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ROLE OF RUTIN IN 1-METHYL-4-PHENYLPYRIDINIUM TOXICITY: THERAPEUTIC IMPLICATIONS FOR PARKINSON'S DISEASE

by

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DECLARATION

I, **Adaze Bijou Enogieru**, declare that "**Role of rutin in 1-methyl-4-phenylpyridinium toxicity: Therapeutic implications for Parkinson's disease**" is my original work and that all the sources that I have used or cited have been indicated and acknowledged by means of complete references, and that this document has not been submitted for degree purposes at any other academic institution.

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Date Signed : August 2018

DEDICATION

This study is dedicated to the Almighty God, for his mercies and goodness over my life. To my parents, Mr and Mrs O.P. Enogieru, for their unconditional love and efforts towards my education.

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LIST OF ABBREVIATIONS

OH	Hydroxyl radical
2-DG	2-deoxy-D-glucose
6-OHDA	6-hydroxydopamine
AD	Alzheimer's disease
AMPK	5' AMP-activated protein kinase
APAF1	Apoptotic protease activating factor 1
APP	Amyloid precursor protein
ATF6	Activating transcription factor 6
ATP	Adenosine triphosphate
BafA1	Bafilomycin
Bcl-2	B-cell lymphoma 2
BDNF	Brain-derived neurotrophic factor
BiP	Binding immunoglobulin protein
Ca^{2+}	Calcium
CAT	Catalase
CCK-8	Cell Counting Kit-8
CHOP	CCAAT-enhancer-binding protein homologous protein
CJD	Creutzfeldt-Jakob disease
CMA	Chaperone-mediated autophagy
CNS	Central nervous system
COX	Cyclooxygenase
CREB	cAMP response element binding
CTLs	Cytotoxic T cells
DA	Dopamine
DAT	Dopamine transporter
DCF	2′,7′-dichlorofluorescein
DCFH-DA	2′,7′-Dichlorofluorescin diacetate
DLP1	Dynamin like protein 1

DMEM	Dulbecco's modified Eagle medium
DMSO	Dimethyl sulfoxide
DOX	Doxorubicin
DßH	Dopamine-β-hydroxylase
ECAR	Extracellular acidification rate
EC-SOD	Extracellular superoxide dismutase
EIF2-α	Eukaryotic Initiation Factor 2 alpha
ER	Endoplasmic reticulum
ERK1	Extracellular signal regulated protein kinase 1
ETC	Electron transport chain
FasL	Fas-ligand
FBS	Fetal bovine serum
FCCP	Carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone
FITC	Fluorescein isothiocyanate
GFAP	Glial fibrillary acidic protein
glycoPER	Glycolytic proton efflux rate
GPx	Glutathione peroxidase
GSH	Glutathione
GSSG	Oxidized GSH
GWAS	Genome-wide association study
H_2O_2	Hydrogen peroxide
HD	Huntington's disease
IKK	Ikβ kinase
IL-1β	Interleukin 1 beta
IL-6	Interleukin 6
iNOS	Nitric oxide synthase
IRE1	Inositol-requiring enzyme 1
kDa	KiloDalton
LAS	Lysosomal autophagy system
LBs	Lewy bodies

LRRK2	Leucine-rich repeat kinase 2
MAO-B	Monoamine oxidase B
MAPK	Mitogen-activated protein kinase
MDA	Malondialdehyde
MMP	Mitochondrial membrane potential
MnSOD	Manganese superoxide dismutase
MPP^+	1-methyl-4-phenylpyridinium
MPPP	I -methyl-4-phenyl-4-propionoxy-piperidine
MPTP	1-methyl-4-phenyl 1,2,3,6-tetrahydropyridine
mtDNA	Mitochondrial DNA
MTPs	Mutant huntingtin proteins
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NDs	Neurodegenerative diseases
NF-κB	Nuclear factor- kappaB
NMDA	N-methyl-D-aspartate
NO	Nitric oxide
NOS	Nitric oxide synthase
O^-_2	Superoxide
OCR	Oxygen consumption rate
OCT3	Organic cation transporter-3
OD	Optical density
$ONOO^-$	Peroxynitrites
PARP	Poly-ADP-ribose polymerase
PBS	Phosphate buffered saline
PCD	Programmed cell death
PD	Parkinson's disease
PE	Phycoerythrin
PER	Proton efflux rate
PERK	Protein kinase RNA-like endoplasmic reticulum kinase
PGs	Prostaglandins

