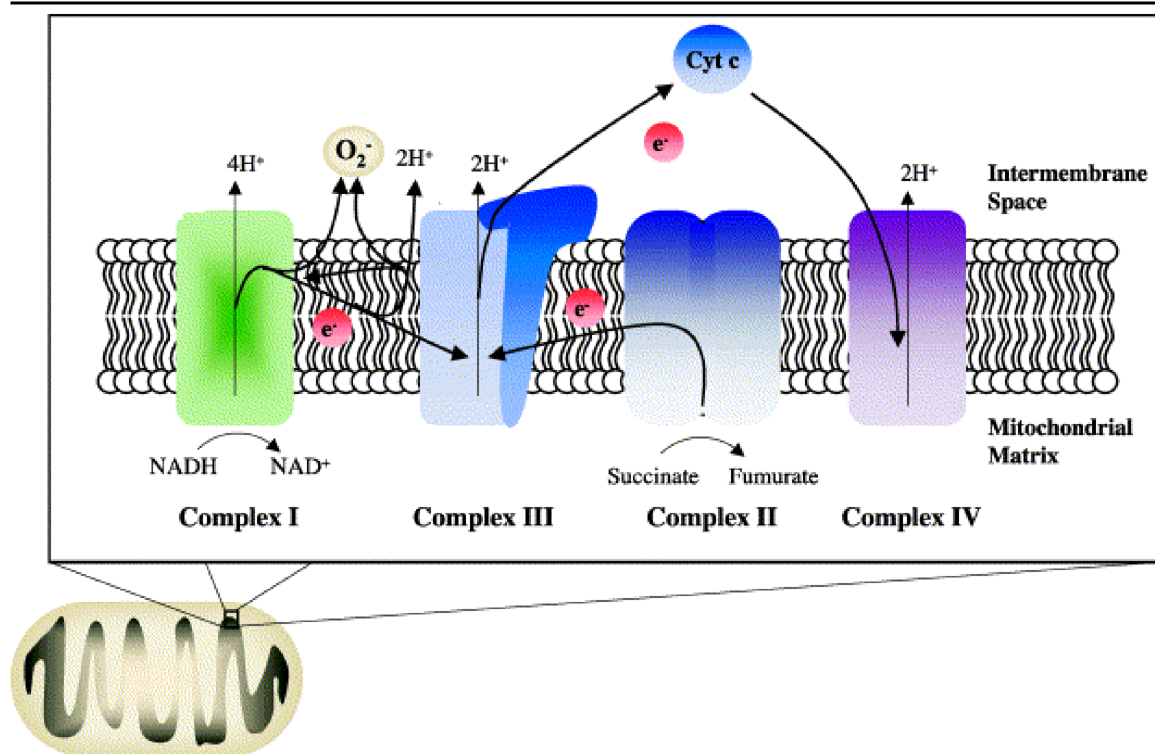


FIG 2.1 *Schematic diagram illustrating the major subunits of the electron transport chain (ETC) and sites of O_2^- production. Electrons enter the ETC at either complex I (NADH-ubiquinone oxidoreductase) or complex II (succinate dehydrogenase) following the oxidation of NADH and succinate, respectively. Ubiquinone is a lipid-soluble electron carrier and carries the electrons from complex I and complex II to complex III (ubiquinol-cytochrome c oxidoreductase). Superoxide ($O_2^{\cdot-}$) can be produced at both complex I and complex III. (Curtin et al., 2002).*

Superoxide toxicity appears through inactivation of proteins that contain iron-sulphur centres, and the ferrous iron released during such inactivation becomes an important reactant in the Fenton reaction (Raha *et al*., 2000). The dismutation of O_2^- generates H_2O_2 and oxygen. This H_2O_2 formed may react with Fe^{2+}, resulting in the formation of the highly toxic hydroxyl radical ($HO^{\cdot}$) via the Fenton reaction. In pH environments of approximately 7.4, superoxide partially protonates to form the hydroperoxyl radical ($HO_2^{\cdot}$), a more reactive oxidising species (Rao & Hayon, 1973). Superoxide can also react with $NO^{\cdot}$, to produce the peroxynitrite anion ($ONOO^-$), which itself can act as an oxidant and cause damage to lipids, proteins and DNA (Beckmann & Koppenol, 1996). $ONOO^-$ can oxidatively damage mitochondrial respiration with further production of $O_2^{\cdot-}$, resulting in a deleterious cascade of events (Radi *et al.,* 1994).

Mitochondrial-derived pathological free radicals have been implicated in numerous diseases and in the aging process itself (Beal, 1995; Dykens, 1997). For example, oxygen radical damage has been specifically implicated in the pathogenesis of PD (Olanow, 1990). The protective measures that the cells employ in response to such damage are usually sufficient except when there is excessive ROS production, causing the biological defenses to be overwhelmed thus leading to oxidative stress (DiFiglia, 1990).

2.2 EFFECT OF MELATONIN ON ROTENONE-INDUCED SUPEROXIDE RADICAL GENERATION IN RAT BRAIN HOMOGENATE

2.2.1 INTRODUCTION

Due to its high dependence on oxidative metabolism and deficient antioxidative measures, the CNS is more susceptible to ROS-induced damage. Rotenone, a highly specific mitochondrial complex I inhibitor, acts by blocking the flow of electrons from the iron-sulphur complexes in the NADH:ubiquinone oxidoreductase complex to ubiquinone. This in turn, results in the promotion of electron flow through the FMN-complex I site and thereby subsequent generation of superoxide anion (Zhang *et al*., 2001).

The aim of these studies was to investigate whether the neurohormone, melatonin is able to offer protection against the induction of superoxide radicals by the environmental neurotoxin, rotenone.

2.2.2 MATERIALS AND METHODS

2.2.2.1 Animals

Adult male, Wistar rats purchased from the South African Institute for Medical Research (Johannesburg, South Africa) were used throughout the study. The animals weighed between 250 and 300g and were housed under artificial illumination with a daily photoperiod of 12 hours. The temperature in the animal house was maintained at 20°C to
24°C with four animals per cage and provided with food and water *ad libitum*. An extractor fan ensured the constant removal of stale air. Protocols for the experiments were approved by the Rhodes University Animals Ethics Committee.

2.2.2.2 Chemicals and Reagents

All reagents were of the highest quality available. Rotenone, melatonin, nitroblue tetrazolium (NBT) and nitroblue diformazan (NBD) were purchased from Sigma Chemical Corporation, St. Louis, MO, U.S.A. Glacial acetic acid was purchased from Saarchem (PTY) Ltd, Krugersdorp, South Africa. 0.1% NBT reagent was prepared by dissolving the NBT in ethanol before making up the solution to the required volume with Milli-Q water.

2.2.2.3 Brain Removal

Rats were sacrificed by cervical dislocation and rapidly decapitated. The brain was exposed by making an incision through the bone on either side of the parietal suture, from the foramen magnum to near the orbit. The calvarium was removed, exposing the brain which was easily removed and either used immediately or stored at –70 ^{0}C.

2.2.2.4 Preparation of Brain Homogenate

The brains were weighed and rapidly homogenised with 0.01M phosphate buffer saline (PBS), pH 7.4, so as to give a final concentration of 10% w/v. This is necessary to prevent lysosomal damage of the tissue.

2.2.2.5 Preparation of Standard Curve

Using NBD as a standard, a series of reaction tubes containing known concentrations of NBD in glacial acetic acid were prepared. The absorbance of each solution at intervals was read at 560 nm and a standard curve was generated (Appendix 1).

2.2.2.6 Nitroblue Tetrazolium Assay

The NBT assay is generally accepted as a simple and reliable method for assaying the superoxide free radical (Ottino and Duncan, 1997). A modification of the assay used by Ottino and Duncan (1997) was used in the following set of experiments.

This assay was carried out in 2 sections which involves the:

(1) *In vitro* exposure to melatonin

(2) *In vivo* administration of melatonin

2.2.2.6.1 In vitro Exposure to Melatonin

Melatonin was dissolved in absolute ethanol and subsequently diluted with Milli-Q water (the final ethanol concentration in the brain homogenate was 0.5%). Rotenone was first dissolved in absolute ethanol and then diluted with water in a ratio of 50:50. The experiments were conducted in which the brain homogenate (1 ml) containing varying concentrations of rotenone (0, 30, 40 or 50μM) alone and in combination with melatonin (0, 30, 40 0r 50μM) was incubated with 0.4 ml of 0.1% NBT in an oscillating water bath for 1 hour at 37°C. Termination of the assay and extraction of reduced NBT was carried out by centrifugation of the samples at 2000g and the resuspension of the pellet with 2 ml glacial acetic acid. The absorbance of the glacial acetic acid fraction was measured at 560 nm and converted to μmoles diformazan using a standard curve generated from NBD.

2.2.2.6.2 In vivo Administration of Melatonin

For the *in vivo* experiments the dose of rotenone selected was lower than melatonin as compared to the *in vitro* one. Melatonin was dissolved in ethyl oleate for the purpose of *in vivo* administration to the rat. The rats were divided into two groups each containing five rats. One set of rats was the test group and were injected intraperitoneally (i.p.) with 5mg/kg of melatonin twice daily. The second group of rats was the control group and was injected i.p. with ethyl oleate at the same time and for the same duration as the test group. The animals were injected for five consecutive days and on the fifth day, were sacrificed and brains removed as described in section 2.2.2.3. The brain homogenate was allowed to incubate with different concentrations of rotenone (10, 20 and 30μM) at 37^{0}C for one hour, after which the NBT assay was performed as described in section 2.2.2.5.1.

2.2.2.7 Statistical Analysis

The results were analysed using a one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls Multiple Range Test. The level of significance was accepted at $p<0.05$ (Zar, 1974).

2.2.3 RESULTS

The final results were corrected for dilutions and expressed as µmoles of diformazan produced per mg tissue. The *in vitro* exposure of rat brain homogenate to increasing concentrations of rotenone caused a significant increase in superoxide radical generation, when compared to the control (Figure 2.2).

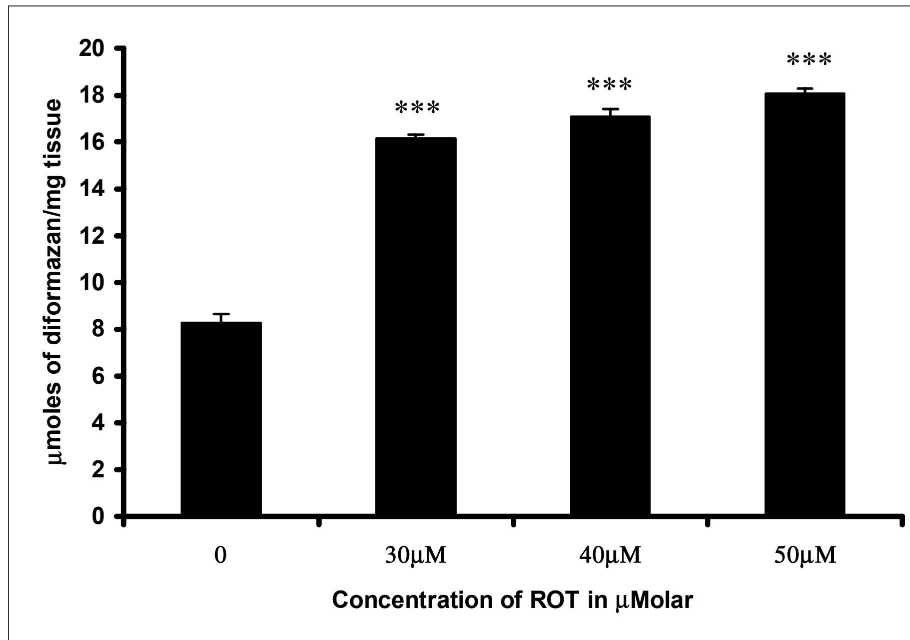


FIG 2.2 *Concentration-dependent effect of ROT on superoxide generation in whole rat brain homogenate. Each bar represents the mean ± SD, n=5; (*** p<0.001 in comparison to control). Student-Newman-Keuls Multiple Range Test.*

Co-treatment of this homogenate with melatonin resulted in a significant reduction in superoxide generation induced by 50µM rotenone (Figure 2.3).

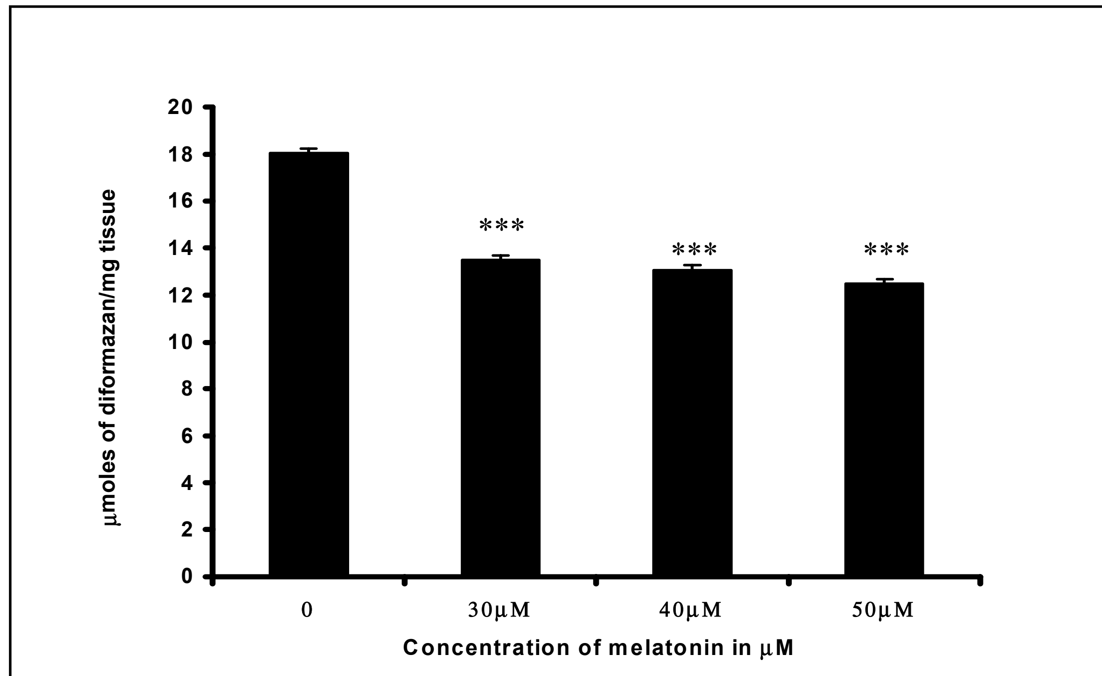


FIG 2.3 *The effect of melatonin on ROT (50µM)-induced superoxide anion generation in whole rat brain homogenate. Each bar represents the mean ±SD, n=5; (*** $p<0.001$ in comparison to control). Student-Newman-Keuls Multiple Range Test.*

Brain homogenate obtained from rats that were pre-treated with melatonin 5mg/kg i.p for five consecutive days showed significant reduction in superoxide generation by increasing concentrations of rotenone (10-30µM) in comparison to the control (Figure 2.4).

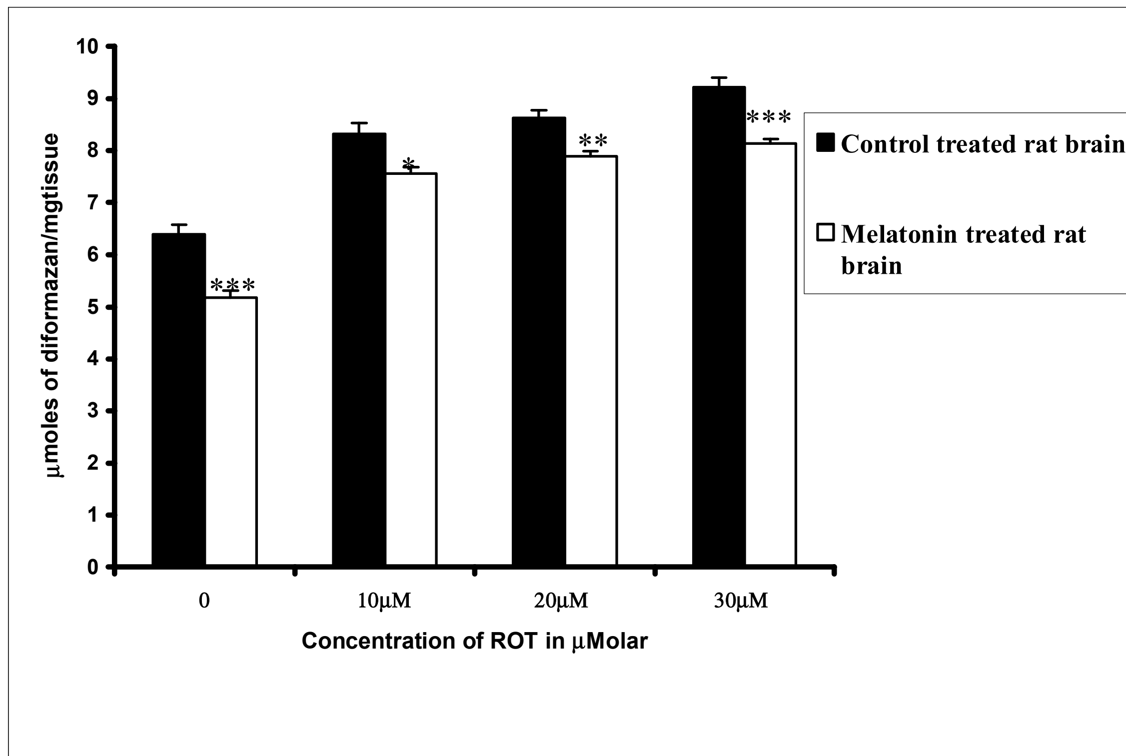


FIG 2.4 *The effect of increasing concentrations of ROT on superoxide generation in rat brain homogenate obtained from rats pretreated with melatonin 5mg/kg i.p. for five consecutive days. Each bar represents the mean ± SD; n=5. (* $p<0.05$; ** $p<0.01$; *** $p < 0.001$); Student-Newman-Keuls Multiple Range Test.*

2.2.4 DISCUSSION

The results of the present study demonstrate that melatonin significantly blunts the concentration-dependent induction of superoxide radicals by rotenone. Brain homogenates obtained from rats that were pre-treated with melatonin also showed significant protection against rotenone toxicity.

Respiring mitochondria represent the major source of superoxide production in most cell types, the major sites being respiratory complexes I and III (Barja, 1999). The oxygen reducing site in

complex I is localized between the ferricyanide reducing site and the rotenone block (Maria *et al*., 2001). Early reports have shown that addition of rotenone to sub-mitochondrial particles could stimulate superoxide formation (Turrens *et al*., 1980; Turrens *et al*., 1982). The generation of superoxide anions by rotenone could be attributed to the ability of rotenone to block mitochondrial respiratory chain complex I, thereby increasing the formation of ubisemiquinone, the primary electron donor in mitochondrial superoxide generation.

Melatonin has been found to be both a direct free radical scavenger and an indirect antioxidant (Tan *et al*., 1993; Reiter *et al*., 1997). Though melatonin directly scavenges a variety of free radicals, it has little ability to directly scavenge the superoxide anions (Reiter, 1998). However, it has a potent role in reducing the intracellular concentrations of superoxide anions by a number of ways. The indolyl cation radical, formed when melatonin donates an electron to a reactive free radical, is believed to secondarily scavenge superoxide anions (Hardeland, 1993). Furthermore melatonin stimulates a number of antioxidative enzymes including superoxide dismutase, glutathione peroxidase, glutathione reductase and catalase (Reiter *et al*., 1999) whereas it inhibits the pro-oxidative enzyme nitric oxide synthase, thus reducing the $NO^{\cdot}$ formation (Pozo *et al*., 1994). Recently melatonin has shown to increase the efficiency of the electron transport chain and, as a consequence, to reduce electron leakage and the generation of free radicals (Martin *et al*., 2002). Apart from these ubiquitous actions, the present study points to a role of melatonin in preventing rotenone inhibition at the level of complex I, thus enabling in the reduction of superoxide generation. More studies need to be conducted to elucidate this hypothesis.

CHAPTER THREE

BIOLOGICAL OXIDATION ASSAY

3.1 INTRODUCTION

Mitochondria play an important role in maintaining cell homeostasis and viability, and consequently are important regulators of cell death processes. In addition to their central bioenergetic task of ATP (adenosine triphosphate) generation, the organelles are the main source both of reactive oxygen species (ROS) and of the cell's antioxidant defenses (Cadenas & Davies; 2000). The mitochondrial electron transport chain (ETC) accounts for a vast majority of oxygen consumed within cells and has been recognized as a major intracellular source of ROS (Chance *et al*., 1979). Under physiological conditions 90% of cellular oxygen consumption occurs during ATP-coupled mitochondrial electron transport and it is estimated that around 1–2% is converted into superoxide anions ($O_2.^-$) due to the "leakage" of unpaired electrons to molecular oxygen as they are being transported down the respiratory chain (Turrens *et al*., 1980). This basal rate of $O_2.^-$ production may be altered in pathological conditions resulting in elevated oxidative stress.

Mitochondrial respiratory chain defects have been implicated in the pathogenesis of AD (Grunewald and Beal, 1999) and mitochondrial dysfunction has been identified in neurodegenerative disorders, including PD and Huntington's disease (Schapira *et al*., 1990). During aging and some neurodegenerative diseases, oxidatively damaged mitochondria are unable to maintain the energy demands of the cell leading to an increased production of free radicals. Both processes, i.e., defective ATP production and increased oxygen radicals, may induce mitochondrial-dependent apoptotic cell death.

3.2 EFFECT OF MELATONIN ON ROTENONE-INDUCED MITOCHONDRIAL RESPIRATORY INHIBITION

3.2.1 INTRODUCTION

Rotenone, being a well established respiratory poison blocks the flow of electrons from complex I to ubiquinone in the electron transport chain thus interfering with the normal respiratory functions (Zhang *et al*., 2001). The consequence is an impaired tissue utilisation of oxygen, and thereby energy depletion. There is a site of 'electron leak' within complex I of the ETC, proximal to the binding site of rotenone which is of particular relevance to PD. Due to the inhibitory effect on complex I, rotenone have the potential to increase the production of superoxide anions as observed in the previous chapter (Turrens *et al*., 1980).

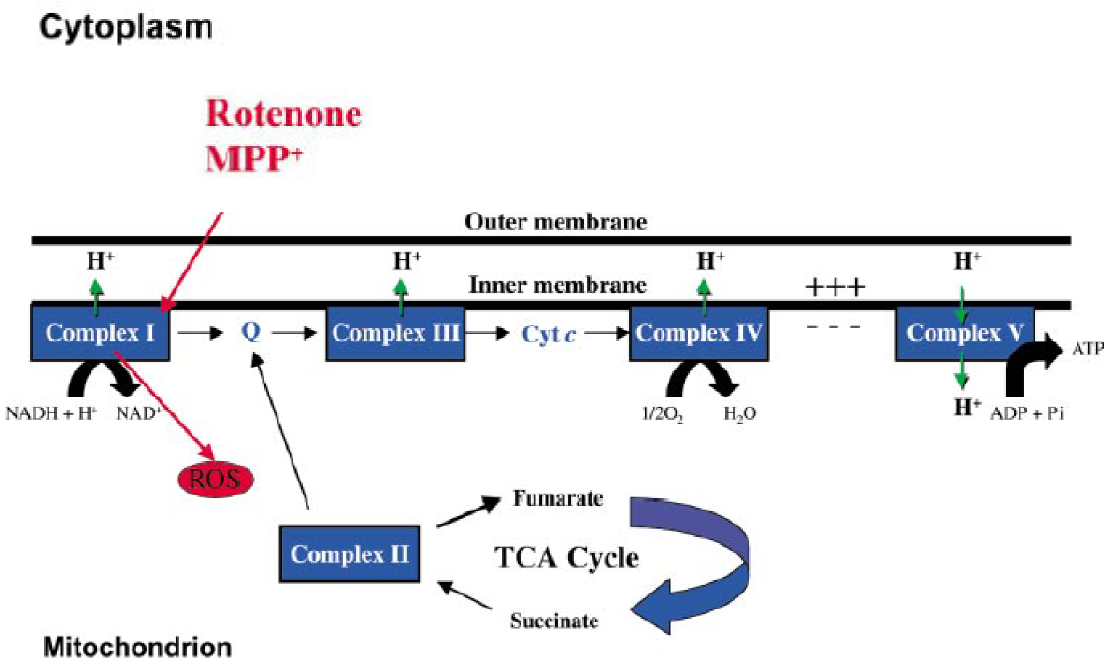


FIG 3.1 *Schematic diagram of the mitochondrial Electron Transport Chain. The site of complex I inhibition by rotenone and MPP^{+}, and ROS production is depicted by red arrows (Betarbet et al., 2002)*

Recent studies suggest an important role of melatonin in mitochondrial homeostasis.