PI	Propidium iodide
PrDs	Human Prion diseases
PrP	Prion protein peptide
PS	Phosphatidylserine
PVDF	Polyvinylidene difluoride
RA	Retinoic acid
RBD	Rapid eye movement sleep behavioral disorder
REM	Rapid eye movement
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SEM	Standard Error of Mean
SNpc	Substantia nigra pars compacta
SOD	Superoxide Dismutase
TBARS	Thiobarbituric acid reactive substances
TBST	Tris-buffered saline with 0.1% Tween-20
TEM	Transmission electron microscope
TFAM	Mitochondrial transcription factor A
TH	Tyrosine-hydroxylase
TNFR	Tumor necrosis factor receptor
TNFα	Tumor necrosis factor alpha
TPA	12-O-tetradecanoylphorbol-13-acetate
UPR	Unfolded protein response
UPS	Ubiquitin proteasome system
VMAT2	Vesicular monoamine transporter 2
WST-8	[2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl-2H-tetrazolium monosodium salt]
WT	Wild-type
XBP	X box-binding protein
α-Syn	α-synuclein

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ABSTRACT

Parkinson's disease (PD) is a common neurodegenerative disorder characterized by the progressive loss of dopaminergic neurons in the substantia nigra pars compacta of the midbrain. Although the etiology of PD is not completely known, it is believed to involve an association of various genetic, cellular, and environmental factors that individually or simultaneously advance neuronal degeneration. Neurotoxins such as 1-methyl-4-phenylpyridinium (MPP^+) and 6-hydroxydopamine (6-OHDA) have been widely used to investigate distinct underlying mechanisms involved in the pathogenesis of PD.

Presently, treatment options for PD are limited, as the available drugs are mainly focused on alleviating symptoms with limited ability to prevent disease progression. Accordingly, there is an increase in the use of natural compounds/products as potential neuroprotective agents. These neuroprotective treatments are believed to intervene in some stages in the pathogenesis of PD to suppress possible mechanisms of dopaminergic neuronal death such as apoptosis, mitochondrial dysfunction, oxidative stress, disturbances of calcium homeostasis, inflammation and autophagy. Thus, novel protective strategies for PD may be designed by targeting these mechanisms or intracellular signaling cascades that participate in PD pathogenesis.

Plant-derived bioactive compounds used in traditional medicine have beneficial effects on some disorders including PD. For example, the bioflavonoid rutin, derives its common name from *Ruta graveolens*, a plant that contains high amounts of rutin. It is present in over 130 registered commercially available medicinal preparations and pharmacological studies have reported the beneficial effects of rutin in many disease conditions. Although rutin has been found to attenuate 6-OHDA toxicity in PC12 cells, its activity in MPP^+-treated SH-SY5Y cells and fibroblasts have not been investigated. Consequently, for the first time, the protective activity of rutin in MPP^+-treated SH-SY5Y cells and primary dermal fibroblasts was investigated, thus revealing possible molecular pathways and mechanisms of action.

Findings from the cell viability studies show that rutin significantly protected SH-SY5Y cells and primary dermal fibroblasts from MPP^+ toxicity. Additional findings further revealed that rutin pretreatment significantly attenuated MPP^+ triggered increase in the production of nitric oxide (NO) and reactive oxygen species (ROS) in SH-SY5Y cells. The attenuation of increased ROS and NO production in SH-SY5Y cells is a crucial mechanism of action for its protection against MPP^+ induced DNA damage and inflammation. This was demonstrated by a significant reduction in the expression levels of DNA damage (γH2AX) and inflammation (COX-2)

markers following rutin pretreatment in SH-SY5Y cells. Also, rutin significantly suppressed MPP^+ induced disruption of antioxidant enzymes and prevented MPP^+ induced damage in nuclear morphology, clearly evidenced by fluorescence images from Hoechst staining showing shrinkage and fragmentation of SH-SY5Y cells.

Meanwhile, the inhibition of p-Akt and p-NF-κB, as well as the activation of p-AMPK in MPP^+ treated SH-SY5Y cells resulted in a cascade of apoptotic, autophagic and endoplasmic reticulum (ER) stress events leading to cell death. The ability of rutin to effectively regulate cell signaling pathways could be responsible for the protection of SH-SY5Y cells from the deleterious effects of apoptosis, autophagy and ER stress. This was demonstrated by a significant increase in the expression of full-length caspase 3 and GRP78/BiP, as well as a significant reduction in the expression levels of cleaved PARP, cytochrome C, LC3-II, p62 and CHOP proteins in pretreated SH-SY5Y cells. In confirmation of the western blot findings on autophagy, transmission electron images revealed abnormal presence/accumulation of numerous autophagosomes in our MPP^+ treated SH-SY5Y cells while there was significantly reduced autophagic vacuoles in SH-SY5Y cells pretreated with rutin, perhaps due to the capacity of rutin to enhance efficient and speedy clearance of these vacuoles.

Furthermore, increased levels of Ca^{2+} and significantly reduced mitochondrial membrane potential in SH-SY5Y cells, as well as significantly reduced maximal respiration and spare respiratory capacity in SH-SY5Y cells and fibroblasts, clearly highlights major characteristics of mitochondrial dysfunction in cells treated with MPP^+. However, these effects were significantly attenuated following rutin pretreatment in SH-SY5Y cells and fibroblasts. Additionally, in SH-SY5Y cells, our findings show that rutin significantly improved basal and compensatory glycolysis as a compensatory response to an impaired oxidative phosphorylation system triggered by MPP^+ which resulted in an insufficient ATP production.

Taken together, proper regulation of the ROS-NO and cell signaling pathways, maintenance of Ca^{2+} homeostasis, mitochondrial protection and efficient autophagy clearance may account for the neuroprotective effects of rutin observed in our dopaminergic SH-SY5Y cells and fibroblasts. These findings further suggest that rutin may be a promising neuroprotective agent for the treatment of PD. Future studies will involve investigating its activity in animal models of PD.

Keywords: Parkinson's disease, rutin, MPP^+, SH-SY5Y cells, fibroblasts, reactive oxygen species, nitric oxide, autophagy, calcium homeostasis, cell signaling, endoplasmic reticulum stress, apoptosis, inflammation.

RESEARCH OUTPUTS

1. **Enogieru, AB**., Omoruyi, SI., Hiss, DC., Ekpo, OE. 2018. Potential antiparkinsonian agents derived from South African medicinal plants. *Journal of Herbal Medicine*. 13, 1-7
2. **Enogieru, AB**., Haylett, W., Hiss, DC., Soraya, B., Ekpo, OE. 2018. Rutin as a potent antioxidant: Implications for neurodegenerative disorders. *Oxidative Medicine and Cellular Longevity*. 2018.
3. **Enogieru, AB**., Omoruyi, SI., Hiss, DC., Ekpo, OE. 2018. Regulation of GRP78/BiP/HSPA5 as a neuroprotective mechanism in experimental models of Parkinson's Disease. *Advances in Pharmacological Sciences.* **(Under review)**
4. Denya, I., Malan, S. F., **Enogieru, A. B**., Omoruyi, S. I., Ekpo, O. E., Kapp, E., Zindo, F. T. & Joubert, J. 2018. Design, synthesis and evaluation of indole derivatives as multifunctional agents against Alzheimer's disease. *Medicinal Chemistry Communications*. 9, 357-370
5. Kapp, E., Visser, H., Sampson, S. L., Malan, S. F., Streicher, E. M., Foka, G. B., Warner, D. F., Omoruyi, S. I., **Enogieru, A. B**., Ekpo, O. E., Zindo, F. T., & Jacques J. 2017. Versatility of 7-substituted coumarin molecules as antimycobacterial agents, neuronal enzyme inhibitors and neuroprotective agents. *Molecules*, 22(10), p.1644
6. **Enogieru, AB**., Haylett, W., van Dyk, H., van der Westhuizen, F., Hiss, DC., Ekpo, OE. Rutin attenuates endoplasmic reticulum stress, impaired calcium homeostasis and altered bioenergetic functions in MPP^+-treated SH-SY5Y neuroblastoma cells. *Neurotoxicology.* **(Under review)**

Manuscripts in Preparation

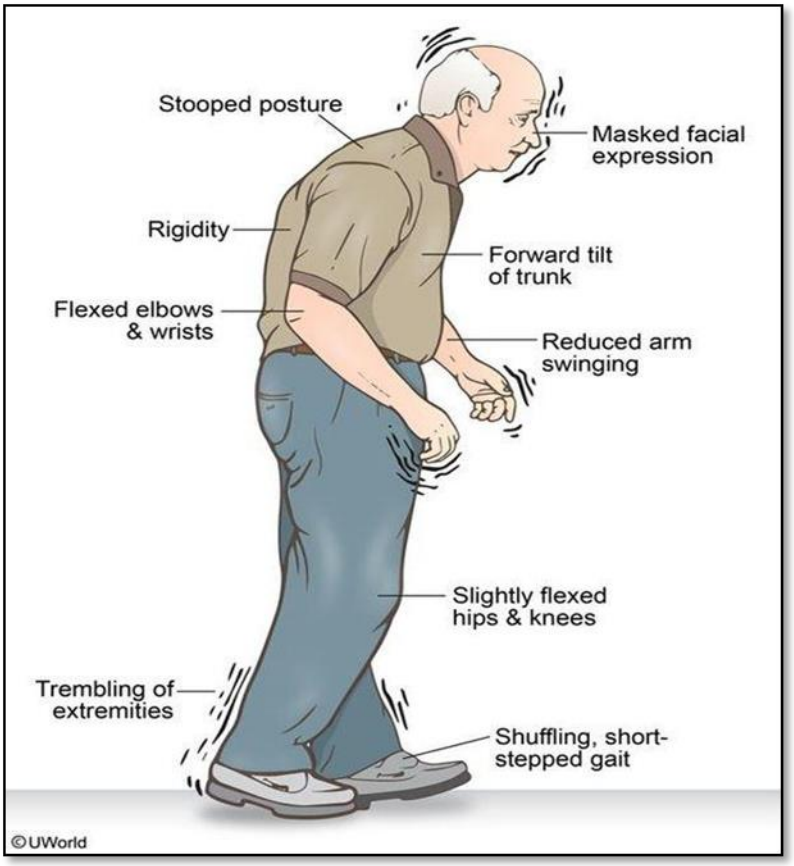
7. **Enogieru, AB**., Haylett, W., Hiss, DC., Ekpo, OE. Rutin Protects SH-SY5Y cells from Toxin-Induced Oxidative stress, DNA damage and Inflammation.
8. **Enogieru, AB**., Haylett, W., Hiss, DC., Ekpo, OE. Rutin mitigates MPP^+ induced neurotoxicity through the regulation of Akt, AMPK and NF-κB signaling pathways.