The present assay was conducted to determine the extent of rotenone toxicity due to impaired respiration and whether melatonin was able to afford protection against ROT-induced mitochondrial alterations. The activity of the respiratory chain was determined spectrophotometrically, which employed the synthetic dye 2,6-dichlorophenolindophenol (DPI) as an electron acceptor following biological oxidation of lactate.

3.2.2 MATERIALS AND METHODS

3.2.2.1 Animals

Adult male, Wistar rats, weighing between 250 and 300g were used for the experiments, and were housed and maintained under the conditions described in section 2.2.2.1.

3.2.2.2 Chemicals and Reagents

All reagents were of the highest quality available. Melatonin, rotenone, nicotinamide adenine dinucleotide (NAD) and 2,6-dichlorophenol-indophenol (DPI) were purchased from the Sigma Chemical Corporation, St. Louis, MO, U.S.A. L-Malate was purchased from Eastman Organic Chemicals and sucrose was purchased from Saarchem (PTY) Ltd, Krugersdorp, South Africa. Rotenone was dissolved in absolute ethanol and melatonin in 50% ethanol for the purposes of the experiment. All other reagents were prepared in 0.1M potassium phosphate buffer, pH 7.4.

3.2.2.3 Isolation of Mitochondria from Rat Brain and Liver

Rats were sacrificed by cervical dislocation followed by decapitation. The brains and livers were removed and chilled on crushed ice.

Mitochondrial suspensions were prepared by differential centrifugation according to Plummer (1978). All procedures were carried out at 4°C. Brain and liver were diced and rinsed in ice-cold saline [0.9% (w/v) NaCl] and homogenized in 0.1M potassium phosphate buffer, pH 7.4 in a manual glass-teflon homogenizer on ice to yield a 10% w/v homogenate. After centrifuging twice at 600×g for 10 min, the supernatants obtained were subsequently centrifuged at 8000×g for 10 min. The pellet was resuspended in half the volume of 0.32M-buffered sucrose and centrifuged at 8000×g for 10 min. This step was repeated twice to obtain the mitochondria rich pellet, which was resuspended in the same buffer and was used immediately.

3.2.2.4 Biological Oxidation Assay

A modification of the spectrophotometric technique described by Plummer (1978) was used to determine the "activity" of the inner mitochondrial electron transport chain. This is determined by the rate of reduction of the synthetic electron acceptor dye, DPI, in the presence of the substrate, L-malate. L-malate and NAD were present in saturating final concentrations of 90mM and 0.5mM respectively. Mitochondrial suspension containing rotenone with and without melatonin was incubated at 37°C in a water bath for varying incubation times of 0, 30 and 60 minutes. Following incubation 1ml of the suspension was removed and incorporated with NAD, L-malate, DPI and potassium phosphate buffer. This was inverted once to mix solutions and the decrease in absorbance over a 5-minute period was read at 30-second intervals on a UV/VIS spectrophotometer at 600nm. All data is expressed as absorbance versus time in minutes and corrected for appropriate controls.

3.2.3 RESULTS

The *in vitro* exposure of brain and hepatic mitochondria to rotenone caused a significant decline in mitochondrial ETC activity in a time-dependent manner. Fig. 3.2 shows the effect of melatonin alone and melatonin in presence of rotenone on the ETC of brain mitochondria, in comparison to the control and rotenone treated mitochondria, at 0 incubation time. ROT at a concentration of 50µM decreased the activity of the mitochondrial electron transport chain at t=0 minute in comparison to the control. When melatonin was used together with rotenone, the inhibitory effect was less, indicating that melatonin is able to prevent ROT-induced respiratory inhibition.

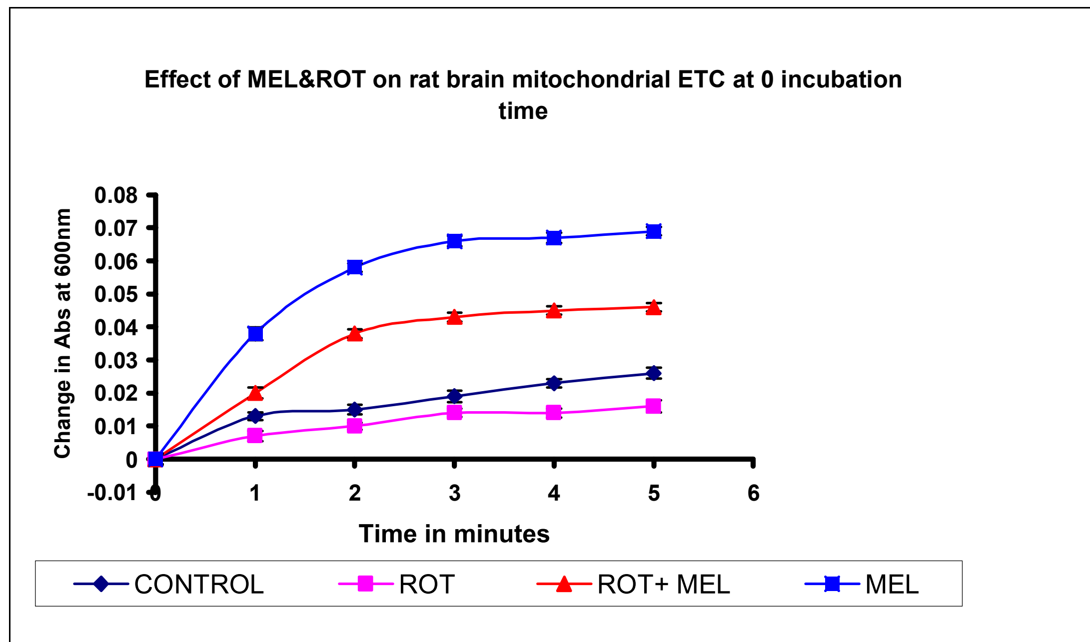


FIG. 3.2 *A comparison of the effect of melatonin (1μM) alone and in presence of rotenone (50μM) to that of rotenone alone and control, on the rat brain mitochondrial ETC using L-Malate as the substrate, after 0 minutes of incubation.[Data represents the mean ±SD; n=5].*

Fig.3.3 represents the ETC activity in liver mitochondria with rotenone, with and without melatonin at 0 incubation time. Mitochondria treated with ROT only showed a decrease in respiratory activity in comparison to the control. With the addition of melatonin, a significant improvement in ETC was observed.

When both rat brain and liver mitochondria were treated with melatonin alone, a marked increase in ETC activity could be observed implying the protective role of melatonin in mitochondrial respiration.

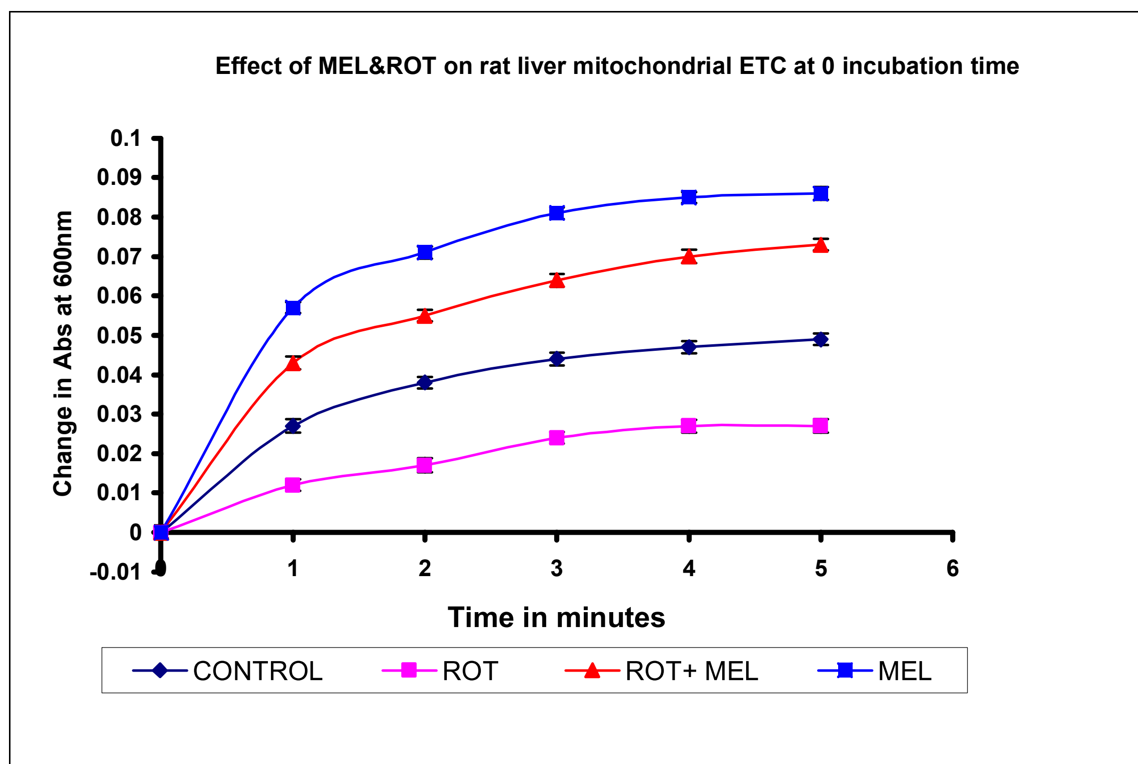


FIG. 3.3 *A comparison of the effect of melatonin (1µM) alone and in presence of rotenone (50µM) to that of rotenone alone and control, on the rat liver mitochondrial ETC using L-Malate as the substrate, after 0 minutes of incubation.[Data represents the mean ±SD; n=5].*

Fig. 3.4 depicts the effect of melatonin alone and melatonin in presence of rotenone on ETC of brain mitochondria, in comparison to control and ROT treated mitochondria after 30 min of incubation. It can be observed that there is a marked decrease in the ETC activity of ROT treated mitochondria when compared to the control. This can be attributed to the increased time of contact between the mitochondria and ROT. Again with ROT/MEL, there is a rise in activity during the first two minutes, thereafter it lowers just below the control but preventing the respiratory inhibition. With MEL alone, an increase in activity can be observed.

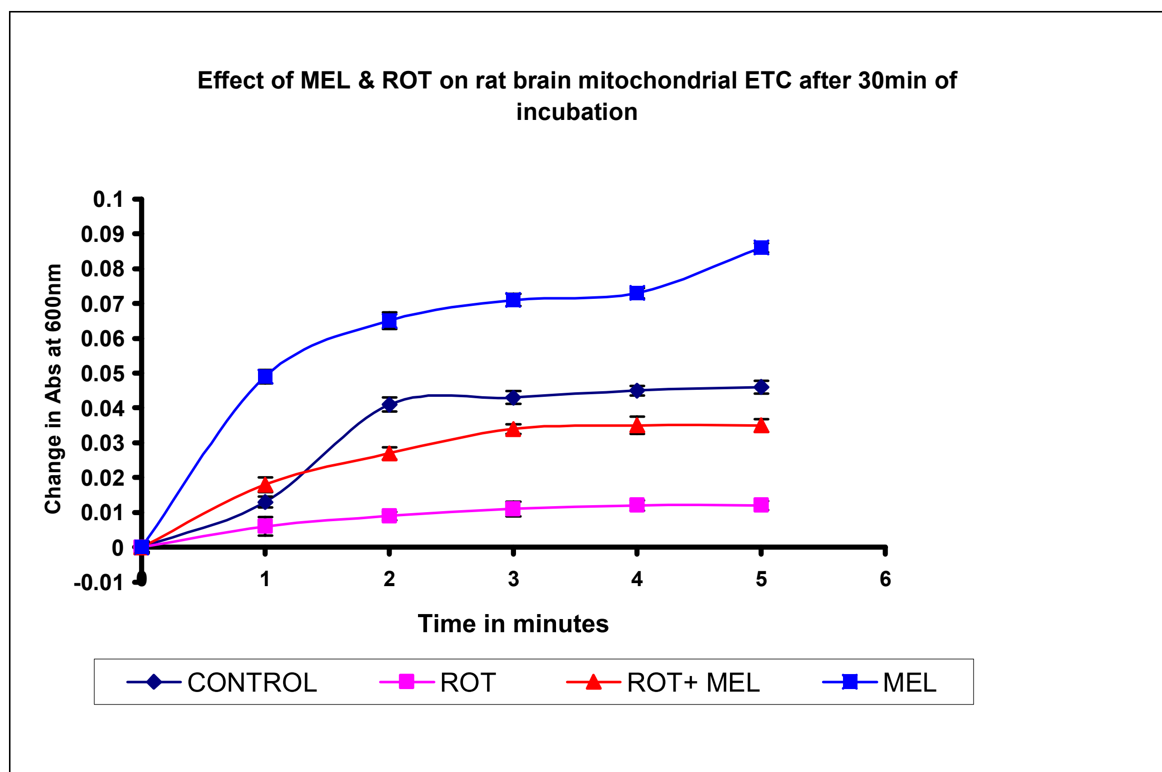


FIG. 3.4 *A comparison of the effect of melatonin (1µM) alone and in presence of rotenone (50µM) to that of rotenone alone and control, on the rat brain mitochondrial ETC using L-Malate as the substrate, after 30 minutes of incubation.[Data represents the mean ±SD; n=5].*

With hepatic mitochondria, similar results were observed with ROT, with and without melatonin as shown in Fig. 3.5. Addition of ROT alone caused a decrease in the respiratory chain activity after 30 min of incubation. On addition of melatonin, a significant rise in ETC activity can be observed in comparison to ROT alone. The treatment of hepatic mitochondria with melatonin alone showed a marked rise in the ETC activity.

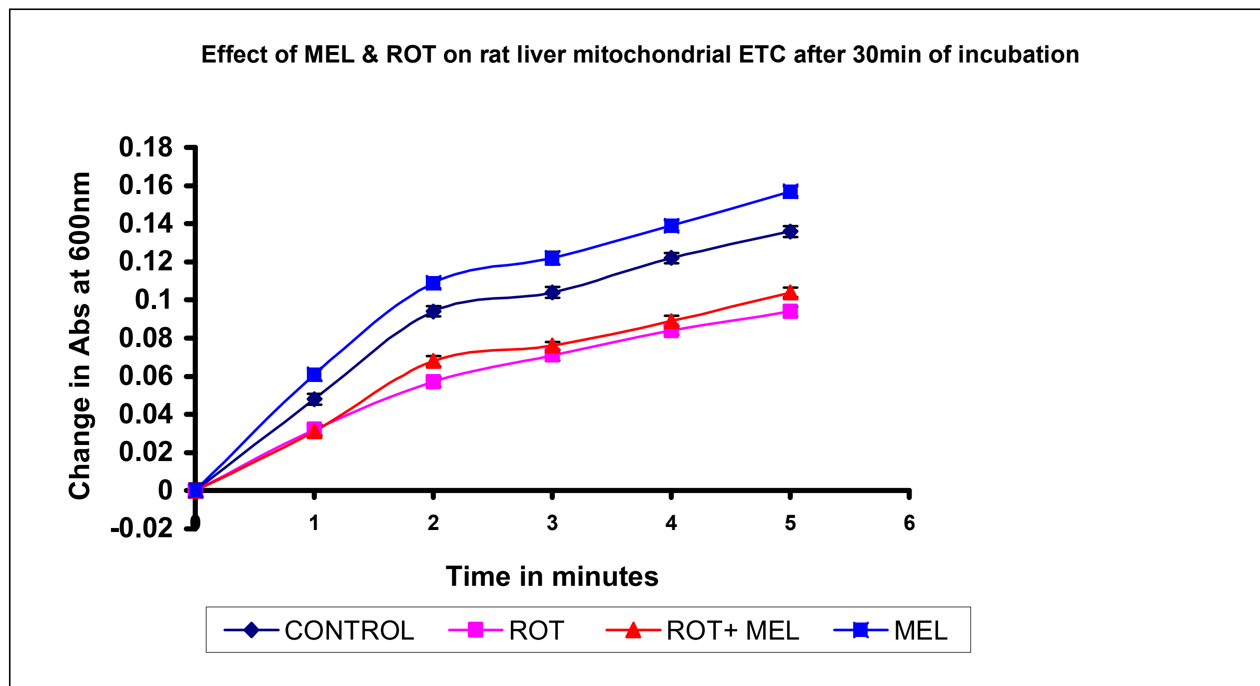


FIG. 3.5 *A comparison of the effect of melatonin (1µM) alone and in presence of rotenone (50µM) to that of rotenone alone and control, on the rat liver mitochondrial ETC using L-Malate as the substrate, after 30 minutes of incubation. [Data represents the mean ±SD; n=5].*

Fig. 3.6 represents the effect of melatonin alone and melatonin in presence of rotenone on ETC of brain mitochondria, in comparison to control and ROT treated mitochondria after 60 min of incubation. Here, ROT shows a marked decrease in electron transport in comparison to control. This could be due to the increased time of contact between the mitochondria and rotenone. When melatonin was used in combination with rotenone, significant protection was observed. The level of protection decreases over time and this can be attributable to the higher vulnerability of the mitochondria to ROT due to increased time of incubation.

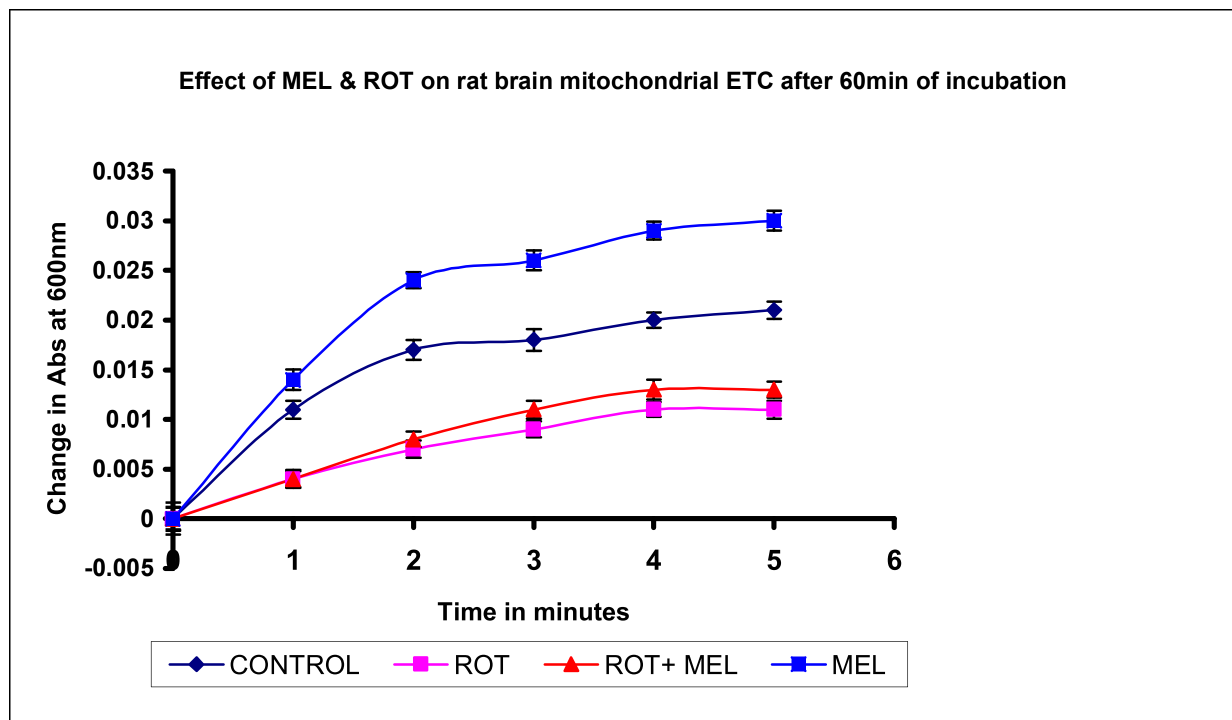


FIG. 3.6 *A comparison of the effect of melatonin (1µM) alone and in presence of rotenone (50µM) to that of rotenone alone and control, on the rat brain mitochondrial ETC using L-Malate as the substrate, after 60 minutes of incubation.[Data represents the mean ±SD; n=5].*

Fig. 3.7 shows the ETC activity in liver mitochondria with ROT, with and without melatonin after 60 min of incubation. Here again, ROT decreases the electron transport in comparison to the control. When melatonin was co-treated, a slight rise in ETC can be observed, in comparison to the toxin, which after 2 minutes decreases indicating that the electron transport operates slower when the mitochondria is incubated for 60 min. The extent of protection offered by melatonin appears to be lower than the previous graphs. This however would be expected considering the fact that this experiment is an *in vitro* one, and that the vulnerability of the mitochondria is bound to increase with an increase in time. Also, melatonin alone showed a rise in ETC activity.

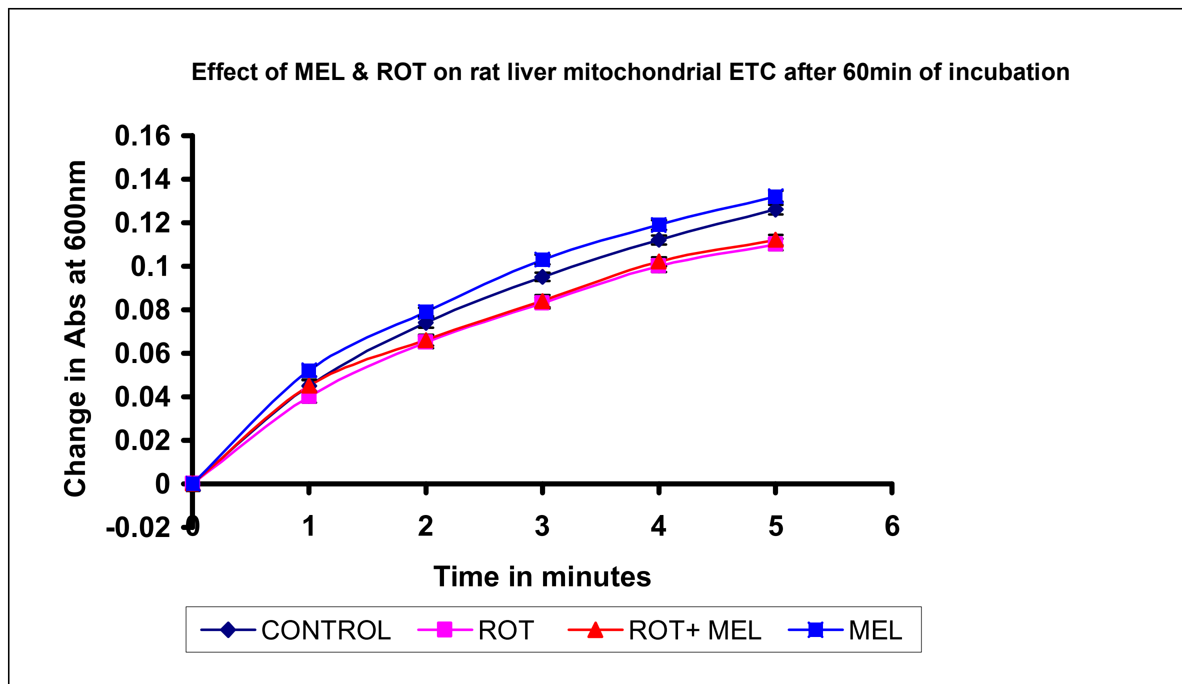


FIG. 3.7 *A comparison of the effect of melatonin (1μM) alone and in presence of rotenone (50μM) to that of rotenone alone and control, on the rat liver mitochondrial ETC using L-Malate as the substrate, after 60 minutes of incubation. [Data represents the mean ±SD; n=5].*

3.2.4 DISCUSSION

Mitochondria largely contribute to the production of ROS, via the respiratory chain. The efficiency of mitochondrial electron transport system decreases with age. Since the brain is dependent on mitochondrial energy supply to maintain normal brain function, any decline in brain ETC activity could have a significant impact on the etiology of neurodegenerative disease.