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1. BACKGROUND

Parkinson's disease (PD) is the second most common neurodegenerative disorder after Alzheimer's disease (Yang et al., 2016). About two centuries ago, PD was first illustrated by James Parkinson in his 1817 monograph 'An Essay on Shaking Palsy' (Parkinson, 2002).



Source: https://www.quora.com/What-are-the-symptoms-of-parkinsons

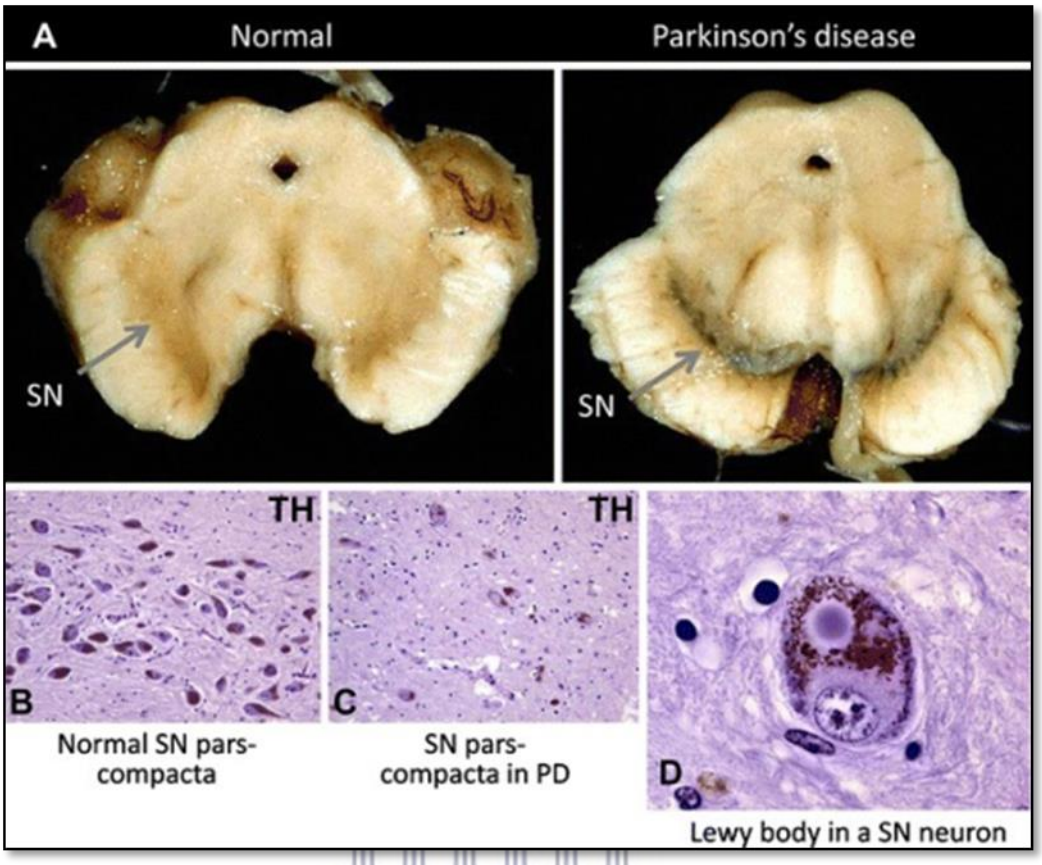
Figure 1.1: An illustration of a typical appearance of Parkinson's disease

In his words, he reported patients with "*Involuntary tremulous motion, with lessened muscular power, in parts not in action and even when supported; with a propensity to bend the trunk forwards, and to pass from a walking to a running pace: the senses and intellects being uninjured".* The motor symptoms that mostly affect the quality of life in PD patients include gait disorders (Figure 1.1), instability and fluctuations arising from motor impairment (Gomez-Esteban et al., 2007, Berganzo et al., 2016). In addition, PD is associated with non-motor symptoms including disorders in sleep, mood and cognitive functions (Barone et al., 2009, Chaudhuri and Schapira, 2009, Gómez-Esteban et al., 2011, Martinez-Martin et al., 2011). Mood and sleep disorders are known to be the most frequent and disruptive symptoms in PD patients (Gómez-Esteban et al., 2006, Barone et al., 2009, Gómez-Esteban et al., 2011, Martinez-Martin et al., 2011).