Cellular energy production, in the form of ATP, begins to fail when the electron transport system is faulty. Impaired electron transport also leads to diversion of electrons from their normal ETC recipients and further formation of damaging free radicals. Inhibition of ATP synthesis as a result of mitochondrial defect would also compromise glutathione production and therefore reduce the antioxidant capacity of the cell. Respiratory failure also results in blockade of nerve impulses.

In the present study, it is evident that rotenone inhibits the mitochondrial respiratory activity in brain and hepatic mitochondria. This is due to the fact that rotenone inhibits the electron flow at the level of Complex I (Heikkila, 1985). In addition to free radical generation, an impairment of mitochondrial respiration and subsequent cellular energy depletion appears to be a likely mechanism for rotenone toxicity.

The results obtained show that melatonin (1µM) is able to prevent the respiratory inhibition induced by 50µM rotenone. This protective effect could be due to melatonin preventing the interaction of rotenone with complex 1. A similar protective effect of melatonin with MPP^{+} has been reported elsewhere (Absi *et al*., 2000). On the other hand, melatonin alone caused an increase in electron transport, suggesting that the indoleamine alone can potentiate the electron transport chain “activity”. Recent reports have shown that melatonin improves the activity of mitochondrial respiratory complexes I and IV, thereby promoting electron flux through the electron transport chain, which in turn results in the rise of ATP production (Martin *et al*; 2002).

A possible mechanism for the stimulatory effect of melatonin on mitochondrial physiology may be due to its ability to increase the respiratory chain complex activities. The potent antioxidant properties of melatonin at the mitochondrial level (Martin *et al*; 2000) also plays a beneficial role in the proficient functioning of the ETC and its coupling with the oxidative phosphorylation. Being extremely lipophilic, melatonin crosses cell membranes thus enabling it to reach the mitochondria, where it stabilizes the inner mitochondrial membranes (Costa *et al*; 1995). Thus the results obtained conclude that melatonin is able to protect against rotenone induced mitochondrial insult. However, further studies are necessary to confirm the protective role of melatonin against rotenone.

CHAPTER FOUR

COMPLEX I ASSAY

4.1 INTRODUCTION

Mitochondria are the source of oxidative phosphorylation (OXPHOS) providing energy in the form of ATP. The mitochondrial inner membrane contains four electron-transporting complexes (I, II, III, and IV) and one H^+-translocating ATP synthetic complex (complex V) that catalyse OXPHOS (Shoffner., 1990). Of the five complexes, NADH:ubiquinone oxidoreductase (complex I) is the largest and the complicated membrane-bound multi-subunit enzyme that serves as the principal starting point of the electron transport chain (ETC). As mentioned earlier this L-shaped complex is composed of 41 subunits, seven of which (ND1, ND2, ND3, ND4, ND4L, ND5 and ND6) are encoded by mitochondrial DNA (mtDNA) (Walker *et al*., 1992).
Complex I initiates the electron transfer by oxidizing NADH and the electrons are transferred to coenzyme Q (ubiquinone) which acts as an electron acceptor (Takahiro., 2002). It catalyzes the following reaction:

$$NADH + Q + H^+ + nH^+_{in} \rightarrow NAD^+ + QH_2 + nH^+_{out} \quad (Q=quinone; n=2\text{-}4) \quad (5)$$

Abnormalities in the respiratory chain complexes have been implicated in a number of diseases such as ischemia/reperfusion, chemical-induced cell injury, neurodegeneration and in the ageing process itself (Schapira *et al*., 1990; Bindoff *et al*., 1990). Of these, complex I has recently taken on particular significance among researchers since the finding that many mitochondrial diseases involve structural and functional defects at the level of this enzyme complex.

Complex I deficiency, either specific or associated with other respiratory chain defects has been identified in a variety of diseases such as myopathies, encephalomyopathies, and in neurodegenerative disorders such as Parkinson's disease (PD), Leber's hereditary optic neuropathy (LHON), Leigh's syndrome and focal dystonia (Schapira.,1998; Robinson., 1998; Morris *et al*.,1996).

Complex I Assay

The discovery that 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP) caused parkinsonism via inhibition of complex I gained attention as to whether complex I defects might play a role in the pathogenesis of typical PD (Langston *et al*., 1983; Nicklas *et al*., 1985). Later it was reported that PD patients express a specific loss of activity in complex I of the ETC (15-30%) in many tissues including brain, muscle, and platelets (Schapira *et al*., 1990; Parker *et al*., 1989., Bindoff *et al*., 1991). LHON is another mitochondrial neurodegenerative disease associated with complex I dysfunction (Brown *et al*., 1992). Mitochondrial DNA mutations in complex I have been identified as being responsible for this genetic disease (Wallace *et al*., 1998).

Complex I deficiency results in the decline of energy production by OXPHOS. In addition, this impairment also leads to enhanced formation of ROS (Turrens *et al*., 1980; Pitkanen *et al*., 1996), which in turn damages DNA, proteins, and lipids (Figure 4.2) thus triggering the onset of various neurodegenerative diseases.

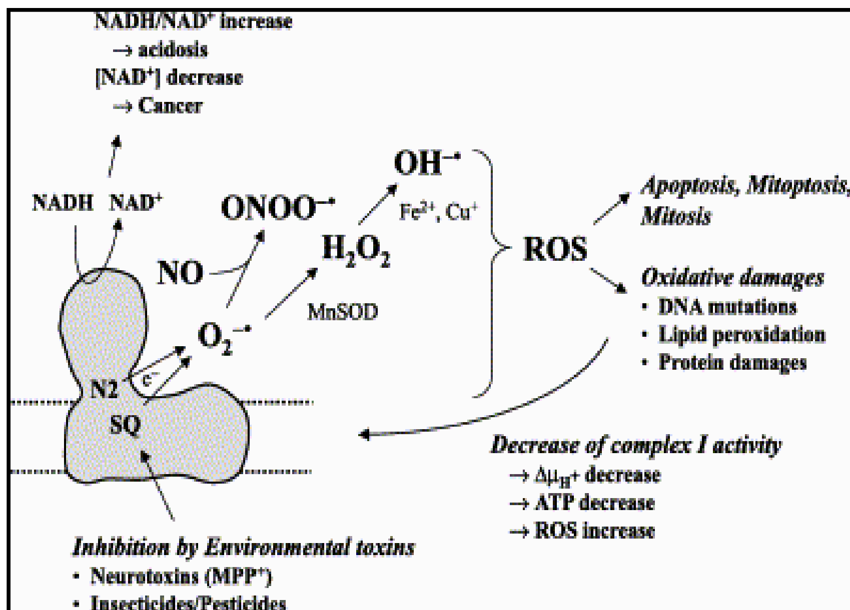


FIG 4.1 *Generation of ROS by complex I and its implicated consequences. O_2^{-} is generated by a single electron reduction of O_2 by ubisemiquinone (SQ)or cluster N2. H_2O_2 and OH^{-} are formed by MnSOD and by Fenton's reaction in the presence of Fe orCu, respectively. NO reacts with O_2^{-} generating $ONOO^{-}$. Changes in [NADH]/[NAD^{+}] ratio and decrease of [NAD^{+}] concentration are suggested to affect several cellular activities (Takahiro, 2002).*

Mitochondrial ROS also triggers programmed cell death (apoptosis). NO also has shown to inhibit complex I activity through peroxynitrite formation, thus suggesting its role in the regulation of this protein complex (Riobo *et al*., 2000).

Long-term exposures to environmental stimuli have shown to reproduce features of PD. This involves various neurotoxins, pesticides, insecticides etc. which bring about their toxic effects by inhibiting the activity of complex I. In order to prevent the oxidative damage in cells with respiratory chain deficiencies, it is important to examine the interventions of neuroprotective agents that modulate mitochondrial function.

4.2 EFFECT OF MELATONIN ON ROTENONE-INDUCED COMPLEX I INHIBITION

4.2.1 INTRODUCTION

The measurement of the activities of individual respiratory chain complexes is an important component of the investigation of diseases due to mitochondrial dysfunction. Previous chapters have shown that rotenone increased superoxide anion formation, followed by ETC inhibition. These effects of rotenone could be attributed to its potent inhibitory action at the level of the mitochondrial complex I enzyme. Hence the present assay was conducted to determine whether melatonin is able to counteract the inhibitory effect of rotenone on the complex I enzyme.

4.2.2 MATERIALS AND METHODS

4.2.2.1 Animals

Adult, male, Wistar rats, weighing between 250 and 300g were used for the experiments, and were housed and maintained under the conditions described in section 2.2.2.1.

4.2.2.2 Chemicals and Reagents

All reagents were of the highest quality available. Rotenone, melatonin, antimycin, CoQ_0, NADH, Tris, were purchased from the Sigma Chemical Corporation, St. Louis, MO, U.S.A. Potassium cyanide and sucrose were purchased from Saarchem, Merck Laboratory Supplies (Pty) Ltd. Gauteng, South Africa. Defatted bovine serum albumin was purchased from Boehringer Mannheim, Germany.

4.2.2.3 Isolation of Mitochondrial P_2 fraction from rat brain

Mitochondrial P_2 fraction from rat brain was prepared by differential centrifugation as described by Mitra (Mitra *et. al*., 1994). Rats were sacrificed by cervical dislocation and the brain rapidly removed, placed on dry ice and processed immediately. The brain was homogenized in ice-cold 0.32M sucrose dissolved in 10mM potassium phosphate buffer, pH 7.2 in a manual glass-teflon homogenizer on ice to yield a 10%w/v homogenate. The homogenate was centrifuged at 1500×g for 10minutes at 4ºC to remove cell debris and nuclei, and mitochondria were separated from the supernatant by centrifugation at 10000×g for 30minutes at 4ºC. The pellet was then resuspended in ice-cold 50 mM Tris in10mM potassium phosphate buffer, pH 7.2 (1:1v/v) and centrifuged again at 10000×g for 30minutes at 4ºC. The supernatant obtained was discarded and the pellet was resuspended in cold 10mM potassium phosphate buffer, pH 7.2. The suspension was sonicated until the pellet was uniformly dispersed to ensure that the mitochondrial lysis was complete. The submitochondrial particles thus prepared were used for estimating the complex I activity.

4.22.2.4 Complex I (NADH:ubiquinone oxidoreductase) Assay

The NADH:ubiquinone reductase activity of complex I catalyses the oxidation of NADH + H^+ to NAD^+, with the ultimate reduction of ubiquinone to ubiquinol.

Complex I activity was assayed as NADH:ubiquinone oxidoreductase according to Shults *et al*, 1995 with minor modifications (Shults *et. al*., 1995). The buffer utilized for complex I was 10mM potassium phosphate pH 7.2, 5mM KCN, and 5mM $MgCl_2$, supplemented with 2.5mg/ml defatted bovine serum albumin (BSA) and 2µg/ml antimycin. An aliquot of the submitochondrial suspension (approx. 30-50µg) and coenzyme Q_0 (an analogue of ubiquinone, 55µM final concentration) were added to the assay buffer. The total assay volume was maintained at 1ml. The reaction was carried out in a 1ml quartz semi-microcuvette, and reagents were pre-warmed for 2minutes at 30ºC. After pre-incubation, the reaction was initiated by the addition of NADH (0.12mM final concentration) and the rate of decrease in absorbance was monitored spectrophotometrically at 340nm for 2minutes. The change in absorbance without NADH was deducted from the change in absorbance with NADH. The above assay was also performed in the presence of rotenone (2µM and 5µM) alone and in combination with melatonin (10µM). The complex I activity was expressed as nanomoles of NADH oxidized/min/mg protein.

4.2.2.5 Protein Determination

The protein concentration of the mitochondrial suspension was determined using the method described by Lowry et al., 1951. A standard curve was generated using BSA as a standard at concentration intervals of 60µg/ml (Appendix 2).

4.2.2.6 Statistical Analysis

The results were analysed using a one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls Multiple Range Test. The level of significance was accepted at $p<0.05$ (Zar, 1974).

4.2.3 RESULTS

Sub-mitochondrial fractions obtained from rat brain incubated with rotenone showed a significant decrease in the complex I activity (Fig.4.2). Rotenone at a concentration of 2µM inhibited the enzyme activity by 50% in comparison to the control whereas 5µM concentration of rotenone further decreased the activity by more than 80%.

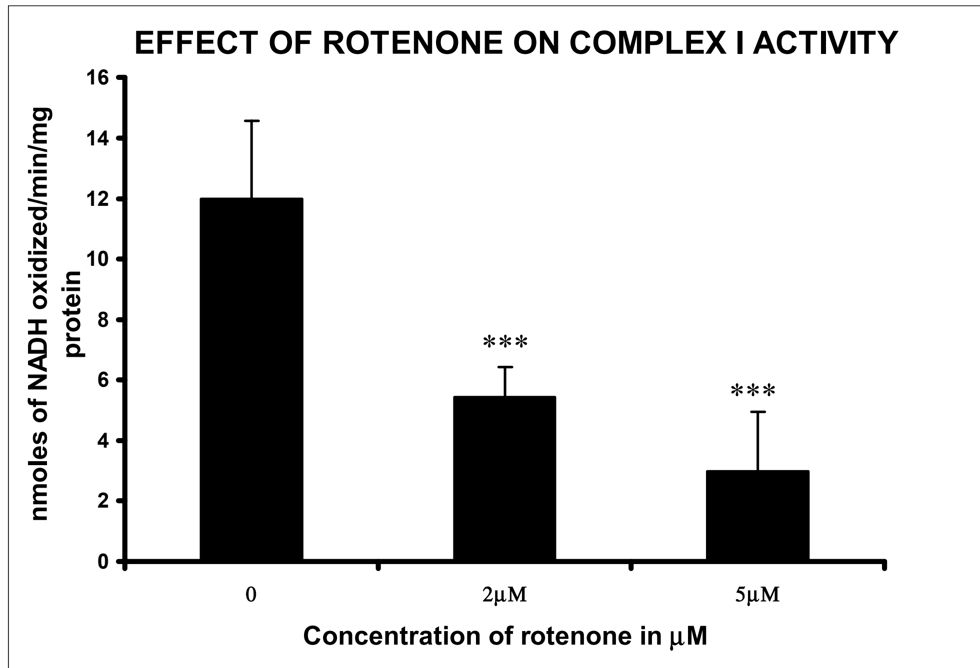


FIG 4.2 *The effect of rotenone on mitochondrial complex I activity in rat brain. Each bar represents the mean ± SD, n=5; (*** p<0.001 in comparison to control). Student-Newman-Keuls Multiple Range Test.*

Fig.4.3 shows the effect of melatonin on ROT-induced complex I inhibition. It can be observed that co-treatment of melatonin with the sub-mitochondrial particles counteracts the toxic effects of rotenone. When melatonin (10µM) was co-treated with rotenone, the inhibitory effect was significantly less as compared to rotenone alone. Melatonin at a concentration of 10µM significantly increased the activity of complex I in comparison to control.

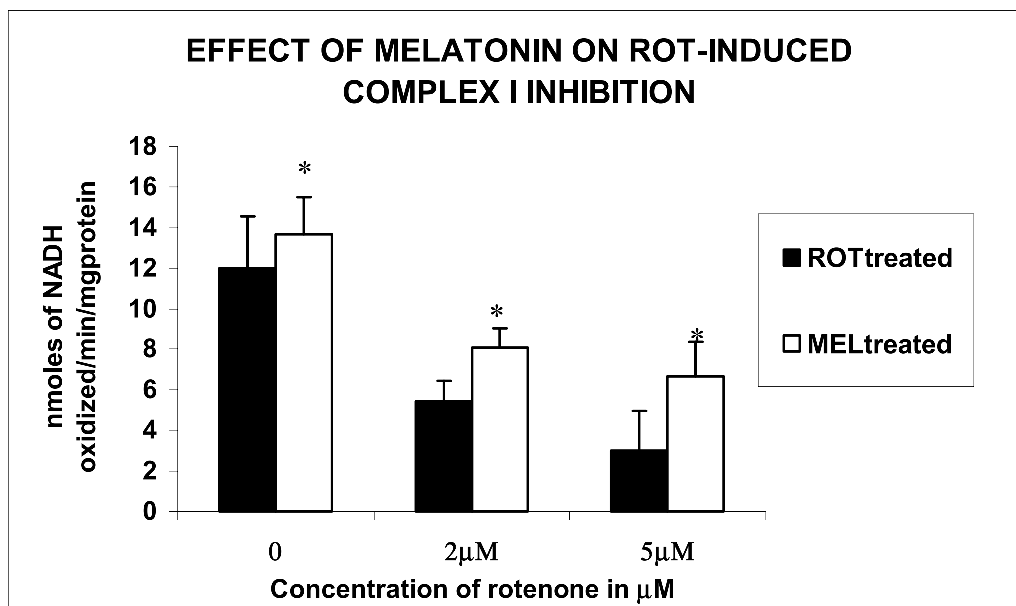


FIG 4.3 *The effect of melatonin (10 M) on rotenone induced mitochondrial complex I inhibition in rat brain. Each bar represents the mean ± SD, n=5; (* p<0.05 in comparison to rotenone treated brain. Student-Newman-Keuls Multiple Range Test*

4.2.2 DISCUSSION

Defects in mitochondrial energy metabolism and oxidative stress have been considered to underlie the pathology of a number of devastating neurodegenerative diseases. Complex I

deficiency has particularly been documented to be associated with several mitochondrial and age-related disorders.

The present study shows that exposure to low concentrations (2 & 5μM) of rotenone results in a drastic decrease in the activity of complex I. It blocks the oxidation of NADH to ubiquinone by binding to complex I. Inhibition of the enzymatic activity of NADH-dehydrogenase by rotenone produces a fall in the intracellular levels of NAD^+ which in turn impairs the normal functioning of the mitochondrial electron transport and subsequent ATP production. Such energetically compromised mitochondria may have detrimental effects on the survival of the cell. Inhibition of complex I by rotenone not only impairs the energy production of OXPHOS but also induces the production of ROS and consequent oxidative damage (Cleeter *et al*., 1992). A defect in complex I enzyme might lead to a defect in proton pumping and decrease in the mitochondrial membrane potential, which results in predisposition of neurons to excitotoxicity and increased risk for apoptosis.

Recent studies have shown evidence that environmental factors, oxidative stress and mitochondrial dysfunction play an important role in the pathogenesis of PD. Chronic exposure to rotenone has shown to produce features of PD indicating that a systemic partial defect in complex I is sufficient to reproduce the behavioural, neurochemical and neuropathological features of PD (Betarbet *et al*, 2000). Schapira *et al*, (1990) also reported that complex I activity was selectively reduced in the substantia nigra of patients with PD.

The results of the present study demonstrate a protective effect of melatonin in counteracting the inhibitory action of rotenone. Melatonin at a concentration of 10μM was able to restore the activity of complex I in comparison to rotenone. An increase in complex I activity was observed when the indoleamine was used alone.

This protective effect of melatonin could be attributed to a specific interaction between melatonin and complex I, thus preventing the binding of rotenone with the enzyme. The antioxidant action exerted by melatonin at the mitochondrial level (Martin et al., 2000) also makes it beneficial in curtailing the oxidative damage induced by rotenone. Previous studies

have shown that melatonin increases the activities of the mitochondrial complexes I and IV of the ETC (Martin *et al*, 2002). Besides, the high redox potential of melatonin (0.94 V) enables it to interact with the respiratory chain complexes, thus improving the electron flow (Martin *et al*, 2002). Absi *et al* (2000) reported that melatonin is able to protect against the parkinsonian neurotoxin, MPP^+ induced complex I inhibition. Furthermore, melatonin was shown to protect against loss of complex I activity in nigral tissue from 6-hydroxydopamine-lesioned rats (Dabbeni-Sala *et al*., 2001). Also, melatonin has been shown to counter the toxic effects of ruthenium red and cyanide at the mitochondrial level and increase the mitochondrial GSH (glutathione) content (Martin *et* al., 2000, Yamamoto and Tang, 1996). Moreover, melatonin easily crosses cell membranes reaching subcellular compartments including mitochondria where it may reach high concentrations, thus rendering it an important target for melatonin action (Martin *et al*., 2000).

In conclusion, the antioxidant role of melatonin and its ability to stimulate the activity of complex I enables it to counteract the toxic effects of rotenone and thereby modulate the mitochondrial metabolism. The data obtained from the previous chapter also supports the current results. These results suggest that melatonin might protect against rotenone-induced mitochondrial dysfunction and oxidative damage.

CHAPTER FIVE

LIPID PEROXIDATION

5.1 INTRODUCTION

Biological membranes function as compartmentalizing structures, and are essential for cell functioning. The membranes are composed of proteins, carbohydrates and lipids, of which the most abundant is amphipathic phospholipids (Campbell, 1996). The CNS is a tissue consisting substantially of membranes and fatty acids (Halliwell and Gutteridge, 1989; Halliwell, 1992). The high content of polyunsaturated fatty acids (PUFAs) in the mitochondrial and plasma membrane of brain cells, and the increasing ratio of PUFAs to monounsaturated fatty acids with aging, renders these structures highly susceptible to oxidation (Yu *et al*., 1992). This oxidative destruction of polyunsaturated fatty acids is termed lipid peroxidation, and is known to be extremely damaging because of the self–perpetuating chain reactions they cause (Reiter, *et al.,* 1996). Increased lipid peroxidation is generally believed to be an important underlying cause of the initiation of oxidative stress related tissue injury, cell death and further progression of many acute and chronic diseases.

Enhanced oxidative stress has been implicated in the toxicity associated with several neurodegenerative disorders (Halliwell, 1992). As mentioned earlier, the central nervous system (CNS) is particularly vulnerable to oxidative damage because of the high levels of polyunsaturated lipids in neuronal cell membranes and poor antioxidant defence (Coyle and Puttfarcken, 1993). In addition, a high metabolic rate and an abundant supply of transition metals, make the brain an ideal target for free radical attack. During pathological insults such as trauma, ischemia, and neurodegenerative diseases, there will be an increase in the cellular load of free radicals, resulting in increased lipid peroxidation and loss of cellular compartmentalization. Many mitochondrial functions are linked directly to the structural integrity of its membranes, which is dependent primarily on the interaction of lipids and proteins within the membranes. Oxidative damage to membranes due to mitochondrial production of ROS results in a well-

established loss of membrane fluidity with aging, which parallels the increase in mitochondrial lipid peroxides and further loss of activity (Hagen, *et al.,* 1997).