One of the frequently applied classifications of PD is based on the age of onset in patients. For example, patients are classified as juvenile PD (onset 20 years of age), early-onset PD (onset 21– 40 years of age), and late-onset PD (onset 40 years of age) (Quinn et al., 1987, Gershanik, 2003, Reider et al., 2003). There are numerous reports on the epidemiology of PD, but dissimilarities in methodologies make it difficult to compare its prevalence (Tysnes and Storstein, 2017). A previous report showed that PD prevalence increased with age, with 41 cases occurring in every 100,000 in 40-49 year olds; 107 in every 100,000 in 50-59 year olds; 173 in every 100,000 in 55-64 year olds; 428 in every 100,000 in 60-69 years; 425 in every 100,000 in 65-74 year olds; 1087 in every 100,000 in 70-79 year olds and 1903 in every 100,000 in ages older than 80 (Pringsheim et al., 2014). In addition, global incidence rates are estimated to be 12.3 per 100 000, and 44.0 per 100 000 precisely for individuals over the age of 50 (Van Den Eeden et al., 2003). Factors that may increase the risk of PD include age, sex, genes and environment (Lee et al., 2016, Savica et al., 2016).

1.2. PATHOLOGY OF PARKINSON'S DISEASE

The core pathological hallmark of PD is represented by degeneration of neuromelanin-containing dopaminergic neurons associated with the presence of intra-cytoplasmatic inclusions of ubiquitin and α-synuclein (α-Syn) denominated Lewy bodies (Goldman et al., 1983) in the substantia nigra pars compacta (SNpc) (Figure 1.2) of the ventral midbrain (Chinta and Andersen, 2005). Although dopaminergic neurons are less than 1% of total brain neurons, they are essential for the regulation of brain function. These neurons constitute a functionally and anatomically diverse group of cells contained in the diencephalon, mesencephalon and the olfactory bulb (Bjorklund, 1984). The most notable dopaminergic cell group is located in the ventral part of the mesencephalon containing nearly 90% of all dopaminergic neurons in the brain (Chinta and Andersen, 2005). A vital part of the mesencephalic dopaminergic system is the nigrostriatal system, which originates in the zona compacta of the SNpc and spreads its fibres into the dorsal striatum (caudate-putamen) to control voluntary motor movement (Chinta and Andersen, 2005). Remarkably, motor symptoms of PD are displayed once degeneration of the dopaminergic nigrostriatal pathway has reached around 50–60% (Fearnley and Lees, 1991).



Source: (Mandel et al., 2010): Abbreviations: SN - Substantia nigra, TH - Tyrosine-hydroxylase

Figure 1.2: Diagram showing Substantia nigra degeneration in PD

It is believed that there is a protracted period before the display of motor symptoms, which involves an impairment of other neurotransmitters such as acetylcholine, noradrenaline, serotonin, glutamate and adenosine (Schapira et al., 2006, Mandel et al., 2010). These impairments are often linked to the non-motor defects in PD which often appear before the motor symptoms occasionally few years prior and are thought to be more devastating to PD patients (Mandel et al., 2010). Common manifestations include recurrent falling, cognitive decline, freezing, anxiety, depression and anosmia (Langston, 2006). This concept is supported by the post-mortem study of Braak and colleagues who mapped the sequence of disease in a large series of individuals suffering from PD (Braak et al., 2003). The distribution of Lewy bodies (LBs) seems to follow a specific temporal (subdivided into phases 1 to 6) and anatomical

distribution. In the pre-symptomatic phases 1–2, the inclusion bodies are restricted to the medulla oblongata/pontine tegmentum and olfactory bulb/anterior olfactory nucleus (Sveinbjornsdottir, 2016); phases 3-4 are associated with the development of the clinical features of the disease, with the SNpc and other nuclei of the midbrain and forebrain being affected (Sveinbjornsdottir, 2016); while in phases 5-6, the neocortex becomes affected and manifests with a wide range of clinical features (Braak et al., 2004).