The oxidative insult to brain cell membranes results in the peroxidation of phospholipids and their breakdown into highly reactive carbonyl fragments, most importantly malondialdehyde (MDA) and 4-hydroxyalkenals (4-HDA) (Esterbauer and Cheeseman, 1990). MDA and 4-HDA are degraded lipid products in cell membranes, and are taken as reliable indicators of oxidative stress (Reiter *et. al.,* 1995). The thiobarbituric acid (TBA) assay was used to determine the extent of lipid peroxidation. The principle of the experiment involves the reaction of one molecule of MDA with two molecules of TBA to yield a pink complex that has an absorption maximum at 532nm (Figure 5.1). The reaction takes place in acidic conditions (pH of 2 – 3) at 90 to 100°C for one hour. Trichloroacetic acid (25% w/v) is required for the formation of the complex and the release of protein-bound MDA. Thus, the measurement of the concentrations of MDA provides a convenient method for estimating oxidative damage to cell membranes.

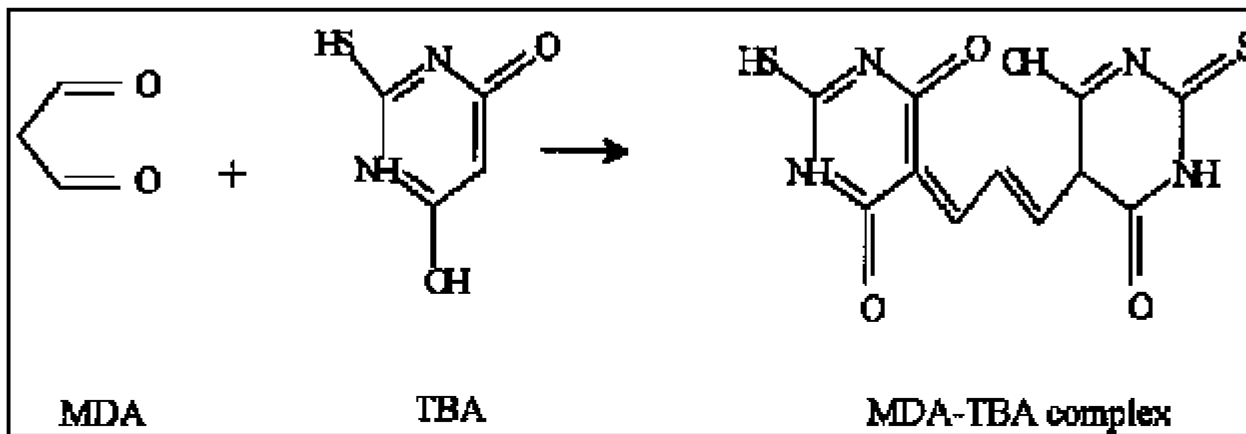


FIG 5.1 *The reaction of MDA with TBA to yield a pink TBA-MDA complex (Mead et al, 1986).*

5.2 EFFECT OF MELATONIN ON ROTENONE-INDUCED LIPID PEROXIDATION IN RAT BRAIN HOMOGENATE

5.2.1 INTRODUCTION

As membranes are vital for proper cell function, any damage to membranes could be toxic to neurons. Under certain abnormal conditions, the components of the electron transport chain can initiate free radical-mediated peroxidation of the mitochondrial lipid membrane system (Vladimirov *et al.,* 1980). Previous chapters have shown that inhibiton of the ETC at the complex I level by rotenone enhances free radical production.

The following experiment was aimed at investigating the potential protective effect of melatonin on rat brain lipid peroxidation induced by the pesticide, rotenone.

5.2.2 MATERIALS AND METHODS

5.2.2.1 Animals

Adult, male, Wistar rats, weighing between 250 and 300g were used for the experiments, and were housed and maintained under the conditions described in section 2.2.2.1.

5.2.2.2 Chemicals and Reagents

1, 1, 3, 3-tetramethoxypropane (98%) was obtained from Fluka AG, Switzerland. 2-thiobarbituric acid (TBA), butylated hydroxytoluene (BHT), rotenone and melatonin were purchased from Sigma Chemical Co, St. Louis, USA. Trichloroacetic acid and butanol were purchased from Saarchem (PTY) Ltd. Krugersdorp, South Africa.

5.2.2.3 Preparation of the Brain Homogenate

The rats were sacrificed by cervical dislocation, and the brains were removed and homogenised in the same manner described in section 2.2.2.4.

5.2.2.4 Preparation of Standard Curve

1, 1, 3, 3-Tetramethoxypropane was used as a standard. A series of reaction tubes each containing appropriate aliquots of water and standard solution were prepared with Milli-Q water to a final volume of 1ml. A calibration curve was generated by measuring the absorbance at 5nmoles/ml intervals. The absorbance was read at 532nm using a GBC UV/VIS 916 spectrophotometer and plotted against the molar equivalent weight of MDA in the complex assayed (Appendix 3).

5.2.2.5 Lipid Peroxidation Assay

Lipid peroxidation was determined using the thiobarbituric acid (TBA) assay. A modified method of Ottino and Duncan (1997) was used in the following set of experiments. This assay was carried out in 2 sections which involves the:

(3) *In vitro* exposure to melatonin

(4) *In vivo* administration of melatonin

5.2.2.5.1 *In vitro* Exposure to Melatonin

Melatonin was dissolved in absolute ethanol and subsequently diluted with Milli-Q water (the final ethanol concentration in the brain homogenate was 0.5%). Rotenone was first dissolved in absolute ethanol and then diluted with water in a ratio of 50:50.

The experiments were conducted in which the rat brain homogenate (1 ml) containing varying concentrations of rotenone (0, 30, 40 or 50μM) alone and in combination with melatonin (0, 30, 40 or 50μM) was incubated in an oscillating water bath for 1hour at 37°C. At the end of the incubation 0.5ml BHT (0.5g/l in absolute ethanol) and 1ml 25% TCA were added to the mixture. The samples were heated at 95°C for 10 min and then centrifuged at 2000g for 20 minutes at 4°C to remove insoluble proteins. Following centrifugation, 2ml of protein free supernatant was removed from each tube and a 0.5ml aliquot of 0.33% TBA was added to this fraction. All tubes were heated for an hour at 95°C in a water bath. After rapidly cooling the tubes on ice, 2ml of butanol was added and the tubes vortexed and centrifuged at 2000g for 10 minutes. A 2ml aliquot of the top layer of the mixture was carefully removed with an auto pipette and placed into separate tubes. This fraction contained the extracted TBA-MDA complexes, which were then read at 532nm. MDA levels were determined from a standard curve generated from 1, 1, 3, 3-tetramethoxypropane.

5.2.2.5.2 *In vivo* Administration of Melatonin

Melatonin was dissolved in ethyl oleate for the purpose of *in vivo* administration to the rat. The rats were divided into two groups each containing five rats. One set of rats was the test group and were injected intraperitoneally with 5mg/kg of melatonin twice daily. The other set was injected with ethyl oleate at the same time and for the same duration as the test rats. The animals were injected for five consecutive days and on the fifth day, were sacrificed and brains removed as described in section 2.2.2.3. The brain homogenate was allowed to incubate with different concentrations of rotenone (10, 20 and 30μM) at 37^0C for one hour, after which the lipid peroxidation assay was carried out as described in section 5.2.2.5.1.

5.2.3 RESULTS

The final results were expressed as nanomoles of MDA produced per mg tissue. Statistical analysis was carried out using ANOVA followed by the Student Newman Keuls multiple range

test. The level of significance was accepted at $p<0.05$. The data represented for these experiments is the mean ± SD of five determinations (n = 5).

The *in vitro* exposure of brain homogenate to increasing concentrations of rotenone (30-50 µM) caused a significant increase in lipid peroxidation when compared to the control (Figure 5.2).

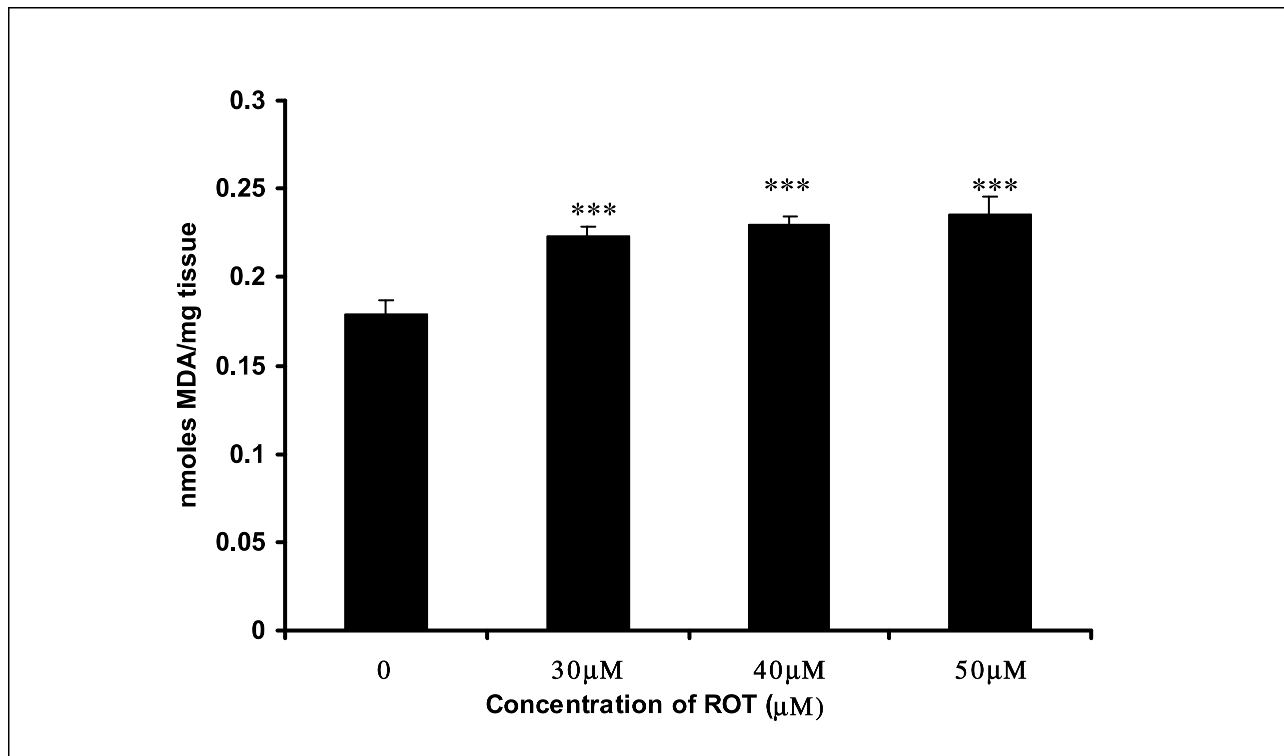


FIG 5.2 *Concentration-dependent effect of ROT on lipid peroxidation in whole rat brain homogenate. Each bar represents the mean ± SD, n=5; (***p<0.001 in comparison to control). Student-Newman-Keuls Multiple Range Test.*

The co-treatment of the homogenate with increasing concentrations of melatonin showed a significant reduction in lipid peroxidation by 50μM ROT (Figure 5.3).

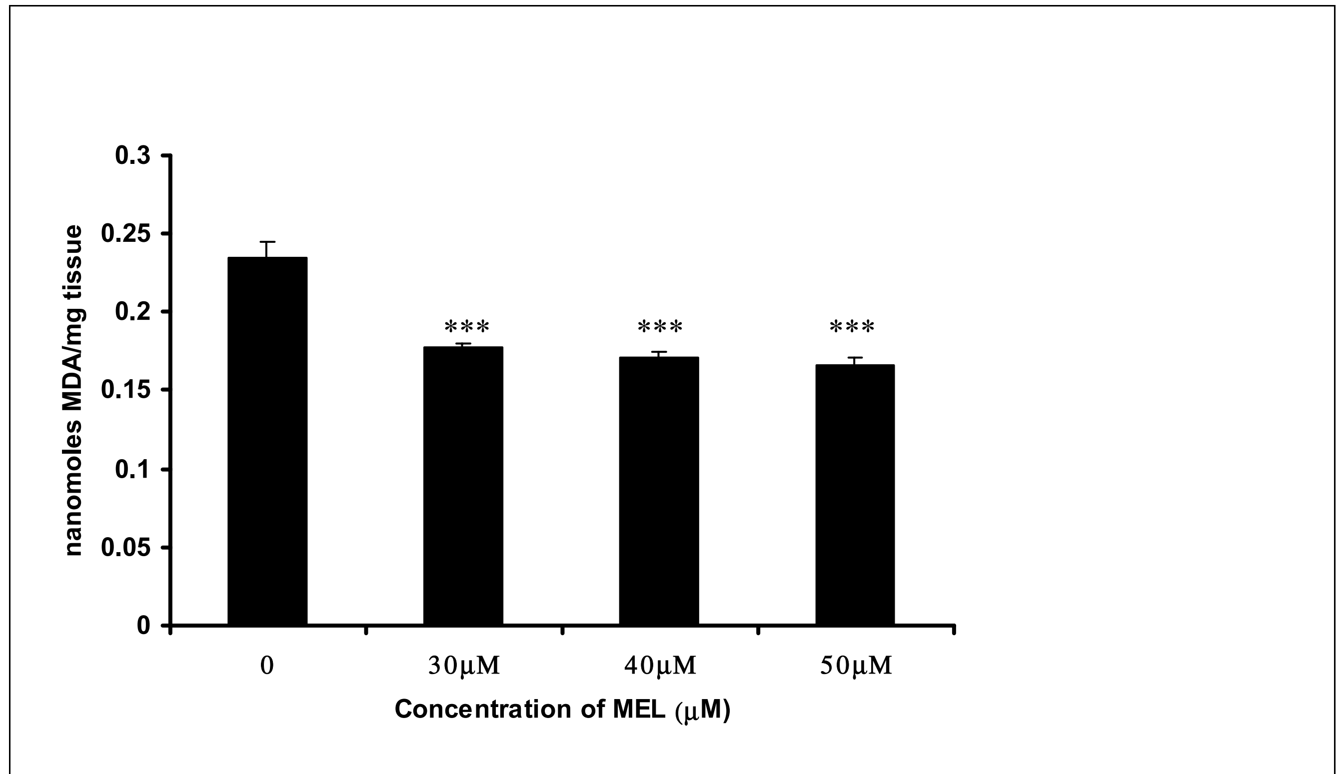


FIG 5.3 *The effect of melatonin (in vitro administration) on ROT (50μM)-induced lipid peroxidation in whole rat brain homogenate. Each bar represents the mean ±SD, n=5; (***p<0.001) Student-Newman-Keuls Multiple Range Test.*

Brain homogenate obtained from rats that were pre-treated with melatonin 5mg/kg i.p for five consecutive days showed significant reduction in MDA produced by increasing concentrations of rotenone (Figure 5.4). Melatonin administered at this dose also showed a significant inhibition of lipid peroxidation produced by the control in absence of rotenone.

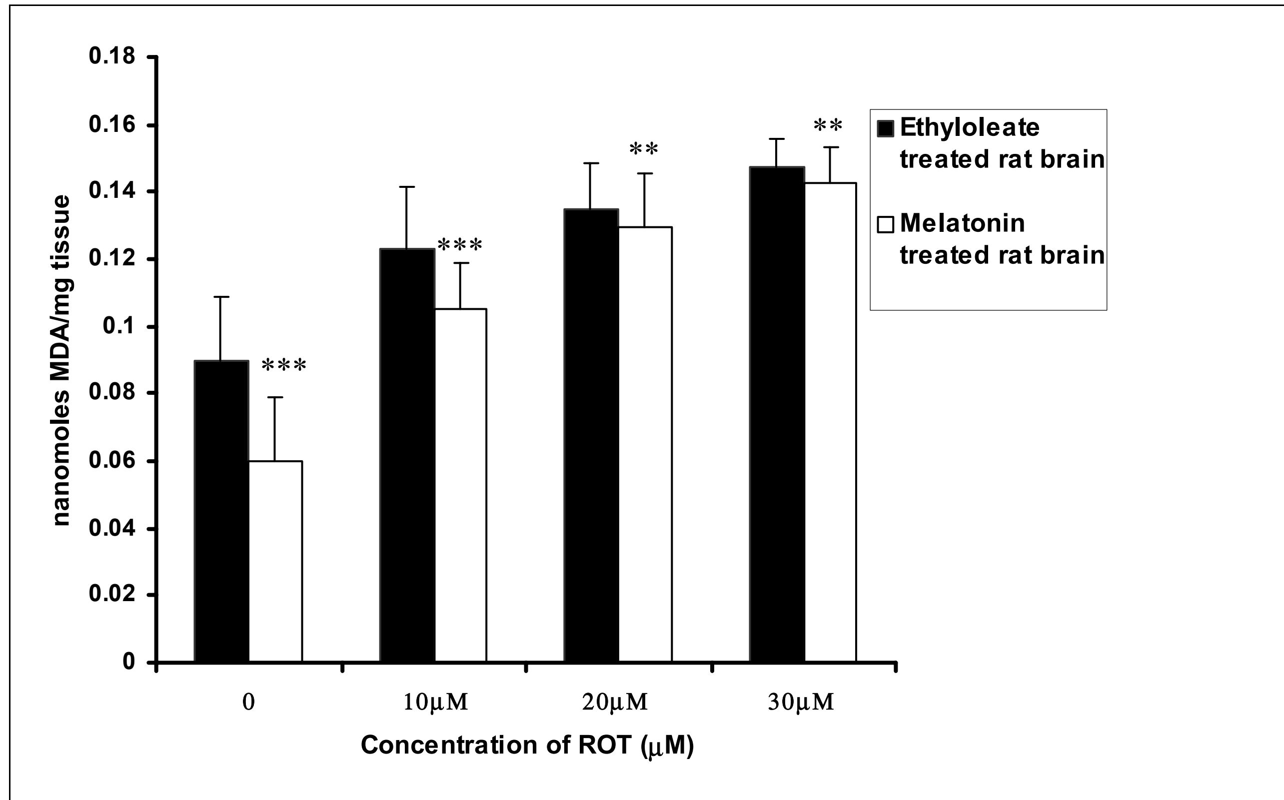


FIG 5.4 *The effect of increasing concentrations of ROT on MDA levels in rat brain homogenate obtained from rats pretreated with 5mg/kg melatonin i.p. for 5 consecutive days. Each bar represents the mean ±SD; n=5. (**p<0.01; ***p<0.001) Student-Newman-Keuls Multiple Range Test.*

5.2.4 DISCUSSION

There is considerable interest in the role played by lipid peroxidation, and other free radical reactions, in human and animal disease, as well as in toxicology (Gutteridge and Halliwell, 1990). Lipid peroxidation, which is not only initiated but also propagated by free radicals, is devastating to the morphologic and functional integrity of cells and is often a late event, accompanying cell death (Kappus, 1987).

The present study shows that rotenone is capable of initiating lipid peroxidation, even in micromolar concentrations. Numerous studies have shown that the mitochondrial complex I is important in the initiation of oxidative stress and lipid peroxidation that leads to toxic cell injury (Zhang *et al.*, 2001). Rotenone enhances the mitochondrial production of ROS and its ability to induce lipid peroxidation can be well attributed to the increase in pro-oxidants generated at complex I (Zhang *et al*, 2001; Nianyu *et al*, 2003). Mitochondrial membrane lipid peroxidation results in irreversible loss of mitochondrial function such as mitochondrial respiration, oxidative phosphorylation and ion transport (Vladimirov *et al.*, 1980). Thus, rotenone is capable of inhibiting mitochondrial functions, thereby causing acute cell death via a lipid peroxidation pathway (Zhang *et al.*, 2001).

Recently, rotenone has gained much interest as a Parkinson inducing agent and oxidative stress related changes have been detected in brains of PD patients (Jenner, 1998). Indices of lipid peroxidation have been found to be elevated in the substantia nigra in PD (Dexter *et al.*, 1989; Hirsch, 1993). 4-Hydroxynonenal (HNE), one of the important products of lipid peroxidation, is reported to be present in Lewy bodies in Parkinson's disease (Kamelija, 2003). In the current experiment rotenone increased lipid peroxidation and thus the presence of rotenone can accelerate many of the pathological symptoms of PD.

Tissue disruption by homogenisation tends to undergo lipid peroxidation more easily than healthy, intact tissue (Halliwell and Gutteridge, 1989). This explains the formation of MDA obtained in the absence of ROT.

The results obtained demonstrate that melatonin, administered both *in vivo* and *in vitro* to the rat brain, is capable of decreasing lipid peroxidation induced by rotenone. Also melatonin was able to reduce the MDA formation that occurred in control samples. The protective effect of melatonin against ROT-induced lipid peroxidation could be due to melatonin preventing the interaction of rotenone with complex I. This was observed in the previous chapters and such an interaction would limit the formation of pro-oxidants by rotenone at the complex I level.

Melatonin is a well-known free radical scavenger and antioxidant and much of its protective effects could also be attributed to these properties.

Melatonin's efficacy as a free radical scavenger depends on the acetyl group on its side chain and the methoxy group at position 5 of the indole nucleus (Tan *et al*, 1993). It is a potent hydroxyl and peroxyl radical scavenger (Tan *et al.*, 1993; Pieri *et al.*, 1994). Melatonin detoxifies the hydroxyl radical (·OH) by electron donation and becomes a radical, which is less reactive and less toxic (Hardeland *et al.*, 1995) (Figure 5.5).

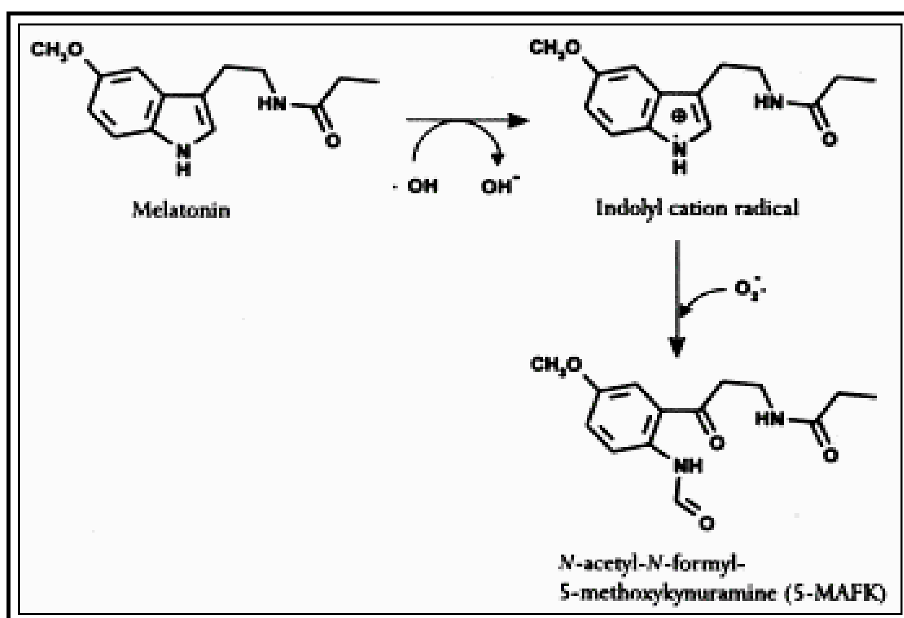


FIG 5.5 *Melatonin scavenges the hydroxyl radical (·OH) via electron donation, thereby neutralizing the ·OH and generating non-toxic nitrogen-centered radical, the indolyl cation radical. It is then believed to scavenge the superoxide anion radical O_2^- to produce 5-MAFK, which is excreted in the urine (Reiter, 1998).*

In addition to these scavenging effects, melatonin also inhibits nitric oxide synthase and stimulates glutathione peroxidase and glutathione reductase (Pozo *et al.,*; Barlow-Walden *et al.,* 1995). Melatonin is a highly effective lipid antioxidant both *in vitro* as well as *in vivo*. The pineal

hormone has been shown to protect against lipid peroxidation induced by the herbicide, paraquat and kainic acid (Melchiorri *et al*., 1994; Melchiorri *et al*., 1995). Also it effectively combats the DNA damage induced by the carcinogen safrole and protects against lipid peroxidation induced by cyanide, H_2O_2 and bacterial lipopolysaccharide (Tan *et al*., 1993; Sewerynek *et al*., 1995).