1.3. CLINICAL CHARACTERISTICS OF PARKINSON'S DISEASE

There are four principal characteristics of PD often classified under the TRAP acronym: tremor at rest, rigidity, akinesia (or bradykinesia) and postural instability (Jankovic, 2008). Due to the distinct profiles and lifestyles of those affected by PD, motor and non-motor symptoms are assessed according to each patient's peculiarity (Jankovic, 2003, 2008). PD was previously considered to be a condition that affects only the motor system but with more research, it is now known to be a multifaceted disorder with diverse clinical features that include sleep disorders, dysautonomia, cognitive and neuropsychiatric disorders (Chaudhuri and Sauerbier, 2016, Krüger et al., 2017).

1.3.1. Motor Symptoms

1.3.1.1. Tremor at Rest

Different forms of tremor affect PD patients but the classic is tremor-at-rest (Hallett, 2012) which refers to a 4- to 6-Hz pill-rolling tremor in the fully resting limb, although repressed throughout commencement of movement, maximum amplitude is attained after 2–3 minutes (Raethjen et al., 2008, Deuschl et al., 2012). Rest tremor is most prominent on one side of the body, affecting the upper and lower limbs, tongue chin and lips, but hardly involves the head (Chen et al., 2017). There are several reports on the challenges in diagnosing tremor disorders due to the lack of biomarkers; thus resulting in frequent errors in differentiating diagnoses between parkinsonian, dystonic and essential tremors (Jain et al., 2006). Tremor is examined

by such characteristic features as activation condition, topography, frequency and amplitude, medical history (onset age, family history, and temporal evolution), and associated systemic or neurological signs (Chen et al., 2017).

1.3.1.2. Rigidity

Rigidity, a cardinal feature of PD, is commonly described as "cogwheel rigidity." in PD patients (Jankovic, 2008, Ruiz et al., 2011, Berardelli et al., 2013). The only sign produced by rigidity is a feeling of stiffness (Berardelli et al., 1983). It manifests as amplified muscle tone or intensified resistance when stretching a muscle passively. Possible mechanisms include an exaggeration of the long-latency and monosynaptic stretch reflexes, the development of a shortening reaction and the development of a tonic stretch reflex (Berardelli et al., 1983). Rigidity in PD is known to affect the face, thus presenting a "masked" appearance (Jankovic, 2008, Munhoz et al., 2010, Reichmann, 2010, Xia and Mao, 2012).

1.3.1.3. Bradykinesia

Bradykinesia is a standard criterion in PD commonly observed as a clinical feature in patients (DeMaagd and Philip, 2015) and is characterized by a decline in the speed and magnitude of action of voluntary movements (Grabli et al., 2012). Bradykinesia manifests later than tremor, although as observed in some circumstances, it may be the preliminary symptom without subsequent development of tremor (i.e., the akinetic-rigid subtype of PD) (Ruiz et al., 2011, Xia and Mao, 2012). In bradykinesia, physical movements are significantly slower than normal, thus resulting in a characteristic slow, shuffling walk with minimal steps (Katzen et al., 2006, Heldman et al., 2011, Shiner et al., 2012, Carlsen et al., 2013, Sage and Mark, 2015). Also, patients may find it extremely difficult to move when confronted by the need to turn or pass through a narrow door (Mendon and Jog, 2008).

1.3.1.4. Postural instability

Postural instability is a marker of disease progression occurring at the end-stages of PD (Rewar, 2015). It is associated with loss of balance, recurrent falls and related injuries, thus impeding the quality of life (Doherty et al., 2011, Kang et al., 2012, Pfeiffer, 2012).

1.3.2. Non-Motor Symptoms

1.3.2.1. Sleep disorders

Reports show that PD patients have sleep disturbances which start early in PD progression (Chaudhuri, 2003, Garcia-Borreguero et al., 2003). The causes of sleep disturbance are multifactorial, but it is believed that the degeneration and impairment of sleep regulation centres in the brainstem and thalamocortical pathways are important mechanisms for this disorder (Chaudhuri et al., 2006). Predominantly frequent is the rapid eye movement (REM) sleep behavioral disorder (RBD) existing in nearly 40% of PD patients (Schenck et al., 1996, Olson et al., 2000, Schenck and Mahowald, 2002). RBD is a parasomnia, defined as an unwanted experience that occurs during entry into sleep, during sleep, or during arousal from sleep (Rodriguez et al., 2017). It is characterized by loss of normal skeletal muscle atonia during REM sleep, thereby allowing PD patients to physically act-out their often miserable dreams (Gagnon et al., 2002). Other common sleep disturbances include frequent awakenings, excessive daytime sleepiness, insomnia and sleep attacks (Maass and Reichmann, 2013).