One of the mechanisms by which melatonin protects against lipid peroxidation is by its ability to stabilize cellular membranes and thereby permit membranes to resist oxidative damage (Garcia *et al,* 1997). Considering its varied and potent antioxidant capability, melatonin is regarded as an essential element of the antioxidant defence system of organisms.

5.3 EFFECT OF MELATONIN ON ROTENONE AND IRON (Fe^{2+}) INDUCED LIPID PEROXIDATION IN RAT BRAIN HOMOGENATE

5.3.1 INTRODUCTION

Free radical generation in the brain is assisted by the presence of large amounts of iron, required by the Fenton reaction (Halliwell, 1992). Free iron or chelates of iron are involved in radical reactions at a number of different levels (Braughler and Hall, 1989). The auto-oxidation of Fe^{2+} results in the formation of O_2- (Braughler and Hall, 1989). Furthermore, Fe^{2+} is also oxidised in the presence of H_2O_2 (Fenton's reagent) to form $^{\cdot}OH$ and a ferryl ion (Fe^{3+}- OH). Both these are very potent oxidants and will react with a wide range of biological substrates such as lipids, DNA, and protein (Dawson and Dawson, 1996; Ottino and Duncan, 1997).

Multiple lines of evidence implicate redox-active transition metals, such as iron and copper, as mediators of oxidative stress and ROS production in neurodegenerative diseases (Sayre *et al.*, 1999; Thompson *et al.*, 2001). The finding of excess iron, decreased glutathione (GSH) and widespread oxidative damage in the substantia nigra of PD patients suggests that oxidative stress plays an important part in the etiology of PD. Iron plays an important role in Parkinson's disease. It has been shown that the iron concentration is significantly increased (around 77%) in the substantia nigra of the brains of PD patients (He *et al.*, 1996). The reason for this localised increase in iron is unknown but it is hypothesised that iron may increase oxidative load because of its ability to stimulate free radical production (Jellinger *et al.*, 1992).

It has been shown that lipid peroxidation in rat brain homogenates increases substantially after addition of ferrous ions with ascorbate, and the observed increase is inhibited by iron chelators, such as desferoxamine (Zaleska and Floyd, 1985). Rotenone was shown to induce lipid peroxidation alone. Thus the following experiment was conducted to determine the effect of rotenone on lipid peroxidation in the presence of iron as well as the role of melatonin on the combined insult induced by ROT and Fe^{2+}.

5.3.2 MATERIALS AND METHODS

5.3.2.1 Animals

Adult male, Wistar rats, weighing between 250 and 300g were used for the experiments, and were housed and maintained under the conditions described in section 2.2.2.1.

5.3.2.2 Chemicals and Reagents

Ferrous sulphate was purchased from BDH Laboratory Supplies UK. Ferrous sulphate was dissolved in deaerated Milli-Q water to give a final concentration of 0.5 mM in the brain homogenate. Ascorbic acid was obtained from Saarchem (PTY) LTD, Krugersdrop and EDTA from Holpro Analytics (PTY) LTD, Johannesburg. All reagents were prepared in deaerated Milli-Q water.

5.3.2.3 Preparation of the Brain Homogenate

Rat brain homogenate was prepared as explained in section 2.2.2.4.

5.3.2.4 Lipid Peroxidation Assay

Aliquots of 1.5 ml rat brain homogenate containing 1 mM ascorbate, 100 µM EDTA, 0.5 mM ferrous sulphate, H_2O_2 and rotenone (0.1 mM and 0.5 mM concentrations respectively) alone or in combination with varying concentrations of melatonin (0.1 mM and 0.5 mM concentrations respectively) were incubated in an oscillating water bath for 1hour at 37°C after which the lipid peroxidation assay was carried out as described in section 5.2.2.5.1.

5.3.2 RESULTS

Figure 5.6 shows the combined effect of rotenone and iron (II) on lipid peroxidation in comparison to control and rotenone alone. A significant increase in lipid peroxidation was observed with 0.1mM and 0.5mM ROT in combination with 0.5mM Fe^{2+} respectively. Rotenone

alone, at concentrations of 0.1mM and 0.5mM also showed a significant rise in lipid peroxidation in comparison to control. The presence of ascorbate in the incubation mixture explains the decreased lipid peroxidation obtained with the control brain.

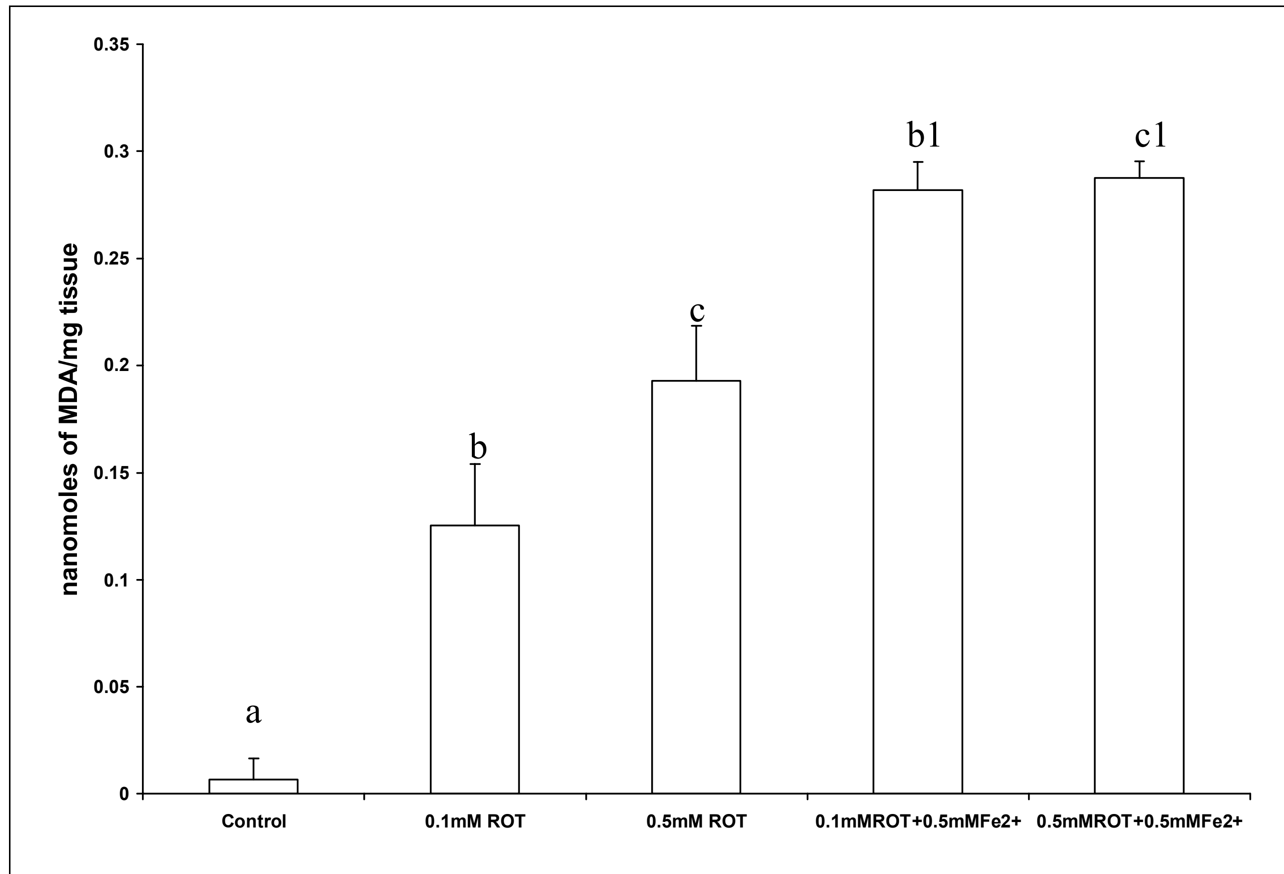


FIG. 5.6 *A comparison of the effect of rotenone (0.1mM and 0.5mM) alone and in presence of iron II (0.5mM) to that of control on lipid peroxidation in whole rat brain homogenate. Each bar represents the mean ±SD; n=3. a vs. b and c (p<0.001); b vs. b1and c vs. c1(p<0.001) Student-Newman-Keuls Multiple Range Test.*

Co-treatment of the brain homogenate (containing ROT and Fe^{2+}) with melatonin showed a decrease in MDA production in comparison to rotenone and iron combination (Fig. 5.7). Melatonin at concentrations of 0.1mM and 0.5mM was able to significantly reduce the lipid

peroxidation induced by 0.1mM ROT + 0.5mM Fe^{2+} and 0.5mM ROT + 0.5mM Fe^{2+} respectively.

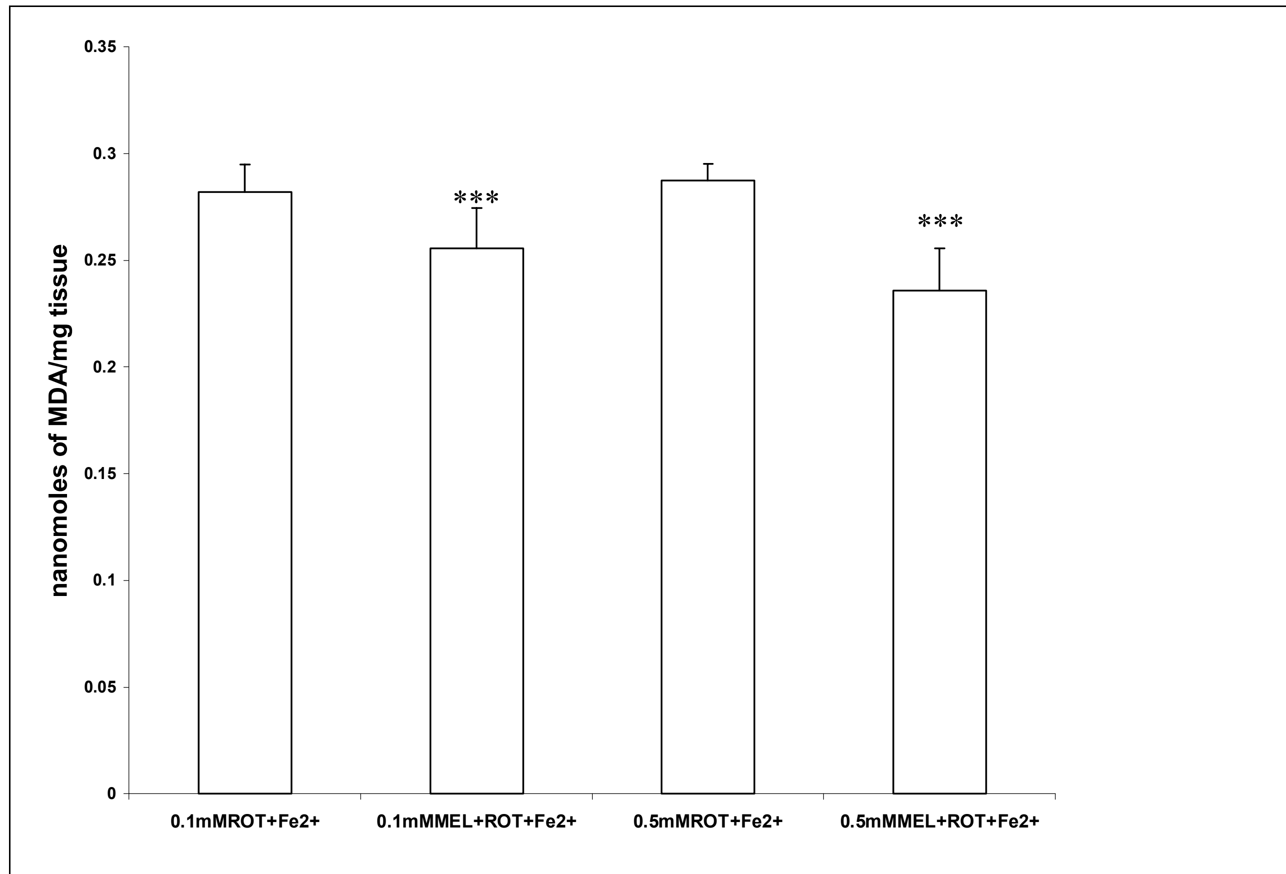


FIG. 5.7 *The effect of melatonin (0.1mM and 0.5mM respectively) on rotenone and iron (II) (combination) induced lipid peroxidation in whole rat brain homogenate. Each bar represents the mean ±SD; n=3. (***p<0.001) Student-Newman-Keuls Multiple Range Test.*

5.3.4 DISCUSSION

Most brain iron is stored in an inactive form encapsulated by intracellular ferritin, (Linert and Jameson, 2000) so the increased amounts of iron in the substantia nigra in Parkinsonism might be a sign of free, redox-active iron, which is able to induce oxidative stress. The iron (II) ion is well known to produce oxygen radicals *in situ* via Fenton's reaction, (Halliwell, 1992) which may in turn result in deterioration of the nigrostriatal dopaminergic system. Even though increased iron is not the primary cause of the acute dopamine cell death in the parkinsonian models of rat and monkey, it could still be an important factor in the cascade of events leading to oxidative stress responsible for the chronic nigral neurodegeneration in parkinsonian patients.

The present study demonstrates that rotenone in combination with iron (II) increases lipid peroxidation significantly in comparison to rotenone alone. The increased lipid peroxidation by the combined treatment indicates that rotenone might accelerate iron induced lipid peroxidation. This could be due to some interaction between iron (II) and rotenone. However, the presence of rotenone together with excess iron in the SN might stimulate free radical reactions and may be responsible for the progression of dopaminergic cell death in Parkinson's disease.

The results obtained suggest that melatonin is able to blunt the lipid peroxidation induced by rotenone-iron (II) combination. In addition to its free radical scavenging actions, a possibility of melatonin's protective role might be its direct chelating effect on iron. Melatonin has been reported to bind with several metals, including iron (III), thus attenuating biological Fenton's reaction and further oxidative damage (Limson *et al*., 1998). Melatonin has been reported to suppress iron-induced neurodegeneration in rat brain (Lin and Ho., 2000). In the current experiment, there is a possibility that melatonin might have bound to rotenone-iron thus inactivating the toxicity of the combination. More investigations need to be done on this aspect to confirm such a role of melatonin. However, the presence of melatonin is able to cause a significant reduction in lipid peroxidation indicating that melatonin is a very potent inhibitor of neuronal lipid peroxidation. The possibility of an interaction between melatonin, rotenone and iron has also been investigated in the following chapter.

CHAPTER SIX

ELECTROCHEMICAL ANALYSIS

6.1 INTRODUCTION

Highly reactive and biologically damaging ˙OH can be formed in biological material when suitable transition metal ion catalysts are available (Halliwell and Gutteridge, 1985; Aust *et al*, 1985). The most abundant metal ion likely to catalyse this reaction is iron.

A chemical reaction in which one or more electrons are transferred from one species to another is the basis for electrochemical analysis (Pecsok *et al*, 1968). Electrochemistry has been used with much success in metal-ligand studies (Limson *et al*, 1998) In the present study, two dynamic electroanalytical techniques, cyclic voltammetry and adsorptive stripping voltammetry were employed to determine the interactions between rotenone, iron (Fe^{3+}) and melatonin *in vitro*.

The electrochemical cell used in these studies consists of a vessel with an inlet port to allow the saturation of a solution with an inert gas, such as nitrogen (N_2) or Ar. This is illustrated in figure 6.1(a). A standard cell consists of three electrodes immersed in the electrolyte solution: a) a working electrode (WE), where the analyte is oxidised or reduced b) a reference electrode (RE) and c) a counter electrode (CE). The reference electrode is the standard electrode against which the potential of the working electrode is referenced (Figure 6.1b). The third electrode used is the counter (auxiliary) electrode, which is usually constructed of an inert material such as platinum, and is used to prevent voltage drop across the working and reference electrodes. A potentiostat monitors and controls the WE potential with respect to the RE. Oxygen is electroactive at almost all electrodes, and seriously interferes with voltammetric and electrochemical measurements (Sawyer and Roberts, 1974). The removal of O_2 is therefore a necessary step to prevent interfering currents that are due to the reduction of O_2.

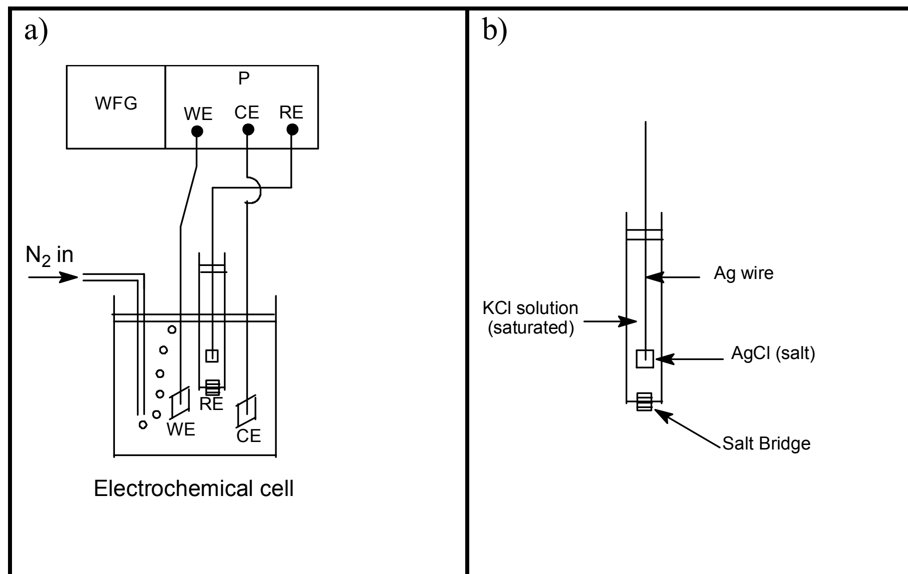


FIG. 6.1 *Schematic representation of a) the basic setup for a cyclic and square wave voltammetric system and b) enlarged schematic of the reference electrode (Ag|AgCl). (WFG = waveform generator, P = potentiostat, WE = working electrode, CE = counter electrode, RE = reference electrode) (Christensen and Hamnett, 1994).*

6.1.1 Cyclic Voltammetry (CV)

One of the techniques used to obtain mechanistic information in redox systems is cyclic voltammetry (CV). During CV, the electrode potential is rapidly scanned in search of redox couples. Once located, a couple can then be characterised from the potentials of peaks on the cyclic voltammogram and from changes caused by variation of scan rate (Kissinger *et al*,. 1996).

In CV, a potential is applied and swept back and forth (where the sweep direction is inverted at a chosen potential) linearly with time. By measuring the resultant current, which is monitored continuously, the subsequent oxidation or reduction of the surface species may then be examined. Invaluable, necessary, information as to the presence of an electroactive species (*i.e.*

the species absorbed onto the surface of the electrode) in solution or at the electrode surface is easily obtained with CV.

Figure 6.2 shows a typical cyclic voltammogram that would be obtained from an electroactive adsorbed species provided that:

a) the electrode has the maximum coverage of an oxidised form (O);
b) the electrochemical reaction can be represented by: $O_{ads} + ne^{-} \leftrightarrow R_{ads}$ and is kinetically fast enough to maintain Nernstian (*i.e.* reversible) behaviour at each point in the CV scan such that the concentrations of O and R near the electrode obey the Nernst equation;
c) the oxidised and reduced species are both strongly adsorbed and have the same enthalpy of adsorption (Christensen and Hamnett, 1994).

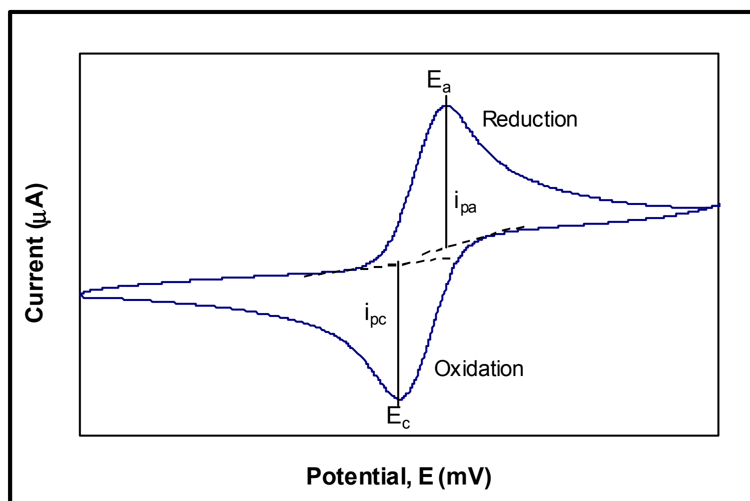
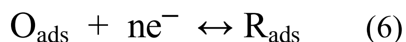


FIG. 6.2 *The current / potential (I / V) curves expected from an electroactive adsorbed species in a reversible redox reaction. E is the potential at which the maxima (E_a) and minima (E_c) in the current are observed (Christensen and Hamnett, 1994)*

6.1.1.1 Reversible Processes

In reversible systems, the species formed in the forward direction is regenerated in the reverse sweep (Figure 6.2), *i.e.*



$$O_{ads} + ne^{-} \leftrightarrow R_{ads} \qquad (6)$$

Information obtained from a reversible system includes (Nyokong, 2002):

i. the $E_{1/2}$, the half-wave potential, which is characteristic of a particular species, *i.e.* where

$$E^{o'} = E_{1/2} = (E_a + E_c) / 2 \qquad (7)$$

$E^{o'}$ is the formal potential, which is corrected to the reference electrode used.

ii. the number of moles of electrons involved in a particular process where:

$$\Delta E = E_a - E_c \qquad (8)$$

$$\Delta E = 2.3\ RT / nF \qquad (9)$$

where:

R = Gas constant

T = Temperature

F = Faraday's constant

n = moles of electrons

iii. stability of a compound (starting compound remains unchanged) and true reversibility of a particular species *i.e.* where

$$i_a / i_c = 1 \qquad (10)$$

i_p is described by the Randles – Sevcik equation:

$$= 2.69 \times 10^5 n^{3/2} A D^{1/2} C V^{1/2} \qquad (11)$$

where:

i_p = peak current (amperes)

A = area of electrode (cm^2)

n = moles of electrons

D = diffusion coefficient (cm^2/s)

C = concentration (mol/cm^3)

V = scan rate (V/s)

CV may thus be used to a) characterise the electroactive system by $E_{1/2}$; b) to determine whether oxidation is occurring; and c) to verify the stability of a complex once oxidation or reduction has taken place.

In an irreversible reaction of the type $O_{ads} + ne^{-} \rightarrow R_{ads}$, the voltammogram shows no inverse peak on reversing the scan direction, *i.e.* the compound is unstable.

6.1.2 Adsorptive Stripping Voltammetry (ASV)

Adsorptive stripping voltammetry exploits the natural tendency of analytes to pre-concentrate at an electrode and is a useful technique for gauging metal-ligand interactions. When a suitable ligand is added to a metal solution, the ligand facilitates movement of the metal to the surface of the electrode. Theoretically then, a ligand which forms a bond with a metal will bring about a change in the current response observed, as well as a shift in potential resulting from the reduction of a new species, the metal-ligand complex at the electrode. The affinity of the ligand toward the metal is expressed by the extent of increase in current response of the metal on addition of the ligand. A lowering in current response indicates possible competition by the ligand for binding sites at the electrode at relatively high analyte concentrations, or formation of strong metal-ligand bonds where the metal is not easily reduced upon scanning. A negative potential shift indicates a strong metal-ligand interaction while a large positive shift is associated with a weaker metal-ligand interaction (Limson *et al.*, 1998).