1.3.2.2. Dysautonomia

The severity of dysautonomia in PD is known to be inconsistent but is however a major feature of multiple system atrophy (Chaudhuri, 2001, Magerkurth et al., 2005). Its complex pathophysiology includes degeneration and impairment of the nuclei facilitating autonomic functions (Benarroch, 1992). Autonomic dysfunction is virtually always present in patients with PD and may include abnormalities of cardiovascular, respiratory, gastrointestinal, bladder and sexual function as well as impairment of thermoregulation and sweating (Kaufmann and

Goldstein, 2013). For instance, Yu and colleagues reported significant dysfunction in sexual stimulation, drive and orgasm in 17 of 21 male PD patients (Yu et al., 2004).

1.3.2.3. Cognitive and neuropsychiatric disorders

Cognitive and neuropsychiatric disorders of PD include anxiety, apathy, depression and dementia (Aarsland et al., 1999, Thanvi et al., 2003). Psychosis is a leading factor prompting the necessity for nursing home placement for PD patients (Chaudhuri et al., 2006). It is reported that 40% of PD patients experience benign visual hallucinations while more threatening indications such as delusions, restlessness and incoherence become more recurrent as PD advances (Diederich et al., 2005). Furthermore, depression inhibits the quality of life (Aarsland et al., 2000, Schrag et al., 2000, Global, 2002), affecting up to 10–45% of PD patients (Burn, 2002). This may result from impairment to serotoninergic neurotransmission as well as limbic noradrenergic and dopaminergic mechanisms (Remy et al., 2005). Similarly, anxiety disorders are common in PD (Shiba et al., 2000, Weisskopf et al., 2003), often presenting as phobias or panic attacks (Chaudhuri et al., 2006). Also, dementia occurs in about 40% of PD patients, amounting to a six-fold higher rate than in healthy individuals (Emre, 2003).

1.4. PARKINSON'S DISEASE RISK FACTORS

The precise cause of PD is yet unknown but theories suggest that a combination of age, genetic and non-genetic risk factors contribute to PD development (Allam et al., 2005).

1.4.1. Genetic risk factors

Earlier studies have shown that family members of affected PD patients are at a 3- to 4-fold increased risk when compared to individuals in the general population (Autere et al., 2000, Kurz et al., 2003). A genetic cause of the disease has been hypothesized for several decades owing to occurrence of PD within families and pedigrees (Alves et al., 2008). Accordingly, several gene loci were found to be linked with autosomal-dominantly (Gasser et al., 1998, Leroy

et al., 1998, Paisán-Ruíz et al., 2004, Zimprich et al., 2004) or recessively (Kitada et al., 1998, Valente et al., 2001, Van Duijn et al., 2001) inherited PD. Most of the known gene mutations cause juvenile or early onset of the disease, while others such as leucine-rich repeat kinase 2 (*LRRK2*) mutations seem to cause parkinsonism resembling sporadic PD with respect to both clinical and demographical features (Aasly et al., 2005, Haugarvoll et al., 2008). The detection of these genetic mutations and dysfunction of abnormally encoded proteins delivered novel understandings into the molecular pathogenesis of PD (Alves et al., 2008). Today, there is convincing evidence to support the deficiency of the ubiquitin-proteasome system, dysfunction of the mitochondria and reduced tolerance of oxidative stress as additional mechanisms in PD pathogenesis (Eriksen et al., 2005, Gandhi and Wood, 2005). Nevertheless, the exact mechanisms are not entirely understood (Olanow, 2007), with monogenetic causes only accounting for 10% of all PD cases while majority appear to be sporadic (Alves et al., 2008). Some confirmed PD-associated loci and genes is summarized in Table 1.1

Table 1.1: Summary of some confirmed PD-linked loci and genes

Locus	Gene	Protein	Disorder	Inheritance	Mutation	Protein function
PARK1	*SNCA*	α-synuclein	Late onset PD	Autosomal Dominant	Point mutations	Maintains a supply of synaptic vesicles in presynaptic terminals by clustering synaptic vesicles
PARK2	*Parkin*	Parkin	Early Onset PD	Autosomal Recessive	Point mutations	Assists in mitophagy and clearance of proteins; Enhances cell survival
PARK4	*SNCA*	α-synuclein	Late onset PD	Autosomal Dominant	Point mutations	Maintains a supply of synaptic vesicles in presynaptic terminals by clustering synaptic vesicles
PARK6	*PINK1*	PTEN putative induced kinase	Early Onset PD	Autosomal Recessive	Point mutations	Protects mitochondria from malfunctioning during periods of cellular stress
PARK7	*DJ-1*	Oncogene DJ-1	Early Onset PD	Autosomal Recessive	Point mutations	Participates in transcriptional regulation, anti-oxidative stress reaction; Regulates chaperone, protease, and mitochondrial activity
PARK8	*LRRK2*	Leucine rich repeat kinase 2	Late onset PD	Autosomal Dominant	Point mutations	Regulation of cytoskeletal reorganization, vesicle trafficking, protein synthesis, protein homeostasis, autophagy, and inflammation
PARK9	*ATP13A2*	P5 subfamily of ATPases	Early Onset PD	Autosomal Recessive	Point mutations	Controls mitochondrial maintenance; Possible role in autophagy-lysosomal activity
PARK14	*PLA2G6*	Phospholipase A2, Group VI	Early-onset dystonia-parkinsonism	Autosomal Recessive	Point mutations	Catalyzes the release of fatty acids from phospholipids
PARK15	*FBX07*	F-box only protein 7	Early-onset parkinsonian-pyramidal syndrome	Autosomal Recessive	Point mutations	Target proteins for ubiquitination.
PARK17	*VPS35*	Vacuolar sorting protein 35	Late onset PD	Autosomal Dominant	Point mutations	Involved in retrograde transport of proteins from endosomes to the trans-Golgi network