6.2 MATERIALS AND METHODS

6.2.1 Chemicals and Reagents

Melatonin and rotenone were purchased from Sigma Chemical Corporation, St. Louis, USA. Ferric (Fe^{3+}) solution was prepared from ferric chloride salt. Ferric chloride was purchased from Merck, Midrand, South Africa. Tris buffer was used and was made by dissolving tris amino methane in Milli-Q water and the pH adjusted to 7.4 by adding hydrochloric acid (HCl). Melatonin and ferric chloride was dissolved in the tris buffer. Rotenone was first dissolved in

DMSO and then serially diluted in tris buffer solution for the analysis. Nitrogen was supplied by MG Fed gas and purified by passing through a Drierite self-indicating mesh 8 (anhydrous $CaSO_4$) from SAAR Chemicals.

6.2.2 Apparatus

Voltammetric measurements were done in a conventional three-electrode system. Both CV and ASV data were collected using a CV-50W Bio-Analytical System (BAS) voltammetric analyser (BAS, Lafayette, Indiana, U.S.A.). A 3mm diameter glassy carbon electrode (GCE) was employed as a working electrode for voltammetric experiments. A silver/silver chloride and a platinum wire were employed as reference and auxiliary electrodes, respectively, in all electrochemical work. Prior to use, the GCE was cleaned by polishing with alumina on a Buehler pad, followed by washing in nitric acid and rinsing in water followed by the buffer solution. GCE was cleaned between each scans. The electrolyte buffer solutions were made up prior to data collection and the solutions saturated with nitrogen before running the experiment. All experiments were carried out at ambient temperature.

6.2.3 Technique

6.2.3.1 Cyclic Voltammetry

For cyclic voltammetric experiments, appropriate concentrations of the metal and ligand in buffer were introduced into a glass cell and degassed for 1 min with nitrogen before scanning a potential window. All potential values quoted are referenced against the silver/silver chloride reference electrode.

6.2.3.2 Adsorptive Stripping Voltammetry

For adsorptive stripping experiments appropriate concentrations of the metal and of the ligand were introduced into an electrochemical cell. The solution was then deaerated with nitrogen for 5 minutes, after which a flow of nitrogen was maintained over the solution throughout the measurement.

The potential was swept between +800 and -1200 mV at a scan rate of 100 mV/s and 25 mV/s during all the CV and ASV experiments respectively. The deposition potential was set at +800

mV, while the deposition time was 60 seconds for the adsorptive stripping voltammetric experiments.

6.3 RESULTS

6.3.1 Voltammetric characterisation of Melatonin, Rotenone and Fe^{3+}

Figure 6.3 and 6.4 shows the cyclic and adsorptive stripping voltammogram of melatonin. One clear reduction potential was observed for melatonin.

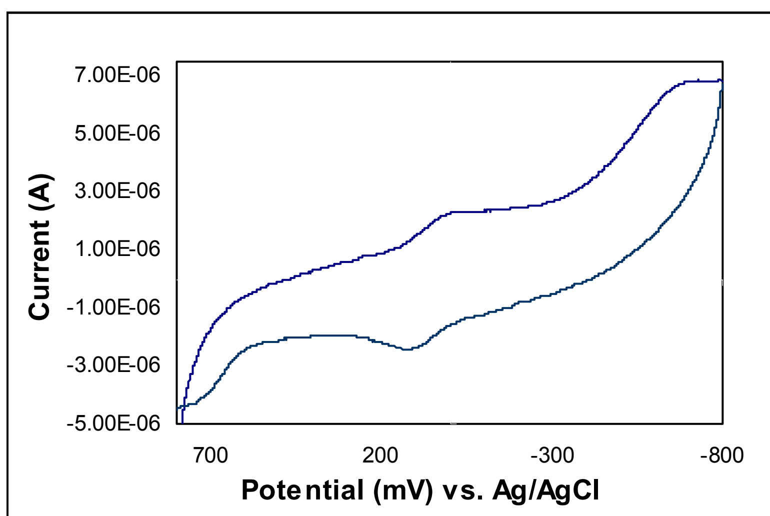


FIG. 6.3 *Cyclic voltammogram of melatonin. Scan Rate 100 mV/s.*

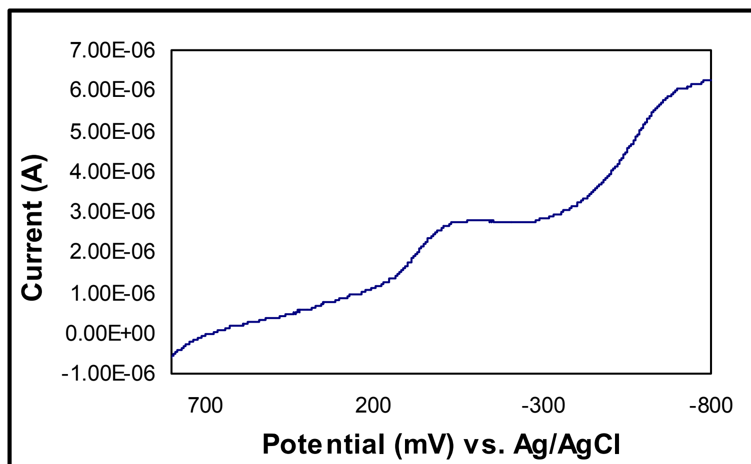


FIG. 6.4 *Adsorptive stripping voltammogram of melatonin Scan Rate 25 mV/s.*

Rotenone (Figure 6.5) displayed a very large ΔE value, which together with the large unity value obtained, implying an irreversible reaction. Irreversible systems are capable of producing both anodic and cathodic waves but the peaks exhibit very large separations *i.e.* > 200 mV (Brett and Brett, 1993). Rotenone displayed a strong reduction but weak oxidation wave during its return scan (Figure 6.5).

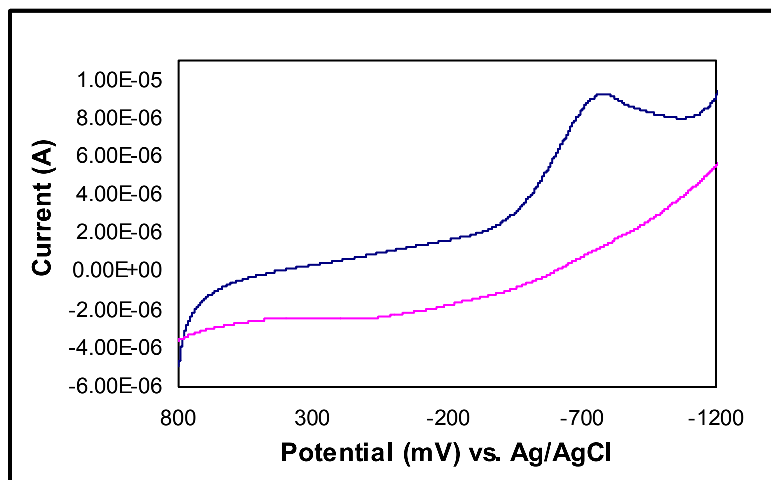


FIG. 6.5 *Cyclic voltammogram of rotenone. Scan Rate 100 mV/s.*

Table 6.1 Data (rate constants and R^2 values) obtained for the plot of peak current (i_a) *versus* square root of the scan rate of melatonin and rotenone.

Compound	**Rate constants / $(mV/s)^{1/2}$**	**R^2 values**
Melatonin	3.29×10^{-7}	0.987
Rotenone	4.52×10^{-7}	0.873
Fe^{3+}	7.03×10^{-7}	0.958

A plot of the variation of the catalytic currents with an increase in square root of the scan rate, for scan rates ranging from 25 to 400 mV/s, run in order to confirm whether a diffusion-controlled movement dominates at the electrode during the reduction of rotenone, indicates that (Table 1.2) a diffusion-controlled process is not as likely for rotenone as for melatonin (as shown by the R^2 value = 0.873. The i_a / i_c value obtained (Table 6.1) for rotenone confirmed the relative instability of rotenone under these conditions.

The dynamics involved could include the lower solubility of rotenone at this pH, adsorption onto the electrode surface between scans, or slow electron transfer between the compound and the electrode surface. Since thorough cleaning of the electrode surface between scans did not improve the peak heights, adsorption onto the electrode surface may be discarded. Figure 6.7 and 6.8 demonstrate the disappearance of rotenone between successive scans. Interestingly, although cleaning of the electrode surface did not restore the original peak heights, substitution of the rotenone solution for a fresh sample did. The possibility thus exists that rotenone degrades or decomposes under these electrochemical conditions. This same phenomenon was observed using ASV.

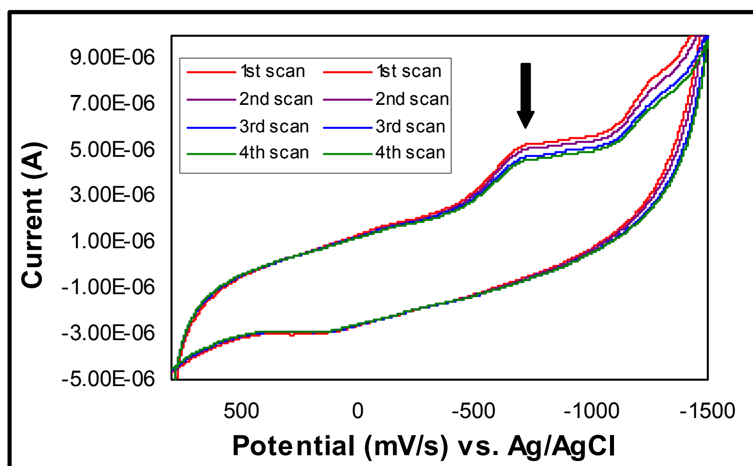


FIG. 6.6 *Successive CV scans for rotenone. Scans were run at 1.5-minute intervals over a period of 6 minutes. Scan rate 100 mV/s.*

6.3.1.1 Effect of melatonin on rotenone

The CV of melatonin and rotenone in solution together is shown in figure 6.7. Scans were taken every five minutes over a total run time of 35 minutes. A substantial decrease in the rotenone peak is seen after the first five to ten minutes. However, this decrease may not be attributed to the influence of melatonin on rotenone, but rather, as evidenced earlier, the instability of rotenone under these electrochemical conditions. The adsorptive stripping voltammograms also showed the same disappearance of rotenone.

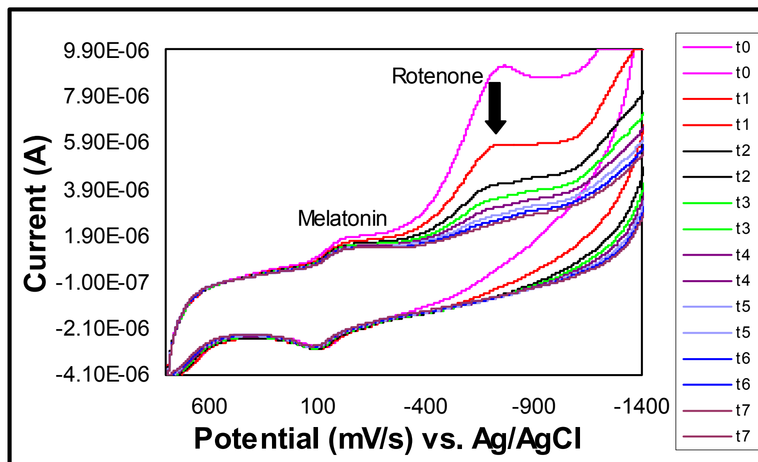


FIG. 6.7 *CV of melatonin and rotenone (in a 1:1 ratio) over a period of 35 minutes. Scan rate 100 mV/s.*

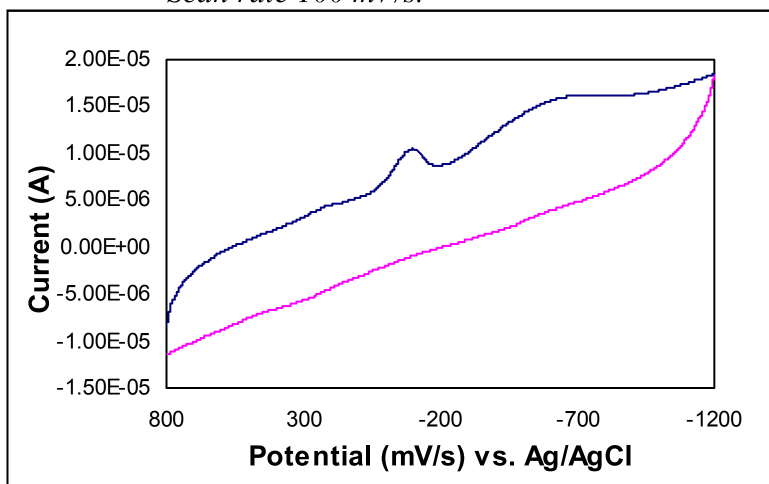


FIG. 6.8 *CV of Fe^{3+}. Scan Rate 100 mV/s.*

The ASV of melatonin and Fe^{3+} in solution are shown in figure 6.9. A slight shift in Fe peak from -30.9 mV (Fe only) to -39.3 mV was observed upon addition of melatonin.

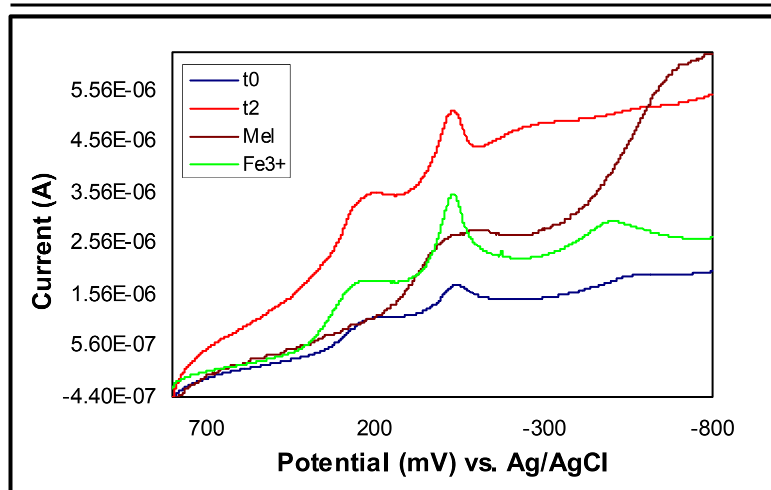


FIG. 6.9 *ASV of melatonin and Fe^{3+} (in a 1:1 ratio). Scan rate 25 mV/s.*

6.3.1.2 Effect of rotenone on Fe^{3+}

The absorptive stripping voltammograms for the effect of rotenone on Fe^{3+} were inconclusive as expected. Once again, the substantial decrease in the rotenone peak seen after the first ten minutes was attributed to the instability of rotenone under these electrochemical conditions. The peak attributed to Fe^{3+} remained unchanged.

6.3.1.3 The effect of rotenone and melatonin on Fe^{3+}

As observed previously, a slight increase in the peak attributed to Fe^{3+} was found which could be attributed to the influence of melatonin (as evidenced in Figure 6.9), while the peak attributed to rotenone disappeared after ten minutes.

6.4 DISCUSSION

Limson *et al* (1998) used electrochemistry to show that melatonin forms complexes with heavy metals such as iron, zinc, copper, aluminium, cadmium, and lead. The electrochemical data from the present study shows that rotenone is unstable under electrochemical conditions. The disappearance of rotenone between successive scans might be due to its degradation or decomposition under electrochemical conditions. The cyclic and adsorptive stripping

voltammograms of melatonin and rotenone also showed a decrease in rotenone peak indicating its disappearance.

The electrochemical data supports the postulation that melatonin binds to Fe^{3+}. Similarly with melatonin, rotenone and iron (III), a slight increase in the peak was observed with Fe^{3+}, but once again a decrease was observed with the rotenone peak. These results do not however, support the possibility that melatonin could be binding to both rotenone and iron due to the unstable behaviour of rotenone. Therefore, the results obtained are inconclusive.

Melatonin appears to have a dual role in protecting against rotenone and iron-induced oxidative damage. Melatonin binds to Fe^{3+}, and thus prevents further free radical formation due to the iron ions. On the other hand, it is more likely that melatonin is acting as a scavenger of free radicals induced by rotenone and thereby reducing oxidative stress.

The electrochemical characterization of rotenone reported above is novel data and future studies are recommended to explore its electrochemical nature.

CHAPTER SEVEN

HISTOLOGICAL ANALYSIS

7.1 INTRODUCTION

Histology is a term derived from the Greek word *histos*, meaning tissue, and *logia*, meaning "the study of" and it involves the examination of preserved, sectioned and stained tissues of both plants and animals. Most of our knowledge of internal tissue structure has come from this branch of science (Hodgeson & Bernard, 1992).

A defining characteristic of most neurodegenerative diseases is selective degeneration of specific cell types in certain brain regions. Apart from this, neuronal inclusions are present in many neurodegenerative diseases and contribute to neuronal dysfunction and death (Goedert *et al*., 2001). AD and PD are the common examples of such disorders. The histopathological signs of AD include the presence of abundant neuritic plaques and neurofibrillary tangles (NFT) (Siegel *et al*., 1989). The senile plaques mostly consist of β-amyloid (Aβ) peptide and the NFTs are intracellular aggregates of tau protein. Parkinson's disease is a late-onset neurological disorder characterized by selective degeneration of dopaminergic neurons of the substantia nigra (SN) (Wooten, 1997). The clinical hallmark of PD is the deposition of intracytoplasmic inclusion bodies called Lewy bodies in brain cells, which contain ubiquitin and α–synuclein (Baba *et al*, 1998).

Dopamine neurons of the SN are selected victims of the actions of a variety of neurotoxins, several of which produce parkinsonism in humans and experimental animals. For example, accidental ingestion of 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP) caused a parkinsonian syndrome in humans (Langston *et al*, 1983).

Previous chapters have shown that rotenone induces oxidative stress in the form of lipid peroxidation and that it is a potent mitochondrial toxin. These undesirable effects of rotenone may lead to neuronal damage and ultimate cell death. Melatonin was shown to offer significant protection against ROT-induced lipid peroxidation and mitochondrial alterations. Since oxidative stress results in the structural and functional disturbances of the neurons, it is important to examine these cells following rotenone administration and to investigate melatonin's role on such changes.

7.2 THE EFFECT OF MELATONIN AGAINST ROTENONE-INDUCED NEURONAL DAMAGE IN RAT BRAIN

7.2.1 INTRODUCTION

The MPTP model of PD suggested a role of mitochondrial dysfunction and environmental exposures in the etiology of PD. This is further delineated by a recent study conducted by Betarbet *et al* (2000). These authors reported that chronic systemic exposure of the pesticide rotenone reproduced many features of PD in rats. These include nigrostriatal dopaminergic lesions and formation of ubiquitin- and α–synuclein-positive inclusions in nigral neurons that resembled the Lewy bodies in PD. Systemic complex I inhibition by rotenone resulted in selective degeneration of the nigrostriatal dopaminergic pathway (Betarbet *et al*., 2000).

The present study was conducted to investigate whether melatonin offers protection against ROT-induced neuronal lesions in the rat brain. After treatment, the brains of rats were sectioned and the nigral neurons were examined microscopically for evidence of any morphological changes.

7.2.2 MATERIALS AND METHODS

7.2.2.1 Chemicals and Reagents

Rotenone, melatonin, haematoxylin and Eosin Y were purchased from Sigma Chemical Co, St. Louis, MO, USA. Paraffin wax was obtained from Lasec (South Africa). Cresyl violet stain was purchased from BDH Chemicals Ltd (England). DPX was purchased from Philip Harris Ltd (England). Haupt's adhesive consisted of the following: 1g gelatin, 100ml water, 2g phenol and 15ml glycerol. All other chemicals were of the highest quality available and were purchased from commercial distributors.

7.2.2.2 Animals

Adult, male, Wistar rats, weighing between 250 and 300g were used for the experiments, and were housed and maintained under the conditions described in section 2.2.2.1.

7.2.2.3 Treatment Regime

The animals were divided into three groups of five animals each (Table 6.1). The animals in group II received intraperitoneal injections of 0.3 mg/kg rotenone in a mixture of dimethyl sulfoxide (DMSO) and polyethylene glycol (PEG), for fourteen days. Group III animals were injected i.p. with 5mg/kg melatonin in ethyl oleate and 0.3 mg/kg rotenone. The animals received melatonin twice a day, the first injection being thirty minutes prior to ROT administration, and the second in the evening. Both agents were injected on the left and right side respectively, so as to avoid interaction at the site of administration. The control group (Group I) received vehicles for melatonin and rotenone, viz. ethyl oleate and a mixture of DMSO and PEG respectively. The duration of all injections was fourteen days.

Table 7.1 Treatment regime for each group of animals

TREATMENT GROUP	Received 30 mins prior to ROT	Intraperitoneal injection	Consisted of:
I. CONTROL (2 WEEKS)	Ethyl oleate	DMSO + PEG	Ethyl oleate
II. ROT ALONE (2 WEEKS)	Ethyl oleate	Rotenone in DMSO + PEG (0.3mg/kg)	Ethyl oleate
III. ROT+ MEL (2 WEEKS)	Melatonin in ethyl oleate (5mg/kg)	Rotenone in DMSO + PEG (0.3mg/kg)	Melatonin in Ethyl oleate

During the treatment regime, the rats were vigilantly monitored for behavioural changes. On the fifteenth day the animals were sacrificed and brains removed as described in section 2.2.2.3.

7.2.2.4 Histological Techniques

7.2.2.4.1 Fixing the brain

Neural tissues are extremely fragile and easily subjected to rapid anoxic and post-mortem changes, hence immediate fixation of these tissues is required (Chang, 1995). Fixation of the nervous tissue functions to preserve cells in their native state in regard to their morphology and localization of chemical constituents.

Here, the brains were immediately and rapidly fixed in a mixture of formol (30%), glacial acetic acid and ethanol in a ratio of 2:1:7 v/v for two hours.

7.2.2.4.2 Specimen Preparation and Embedding

Most tissues, after fixation must be supported during the process of cutting. Embedding involves the infiltration and orientation of tissue in the paraffin wax support medium. The tissue was dehydrated by bathing them successively in graded series of mixtures of ethanol, and this step was followed by immersing the tissue in xylene twice for one hour each. The tissue was then submerged in molten paraffin wax at 57°C twice for one hour each, which removed the xylene, while infiltrating the tissue. This stage provides the hardness and support that the tissue requires for sectioning.

7.2.2.4.3 Blocking Out

This procedure was performed to form a support that would facilitate sectioning using the rotary microtome. This was done by fixing the brain material into a block using a mould. A plastic ice tray coated with ethanol-glycine was used as the mould. Molten wax was added to the mould and allowed to harden. The brain was then removed from the final molten wax stage and placed carefully onto the mould with warmed forceps. Molten wax was then poured onto the brain so that it was completely submerged. Air was then gently blown over the surface of the wax to allow the top to solidify. The entire mould was then immersed in cold-water overnight to facilitate quicker solidification and to prevent the formation of crystals that might disrupt the tissue.

7.2.2.4.4 Sectioning

Sectioning was done using a rotary microtome. This is an instrument, which consists of a sharp metal knife held in a fixed position, and a chuck in which a block of wax with the tissue is held. Depending on the type of microtome, a particular mechanism oscillates the chuck up and down and with each oscillation, the chuck is brought closer to the knife by a fixed distance. In this way sections are cut from the wax block (Hodgson, 1992).

The wax block was trimmed with a razor blade so that two of the sides were parallel, while the other two converged slightly. The sides were cut so as to leave about 2mm of wax around the tissue. The wax block was attached to a small wooden block with a small amount of molten wax. The microtome was set to cut sections of 10µM thickness. As sections were cut these would stick to one another, so as to form long ribbons. Once the sections were cut, they were floated on a warm water bath (40ºC), which smoothens out the wrinkles.

7.2.2.4.5 Transferring Sections to Slides

The sections were removed from the water bath and placed on a glass microscope slide using a thin paint brush. The glass slide was initially brushed with a thin layer of Haupt's adhesive before the sections were mounted. The slides were left overnight in an oven at 40ºC to enable the section to adhere to the slide.

7.2.2.4.6 Staining

The coronal sections of the midbrain were stained using two different stains:

(1) Nissl staining and

(2) Haematoxylin and Eosin staining (H & E staining)

Before the section could be stained, it first had to be dewaxed and rehydrated as the stains are water soluble. The paraffin wax was removed by running the slides through xylene twice for five minutes each, followed by immersion in a mixture of xylene and absolute ethanol (1:1) for three minutes. This step was followed by immersion in absolute ethanol for five minutes, and then re - immersion in absolute ethanol overnight.

7.2.2.4.6.1 Nissl Staining

The Nissl stain is commonly used to study neurons under the light microscope and is extremely useful since it distinguishes neurons and glia from one another. The sections were Nissl stained using cresyl violet. This stains the Nissl substances intense purple and the nuclei purple. The background is left clear (Bauer, 1974).

Sections were stained by placing the slides in 0.1% cresyl violet solution for two hours. The cresyl violet solution contained 0.25g cresyl violet, 250ml milliQ water, 0.75ml glacial acetic acid and 0.0512g sodium acetate. The pH was adjusted to 3.5 before use. The slides were differentiated rapidly in 95% ethanol by rinsing in a flat dish until the background was clear. The sections were then dehydrated by processing in absolute ethanol twice and xylene twice as described in section 6.2.2.4.2 for five minutes each.

7.2.2.4.6.2 Haematoxylin and Eosin staining (H & E)

H & E is the most commonly used stain in histology and is effective in screening for neuronal losses and neuronal oedema (cytoplasmic swelling). Here, the nuclei are stained blue and cytoplasm pink.

The sections were stained in Harris haematoxylin solution for twenty minutes and rinsed in running tapwater. This is followed by 1-6 dips in differentiating solution and then blued in Scott's tap water substitute solution for two minutes. The slides were differentiated in 95% ethanol and then counterstained in alcoholic eosin Y solution for twenty minutes. The sections were then dehydrated using absolute ethanol and xylene as per mentioned in 7.2.2.4.6.1. (Appendix H & E staining)

7.2.2.4.7 Mounting of Slides

The stained section on the slide must be covered with a thin piece of plastic or glass to protect the tissue from external damage like scratching, and to provide better optical quality for viewing under the microscope. While the slides were moist with xylene, sufficient DPX was added to just

cover the tissue. A cover slip was then placed over the tissue. The slides were allowed to dry over a flat surface for 48 hours.

7.2.2.4.8 Photo-microscopy

All the slides were viewed under the light microscope and photographed using a digital camera.

7.2.3 RESULTS

7.2.3.1 Behavioral changes

It was interesting to note that all rotenone treated rats showed reduced activity in comparison to the control and melatonin treated groups. Some of the rotenone-exposed rats had unsteady movement and flexed posture and were rigid. Another interesting feature was that the tail of one rat stood upright occasionally, and one rat had a shaking paw that was observed after one week of ROT administration. Though the rats treated with rotenone and melatonin showed similar behaviour, it was less frequent, and these rats seem to be more active that those treated with the pesticide alone.

7.2.3.2 Histological Analysis

The nigral neurons in the mid brain were carefully examined.

7.2.3.2.1 Nissl staining

The Nissl staining of the nigral neurons of the control animals showed optimally sized cells with a prominent nucleus and pigmented neurons can be observed (Figure 7.1).

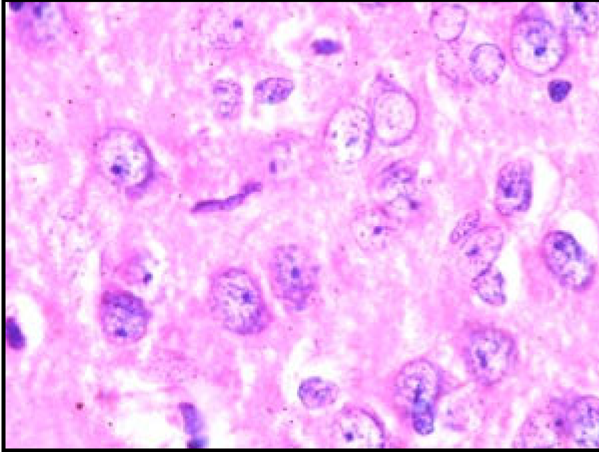


FIG. 7.1 *Nissl stained cells of the nigral region in the midbrain from an animal of the control group. Magnification x 1000*

The cells of rotenone treated animals showed extensive neuronal damage by virtue of loss of pigmented neurons (Figure 7.2). The cells appear to be smaller and shrunken compared to the control cells, indicating that apoptosis has occurred. Also the cells are small in number.

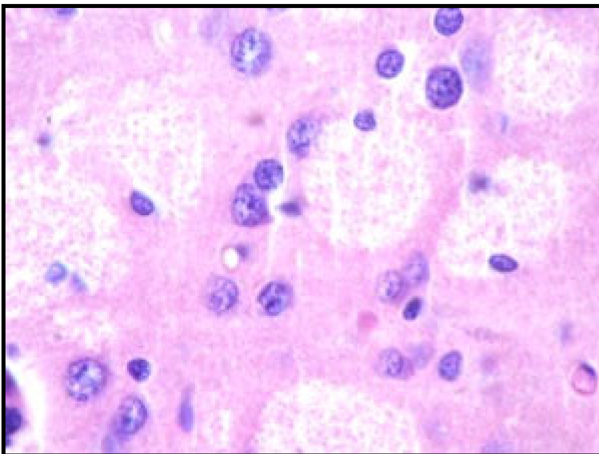


FIG. 7.2 *Nissl stained cells of the nigral region in the midbrain from an animal treated with rotenone (0.3mg/kg i.p.) for fourteen days. Magnification x 1000*

Figure 7.3 shows the neuronal cells from rats treated with rotenone and melatonin. In comparison to sections treated with ROT alone, the neurons of ROT and MEL treated rats showed significant protection. Most of the cells appear to have regained their size and are not that shrunken as those seen in ROT alone treated sections. Large numbers of cells are present. Though loss of neurons can be seen, it is not that pronounced as seen in rotenone alone treated rats.

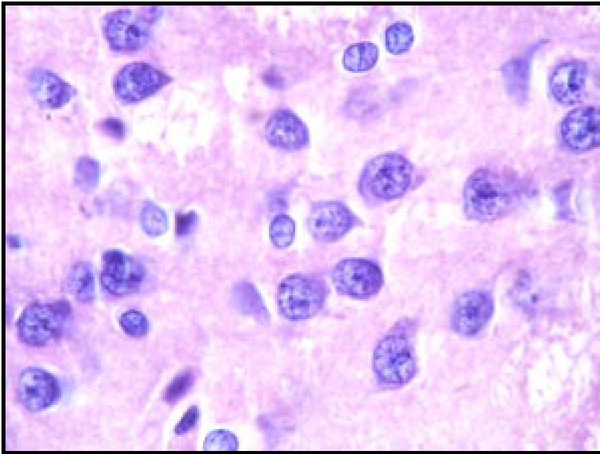


FIG. 7.3 *Nissl stained cells of the nigral region in the midbrain from an animal treated with rotenone (0.3mg/kg i.p.) and melatonin (5mg/kg i.p.) for fourteen days. Magnification x 1000*

7.2.3.2.2 Haematoxylin and Eosin staining

The nigral neurons in the midbrain were examined by light microscope. Figure 7.4 shows the cells from a control animal stained by Harris haematoxylin and eosin. Here, the nuclei are stained blue and the cytoplasm pink.

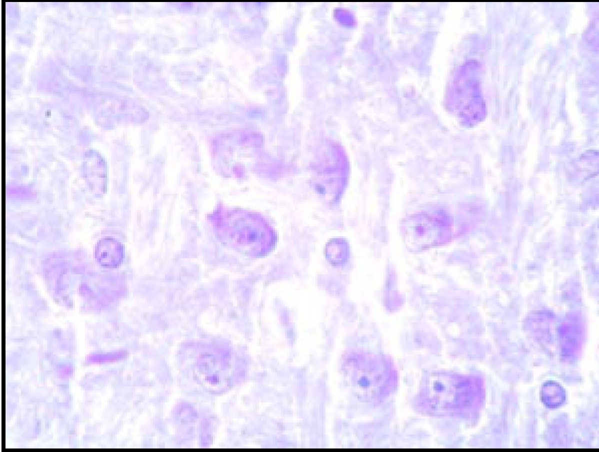


FIG. 7.4 *Cells of the nigral region in the midbrain from an animal of the control group. Haematoxylin and eosin staining. Magnification x 1000*

Neurons from the rotenone treated animals showed signs of damage as evident by disappearance of cells and the presence of cytoplasmic inclusions (Figure 7.5). Structural alterations of the surviving neuronal cells can be observed.

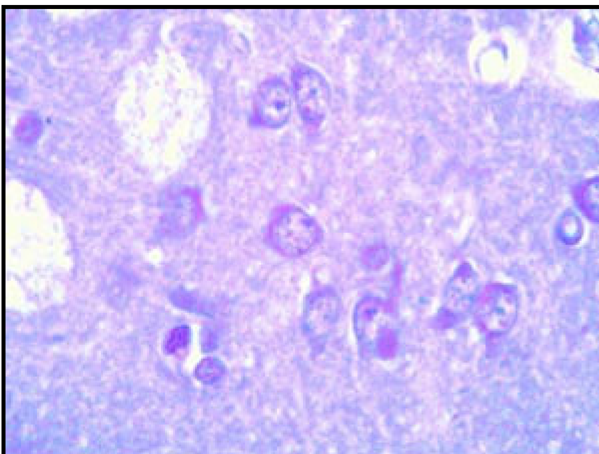


FIG. 7.5 *Cells of the nigral region in the midbrain from an animal treated with rotenone (0.3mg/kg i.p.) for fourteen days. Haematoxylin and eosin staining Magnification x 1000*

The sections from rats treated with rotenone and melatonin appear to have more neuronal cells than with rotenone alone (Figure 7.6). Pale eosinophilic inclusions are present in these sections.

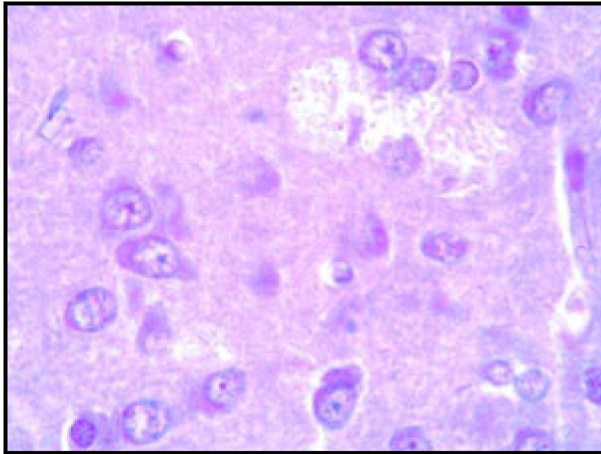


FIG. 7.6 *Cells of the nigral region in the midbrain from an animal treated with rotenone (0.3mg/kg i.p.) and melatonin (5mg/kg i.p.) for fourteen days Haematoxylin and eosin staining. Magnification x 1000*

7.2.4 DISCUSSION

Neuronal cell death is a critical consequence of a variety of brain insults. The loss of key brain cells is presently irreversible and worsens over time, eventually leading to cell death. Parkinson's disease is characterized by loss of pigmented neurons from the substantia nigra in the midbrain and from the locus ceruleus in the pons.

The results of the present study clearly demonstrate that chronic treatment of low doses of rotenone (0.3 mg/kg) causes extensive neuronal damage within the cells. This is characterised by the loss of neuronal cells and signs of apoptosis. The neuronal cells in the nigral region seem to be more vulnerable to rotenone toxicity. External observations showed that rotenone produces many of the behavioural symptoms associated with PD.

Recent research has reported a possible link between rotenone and PD (Betarbet *et al*, 2000). These reports postulate that the rotenone model is capable of reproducing features of PD by

systemic complex I inhibition. This indicates that the nigrostriatal dopaminergic pathway is selectively sensitive to complex I dysfunction (Betarbet *et al*, 2000). Though the MPTP model causes selective nigrostriatal degeneration by inhibiting complex I, the inhibition is not systemic and uniform. Rotenone, being extremely lipophilic, crosses biological membranes easily, and unlike MPTP it does not depend on the dopamine transporter for access to the cytoplasm (Heikkila *et al*, 1985). Behaviourally, the rotenone exposed animals developed motor and postural deficits characteristic of PD (Betarbet *et al*, 2000). Chronic systemic exposure to rotenone produces cytoplasmic inclusions containing α–synuclein and ubiquitin in the nigral neurons, which is a pathological hallmark of PD (Betarbet *et al*, 2000, Sherer *et al*, 2003). Previous studies have shown that rotenone, administered either stereotactically or via intrajugular or intramuscular or intraperitoneal routes, are capable of inducing PD symptoms (Betarbet *et al*, 2000, Sherer *et al*, 2003, Alam and Schmidt, 2002). Hence, in the current study the chronic exposure of low doses of ROT by i.p. injection is comparable to chronic environmental exposure.

Rotenone has been shown to induce lipid peroxidation in chapter five and these processes can damage the critical biomolecules and can signal the initiation of apoptosis. One of the mechanism by which rotenone induces damage to the brain is through the induction of reactive oxygen species and further oxidative damage. Complex I inhibition by rotenone renders the dopaminergic neurons more susceptible to free radical attack and triggers the apoptotic process. Thus rotenone seems to be useful to model the pathogenesis of PD.

From the histology studies, it appears that melatonin attenuates ROT-induced insult in the rat brain. The relatively large number of cells in the melatonin treated sections indicates that this neurohormone, to some extent, prevents the loss of neuronal cells. The presence of cytoplasmic inclusions and absence of complete recovery in the melatonin treated groups can be attributed to the fact that the damage caused by rotenone is of an irreversible nature and melatonin was unable to overcome this damage fully. Probably, pre-treatment with melatonin before starting the rotenone regime might enhance its beneficial effects. Also, the rats treated with melatonin appear to be much healthier and active in comparison to the rotenone group, as observed during the

chronic treatment period. The results of the current study show that administration of melatonin reduced the extent of brain damage caused by rotenone.

The free radical scavenging and antioxidant activity of melatonin is already well established and its protective action against rotenone induced oxidative stress has already been demonstrated in chapter two and chapter five. Since complex I inhibition and oxidative stress has been postulated as possible mechanisms of ROT toxicity, melatonin could be scavenging the free radicals generated by complex I inhibition. Melatonin, *in vivo* and *in vitro* studies, has been shown prophylactically to reduce oxidative damage in several models of Parkinson's disease (dopamine auto-oxidation, 1-methyl-4-phenyl-1, 2, 3, 6- tetrahydropyridine and 6-hydroxydopamine), and to protect against glutamate excitotoxicity (Iacovitti *et al*, 1997, Antolin et *al*, 2002). It rescues dopamine neurons from cell death in tissue culture treated with 1-methyl-4-phenylpyridinium (MPP^+) (Iacovitti *et al*, 1997). Melatonin has also been shown to produce in vivo neuroprotective effects in rats with a unilateral lesion of SN caused by 1-methyl-4-phenylpyridinium (MPP^+) (Jin *et al*, 1998). Also, melatonin increases the mRNA levels and prevent apoptosis induced by 6-hydroxydopamine (6-OHDA) in neuronal PC12 cells (Mayo *et al*, 1998). This pineal hormone was shown to suppress the oxidative damage induced by the herbicide, paraquat (Melchiorri *et al*, 1994). These data indicate that melatonin might possess a remarkable ability to protect organisms from ROT-induced oxidative damage and associated tissue destruction.

CHAPTER EIGHT

APOPTOSIS

8.1 INTRODUCTION

Apoptosis or "programmed cell death" (PCD) is a physiological suicide mechanism that preserves homeostasis, in which cell death naturally occurs during normal tissue turnover (Wyllie *et al.*, 1980). In the nervous system, programmed cell death of sensory and motor neurons occurs in embryonic stages. It has been estimated that 50% or more of vertebrate neurons in the CNS die during embryonic development. Also, maturation of the central and peripheral nervous system is accompanied by extensive deletion of neurons (Batistatou and Greene, 1993). Neuronal cell death is not only part of normal brain development but also occurs in neurodegenerative disorders.

Morphologically, apoptosis is characterized by a series of structural changes in dying cells such as cell shrinkage, blebbing of the plasma membrane, condensation of the cytoplasm and nucleus, and cellular fragmentation into membrane apoptotic bodies (Wyllie *et al.*, 1980). The biochemical hallmark of apoptosis is the fragmentation of genomic DNA, an irreversible event that commits the cell to die. This process of specific DNA fragmentation leads to oligonucleosomes of 180-200bp corresponding to DNA content.

Apoptosis may be triggered pathologically in the adult CNS and may mediate the nonphysiological death of neurons characteristic of various neurodegenerative disorders, such as Alzheimer's disease and Parkinson's disease. Post mortem studies show that the dying cells in PD brains bear not only signs of necrosis but also of apoptosis, in particular chromatin condensation, DNA fragmentation, oxidative damage, mitochondrial dysfunction, and caspase activation (Mochizuki *et al.*, 1996).

It has been suggested that ROS play an important role in programmed cell death (Sastre *et al.*, 2000; Nianyu *et al.*, 2003). Mitochondria, a major sub-cellular source of ROS are often centrally involved in the development of apoptosis. Leakage of high-energy electrons along the mitochondrial electron transport chain causes the formation of superoxide anion radicals, which can interact with nitric oxide (NO) to form peroxynitrite, which may damage cells by promoting

membrane lipid peroxidation and nitration of proteins on tyrosine residues (Sastre *et al*., 2000). Both complex I inhibition and oxidative stress may cause apoptotic cell death. Nianyu *et al* reported that rotenone induces apoptosis via enhancing mitochondrial ROS production (Nianyu *et al*., 2003). Antioxidants decrease the level of ROS-dependent apoptosis by restricting oxidation and quenching free radicals. The antioxidant vitamin E and C as well as exogenous catalase has been shown to prevent apoptosis in some system (Buttke and Sandstom, 1994).

Loss of mitochondrial inner membrane potential by opening the permeability transition pore is a critical event in neuronal apoptosis (Sims and Anderson, 2002). Mitochondrial membrane potential is generated by the pumping of protons out across the inner mitochondrial membrane at the mitochondrial complexes, particularly complex I. So a decrease in complex I activity could result in a decrease in mitochondrial membrane potential that would render PD substantia nigra neurons vulnerable to apoptosis.

A commonly used method to identify apoptosis is the terminal deoxynucleotidyltransferase-mediated dUTP–biotin nick end labelling (TUNEL) method. Gavrieli *et al*., (1992) were the first to report a technique for identifying cells with DNA damage in tissue sections. TUNEL staining detects DNA fragmentation, coupled with the identification of relevant morphological changes including a regular pattern of chromatin condensation and apoptotic bodies.

8.2 THE EFFECT OF MELATONIN ON ROTENONE-INDUCED APOPTOSIS IN RAT BRAIN

8.2.1 INTRODUCTION

The brain treated with rotenone exhibited signs of apoptosis in the previous chapter. To confirm the results obtained, it was decided to investigate whether rotenone induces apoptosis and the role of melatonin thereof. In the current experiment, the *in situ* cell death detection kit, Fluorescein was used for the detection of apoptosis at single cell level based on labeling of DNA strand breaks (TUNEL method).

The assay uses an optimized terminal transferase (TdT) to label free 3'OH ends in genomic DNA with fluorescein d-UTP. The procedure involves:

- fixing and permeabilizing apoptotic cells
- incubating the cells with the TUNEL reaction mixture containing TdT and fluorescein-dUTP. During this incubation step, TdT catalyzes the attachment of fluorescein-dUTP to free 3'OH ends in the DNA.
- Visualizing the incorporated fluorescein with a flow cytometer and / or a fluorescence microscope.

8.2.2 MATERIALS AND METHODS

8.2.2.1 Chemicals and Reagents

The *in situ* cell death detection kit, Fluorescein was purchased from Roche Diagnostics, Mannhein, Germany. DNase and Proteinase K were also purchased from Roche Diagnostics, Mannhein, Germany. TESPA was purchased from Sigma Chemical Co, St. Louis, MO, USA. Paraffin wax was obtained from Lasec (South Africa). All other chemicals were of the highest quality available and were purchased from commercial distributors.

8.2.2.2 Animals

Adult, male, Wistar rats, weighing between 250 and 300g were used for the experiments, and were housed and maintained under the conditions described in section 2.2.2.1.

8.2.2.3 Treatment Regime

The animals were divided into three groups of five animals each (Control group, Rotenone group, Rotenone and Melatonin group). The treatment regime was carried out as outlined in section 7.2.2.3 of the previous chapter except that the duration of injection was seven days. On the eighth day the animals were sacrificed and brains removed as described in section 2.2.2.3.

8.2.2.4 Techniques involved in apoptosis

The detection of apoptosis is a sensitive experiment and therefore some of the protocols involved for processing the tissue is different from that mentioned in the previous chapter.

8.2.2.4 .1 Fixing the brain

The role of the fixative is to maintain the morphology of the tissues as close to *in vivo* morphology as possible and to prevent post-sampling necrosis. Davidson's Alcohol Formalin Acetic Acid Fixative (Davidson's AFA) is an excellent choice for preserving the structure of the tissues (Appendix 5a). Here, the brains were immediately and rapidly fixed in Davidson's solution for forty-eight hours.

8.2.2.4 .2 Specimen Preparation and Embedding

The embedding of samples in paraffin requires several steps during which the water contained in the tissues is progressively replaced, first by alcohol, then by xylene, and lastly by paraffin. A modification of the protocol outlined by Judes (1997) was used.

After having fixed the samples in Davidson's solution, these were transferred through graded alcohols series (50-96 [v/v]) for two hours each, followed by a final dehydration in absolute ethanol thrice, for two hours each. The tissue was then immersed for two hours in a mixture of ethanol : xylene (50:50), followed by immersing in xylene (two hours) which eliminates the alcohol contained in the tissues. The tissues are then impregnated with molten paraffin wax at 60°C thrice for one hour each.

8.2.2.4 .3 Blocking Out

This procedure was carried out as described in section 7.2.2.4.3.

8.2.2.4 .4 Sectioning

This was carried out as outlined in section 7.2.2.4.4. The coronal sections of the mid-brain were chosen and the sections cut were of 5µM thickness.

8.2.2.4.5 Transferring Sections to Slides

When mounting paraffin sections on slides, it is important to use an appropriate adhesive to avoid loss of sections during the subsequent washing procedures. For TUNEL staining, sections can be mounted on Superfrost slides or on glass slides that have been coated with either 3-aminopropyl triethoxysilane (TESPA) or poly-L-lysine. TESPA has been shown to be superior to poly-L-lysine in preventing tissue detachment from the glass (Ben-Sasson *et al*., 1995).

Here, the glass slides were coated with TESPA two days prior to application of paraffin sections. This was done by dipping the slides initially in detergent and then in distilled water followed by 95% ethanol. The slides were then dried in an oven at 150°C for 15 minutes and then cooled. This was followed by dipping the slides in 2% TESPA in acetone for 10 s. Then the slides were washed twice with acetone and dipped in distilled water and dried at 42°C.

8.2.2.5 TUNEL STAINING

Apoptotic cells were detected by immunohistochemistry using the *in situ* TUNEL kit. The procedure was carried out in accordance with the manufacturer's recommendations. In the TUNEL method, TdT is used to incorporate biotinylated deoxyuridine at the sites of DNA breaks. The TUNEL reaction mixture was prepared immediately before use and was kept on ice until use.

8.2.2.5.1 Pre-treatment of paraffin-embedded tissue

Before the commencement of TUNEL staining, the paraffin-embedded tissue was dewaxed and rehydrated according to standard protocols. The slides were deparaffinized by heating for 15 minutes at 60°C. Hydration of the tissue was then accomplished by transferring the slides through xylene twice for five minutes each, followed by immersion in absolute ethanol twice for three minutes and then through a graded series of ethanol (95-80%) and distilled water for three minutes.

The slides were then rinsed twice in PBS and the tissue sections were incubated with Proteinase K (20µg/ml in 10mM Tris/HCl, pH 7.4-8.0) for 15-30 minutes at 21-37°C. This step is done to partially deproteinize the tissue sections. Proteinase K digests cross-linked proteins and thereby increases cell permeability and access to the nucleic acid targets (DNA).

8.2.2.5.2 Labeling

After incubating with Proteinase K, the slides were rinsed twice with PBS. TUNEL reaction mixture was prepared immediately and 50µl of the reaction mixture was added to each of the

samples (Appendix 5b). The slides were then incubated in a humidified chamber for one hour at 37°C in the dark. The samples were covered with parafilm during incubation to ensure a homogenous spread of the TUNEL reaction mixture and to avoid evaporative loss. After incubation, the slides were rinsed thrice with PBS and then mounted with aqueous mountant and coverslipped.

In addition to the experimental control, rotenone, rotenone and melatonin group, two negative controls and a positive control were included in the experimental set up.

For the negative control, the fixed and permeabilized cells were incubated with 50µl/well Label solution (without terminal transferase) instead of TUNEL reaction mixture (Appendix 5b).

For the positive control, the fixed and permabilized cells were incubated with DNase I (3000 U/ml-3 U/ml in 50mM Tris/HCl, pH 7.5, 1mg/ml BSA) for 10 minutes at 15-25°C to induce DNA strand breaks, prior to labeling procedures.

The samples were then analysed by fluorescence microscopy using an excitation wavelength in the range of 450-500nm and photographed using a digital camera.

8.2.3 RESULTS

Figure 8.1 demonstrates TUNEL staining in the experimental control group that received the vehicle. The TUNEL positive cells are indicative of DNA fragmentation.

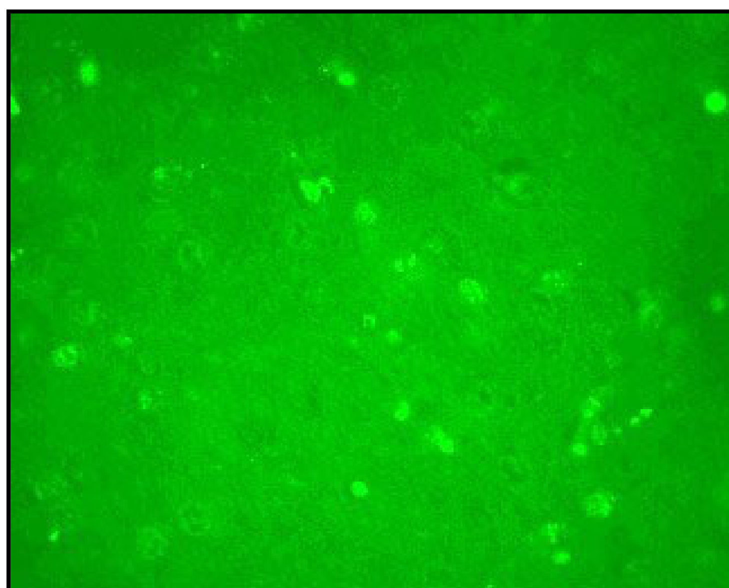


FIG 8.1 *TUNEL stained neurons from a rat treated with vehicle (control)*

Figure 8.2 represents the TUNEL staining in midbrain of the rat treated with rotenone (0.3mg.kg) for seven days. Intense TUNEL staining can be observed in these sections, which indicates the presence of apoptotic bodies. Numerous shrunken neurons can be observed.

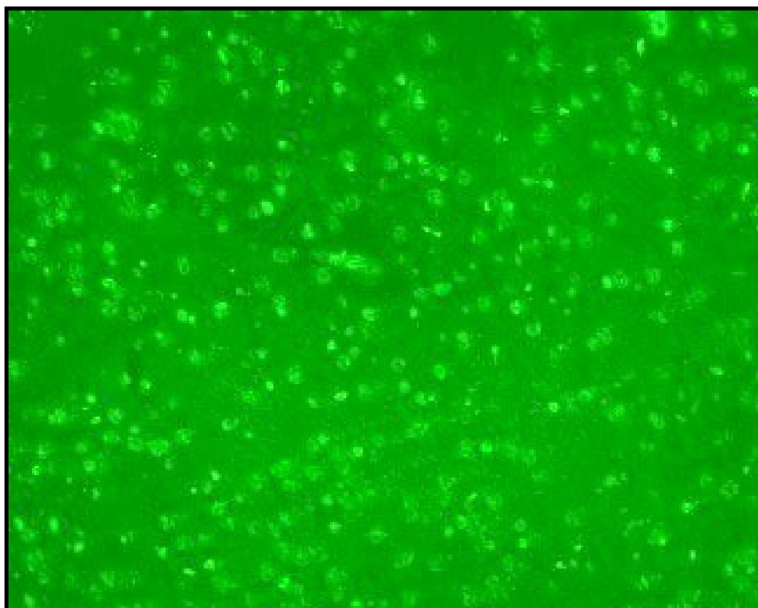


FIG 8.2 *TUNEL stained neurons from a rat treated with rotenone (0.3mg/kg)*

Figure 8.3 shows the TUNEL staining from the midbrain of rats treated with rotenone (0.3mg/kg i.p.) and melatonin (5mg/kg i.p.) for seven days. Although the TUNEL positive cells are present in these sections, it is less compared to that treated with rotenone alone.

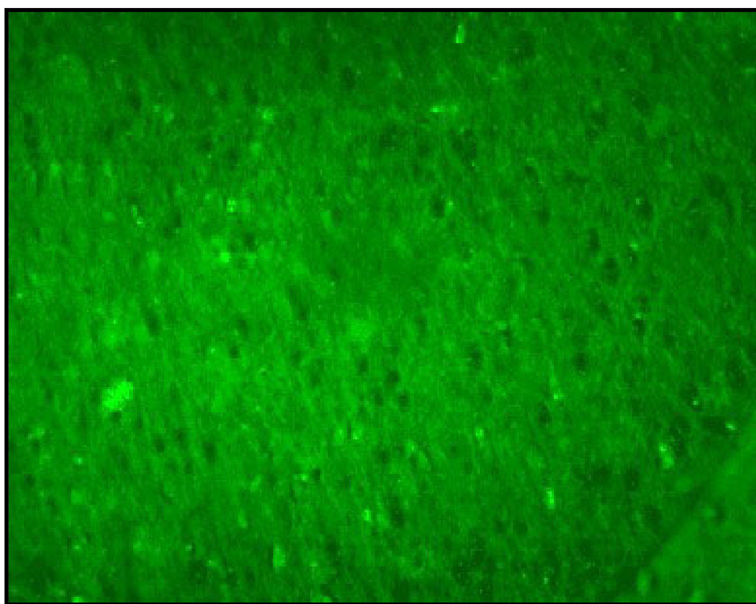


FIG 8.3 *TUNEL stained neurons from a rat treated with melatonin (5mg/kg)*

8.2.4 DISCUSSION

The death of neurons by apoptosis contributes to physiologically appropriate neuronal loss during development and to physiologically inappropriate neuronal loss in pathologic conditions. Brains from PD patients show evidence of apoptosis, including fragmented nuclei and caspase activation (Mochizuki *et al*., 1996).

In the current study, the presence of a large number of TUNEL positive neurons in the brain sections treated with rotenone show that rotenone is capable of inducing apoptotic cell death. Previous experiments have demonstrated that rotenone enhances free radical production by inhibition of mitochondrial complex I enzyme. Inhibitors of mitochondrial complex I induce apoptosis through increased free radical generation. Rotenone has been reported to cause cell death in a variety of cell lines (Sherer *et al*, 2002). However, whether this cytotoxicity leads to apoptosis or necrosis depends upon the cell type. Rotenone, MPP^+ and tetrahydroisoquinoline were reported to induce apoptosis in PC12 cells (Seaton, 1998). Nianyu *et al* reported that rotenone induced apoptosis in HL-60 cells (2003). This author further suggests that induction of mitochondrial ROS is the most significant mechanism of rotenone-induced apoptosis.

The TUNEL assay also reveals the presence of apoptotic bodies in brains treated with both melatonin and rotenone. However, the extent of DNA damage appears to be less compared to rotenone treated sections. Also, the neurons were not that shrunken. These results show that melatonin administered i.p. to rats is able to reduce DNA damage and apoptosis that was triggered in the brain by the administration of rotenone.

Oxidative stress is a mediator of apoptosis and antioxidants limit further cell damage by restricting oxidation and quenching free radicals. In the present study, the protective role of melatonin can be attributed to its antioxidative and free radical scavenging ability. NO has been shown to trigger apoptosis by a variety of mechanisms such as excitotoxicity and by inhibiting the activity of mitochondrial respiratory chain and thus decreasing the cellular ATP content (Wei *et al*, 2000). Pozo *et al* reported that physiological concentrations of melatonin inhibit NO production in the rat brain (Pozo *et al*, 1997) thus suggesting its protective role against NO-induced apoptosis. Melatonin has been reported to protect against the apoptotic damage induced by MPTP in nigral neurons (Antolin *et al*., 2002). The ability of melatonin to stimulate brain glutathione peroxidase activity and its protective effects against the uncoupling of the electron transport chain of several toxins indicate some possible explanations of melatonin's neuroprotective role in apoptosis.

CHAPTER NINE

GENERAL CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE STUDIES

9.1 SUMMARY OF RESULTS

CHAPTER TWO

The effect of melatonin on rotenone – induced oxidative stress in the form of superoxide radicals was investigated in the rat brain *in vivo* and *in vitro*. The results of these pilot studies indicate that melatonin affords significant protection against the superoxide radical generation by rotenone. Brain homogenate that was obtained from rats pre-treated with melatonin also showed significant protection.

CHAPTER THREE

Mitochondria, being the major source of ROS production, and considering the fact that rotenone is a mitochondrial poison, the effect of melatonin on rotenone – induced depression of the mitochondrial ETC was investigated by biological oxidation assay. The experimental results demonstrated an increase in ETC activity in melatonin treated samples compared to rotenone treated samples. Melatonin also showed an increase in basal level of respiration within the brain and hepatic mitochondria.

CHAPTER FOUR

Most of the toxic effects of rotenone is attributed to its potent inhibitory action on mitochondrial complex I enzyme. Since melatonin showed protection against rotenone induced mitochondrial oxidative damage, the effect of melatonin on complex I enzyme was further investigated. The results obtained showed that melatonin was successful in counteracting the complex I inhibition by rotenone. Considering the fact that complex I inhibition of mitochondria by rotenone is the basis for its oxidative damage and ATP depletion, it can be concluded that the protection

offered by melatonin was not only due to its free radical scavenging and antioxidant properties, but also due to its direct interaction with complex I thus preventing rotenone insult.

CHAPTER FIVE

Chapter five investigated the neuroprotective effects of melatonin on the levels of rotenone induced lipid peroxidation in the rat brain *in vivo* and *in vitro*. Melatonin was found to significantly reduce ROT-induced lipid peroxidation *in vivo* and *in vitro*. Iron plays an important role in accelerating lipid peroxidation and is itself a free radical. The effect of melatonin on lipid peroxidation induced by both rotenone and iron was examined and the results demonstrated a protective role for melatonin. It is suggested that the protective nature of melatonin was not only attributable to the antioxidant and free radical scavenging properties but also due to a possible binding to iron and thus inactivating its toxicity.

CHAPTER SIX

Binding studies were conducted to determine the effect of melatonin on rotenone and iron (III) complex using electrochemical techniques. The results implied an interaction between melatonin and iron (III). An interaction between melatonin and rotenone was not confirmed due to the unstable behaviour of rotenone under electrochemical conditions.

CHAPTER SEVEN

Histological investigation of the nigral neurons in rat brain was performed following treatment with rotenone alone or in combination with melatonin. Microscopic examination of nervous tissue within the nigral region revealed loss of neurons, extensive neuronal damage and signs of apoptosis in rats treated with rotenone whereas less damage was observed in rats treated simultaneously with rotenone and melatonin. Moreover external observations in the rats treated with rotenone and melatonin showed a relatively greater activity as compared to those that were treated with rotenone alone.

CHAPTER EIGHT

The results of chapter seven showed signs of apoptosis following rotenone administration. So chapter eight was devoted to determine the role of rotenone and melatonin on apoptosis. The TUNEL staining was employed to detect the apoptotic damage based on labeling of DNA strand breaks. The results obtained showed more TUNEL positive neurons with rotenone whereas less TUNEL positive neurons with melatonin. The results suggest a protective role of melatonin against rotenone-induced apoptosis.

9.2 CONCLUSION

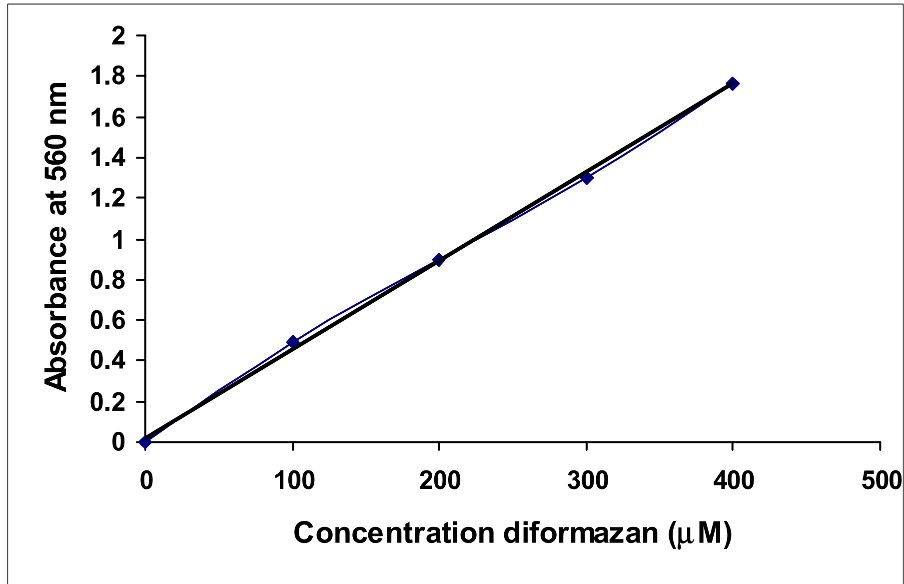
The experiments conducted in this study point conclusively that melatonin affords protection against rotenone – induced neurotoxicity. Chronic exposure to low levels of environmental toxin may cause cumulative damage to the dopaminergic system of the brain, eventually leading to the progression of Parkinson's disease. Inhibition of complex I by one such environmental toxin, rotenone, leads to impaired ATP production, formation of highly reactive oxygen free radicals, induction of mitochondrial permeability transition pore, and apoptosis. All these events have been linked with the pathogenesis of PD. Melatonin, an already well–established neuroprotectant has shown to significantly curtail the insults induced by rotenone. The antioxidant effects of melatonin and its protective effects on mitochondria have been well documented.

No effective cure has so far been reported to treat neurological disorders associated with ageing other than therapeutic interventions that could forestall or delay the normal and pathological ageing process. Although the results of this study point to a possible neuroprotective role of melatonin on rotenone, further research needs to be conducted in order to confirm the deductions made. The rotenone model of PD has gained considerable interest among researchers in that this model reproduces most of the important features in PD pathogenesis and thus neuroprotective drug treatment trials in this model maybe more relevant. Since rotenone treatment results in the formation of α-synuclein inclusions, the role of melatonin on such mutational changes would be an ideal area for future investigation. Another area for future study would be the utilisation of cell culture models to test the combined effect of rotenone and

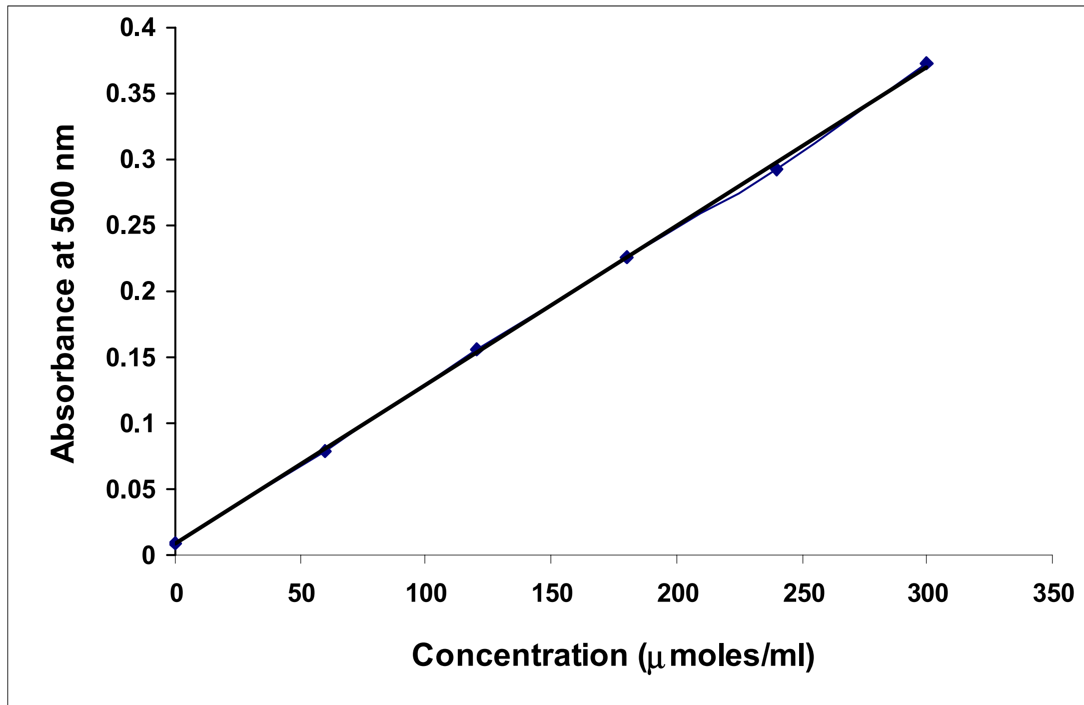
melatonin, which will provide further insights for neuroprotection and may lead to the development of effective therapeutic strategies.

There are still many unknowns that must be explored and since rotenoid compounds are widely used as pesticides, studies with rotenone may provide clues on environmental exposures that could increase the risk for PD and also the role of this endogenous neurohormone in alleviating the progression of such diseases.

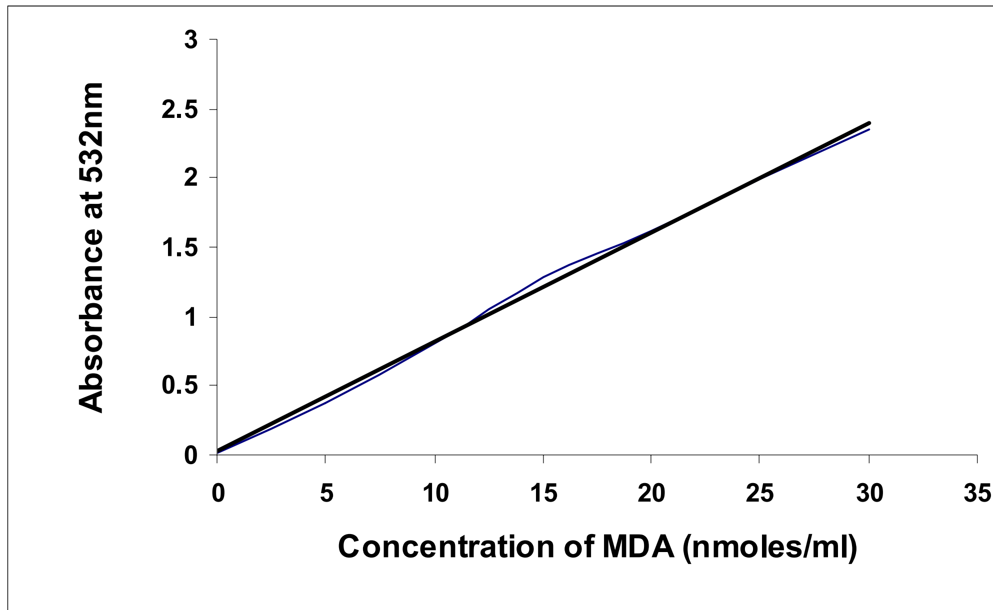
APPENDICES



APPENDIX 1 CALIBRATION CURVE FOR DIFORMAZAN (R^2 =0.9989)



APPENDIX 2 PROTEIN STANDARD CURVE ($R^2 = 0.9995$)



APPENDIX 3 MALONDIALDEHYDE STANDARD CURVE ($R^2 = 0.9977$)

APPENDIX 4

HARRIS HAEMATOXYLIN AND EOSIN STAINING

Preparation of Harris Haematoxylin

Haematoxylin crystals	5.0 gm
Alcohol, 100%	50.0 ml
Ammonium or potassium alum	100.0 gm
Distilled water	1000.0 ml
Mercuric oxide (red)	2.5 gm

Dissolve the haematoxylin in the alcohol, the alum in the water by the aid of heat. Remove from heat and mix the two solutions. Bring to a boil as rapidly as possible. Remove from heat and add the mercuric oxide slowly. Reheat to a simmer until it becomes dark purple, remove from heat immediately and plunge the vessel into a basin of cold water until cool. The stain is ready for use as soon as it cools. Addition of 2-4 ml of glacial acetic acid per 100 ml of solution inceases the precision of the nuclear stain. Filter before use.

1% Stock Alcoholic Eosin

Eosin Y, water soluble	1.0 gm
Distilled water	20.0 ml
Dissolve and add:	
Alcohol, 95%	80.0 ml

APPENDIX 5

a. Fixation with Davidson's Alcohol Formalin Acetic Acid Fixative (Davidson's AFA)

The Recipe:

330ml 95% Ethyl alcohol
220ml 100% Formalin (37-39% solution)
115ml Glacial acetic acid
335ml Distilled water
Store at room temperature.

b. Preparation of TUNEL reaction mixture

One pair of tubes (vial 1: Enzyme Solution and vial 2: Label Solution) is sufficient for staining 10 samples by using 50µl TUNEL reaction mixture per sample and 2 negative controls by using 50µl Label solution per control.

The TUNEL reaction mixture should be prepared immediately before use and should not be stored. Keep TUNEL reaction mixture on ice until use.

Step I: Remove 100µl Label solution (vial 2) for two negative controls.
Step II: Add total volume (50µl) of Enzyme solution (vial 1) to the remaining 450µl Label solution in vial 2 to obtain 500µl TUNEL reaction mixture.
Step III: Mix well to equilibrate components.

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