Source: Modified from (Klein and Westenberger, 2012).

Abbreviations: *FBOX7* - F-box only protein 7; *LRRK2* - leucine-rich repeat kinase 2; *PINK1* - PTEN-induced kinase 1; *SNCA* - α synuclein *VPS35* - vacuolar protein sorting 35

1.4.2. Non-Genetic risk factors

Previously, environmental, lifestyle and occupational risk factors have been implicated in PD; however, studies have revealed conflicting results (Lai et al., 2002, Elbaz and Tranchant, 2007). Most notably, pesticides are associated with a greater risk for PD development (Alves et al., 2008).

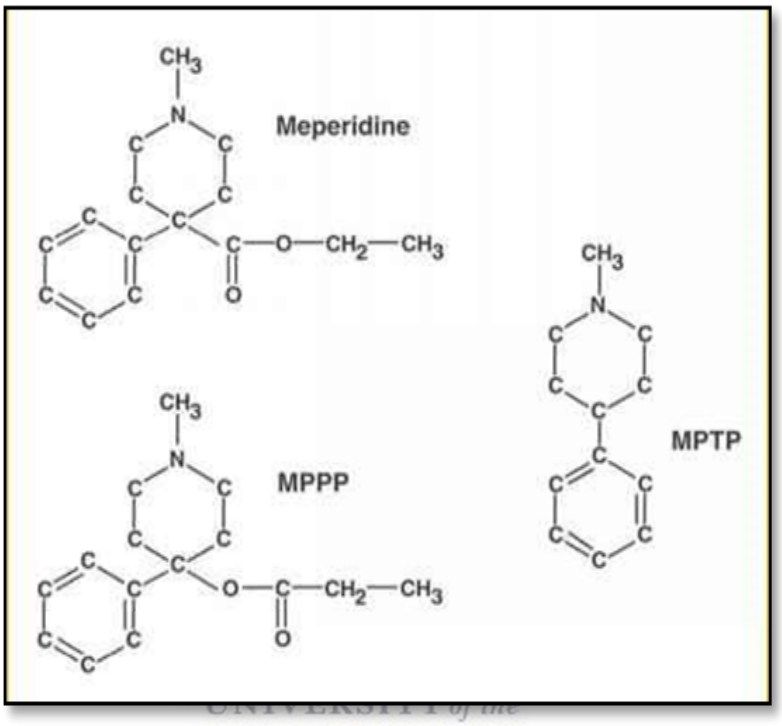
1.4.2.1. Environmental factors

In the absence of a clear genetic cause for the common cases of sporadic PD, several epidemiological studies have been performed to determine the important factors (Warner and Schapira, 2003). For example, rural living (in Quebec and Sweden) has been acknowledged by studies to increase the relative risk of PD development, even though this observation has not been seen consistently (Barbeau et al., 1987, Semchuk et al., 1992, Gorrell et al., 1996, Fall et al., 1999). A case-control study showed that the link between rural living and the agricultural industry in Germany increases the risk of PD in workers from this environment (Seidler et al., 1996). Also, the revelation that 1-methyl-4-phenyl 1,2,3,6-tetrahydropyridine (MPTP), a toxin of a synthetic opiate, causes parkinsonism through its neurotoxic metabolite, 1-methyl-4-phenylpyridinium (MPP^+), elicited interest in environmental chemical exposures as risk factors for PD (Langston et al., 1983). Numerous studies investigating the link between PD and pesticide use have established an association (Priyadarshi et al., 2000, Zorzon et al., 2002, Firestone et al., 2005). Similarly, the use of well water has also been implicated as a risk factor (Lai et al., 2002, Zorzon et al., 2002).

1.4.2.1.1. 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)

In the 1980s, drug abusers who unintentionally self-administered MPTP, a by-product in the synthesis of 1-methyl-4-phenyl-4-propionoxy-piperidine (MPPP), a meperidine analog (Figure 1.3), developed a severe motor disorder which closely resembles an advanced stage of PD (Langston et al., 1983). The toxicity of MPTP was not recognized until the manifestation of a

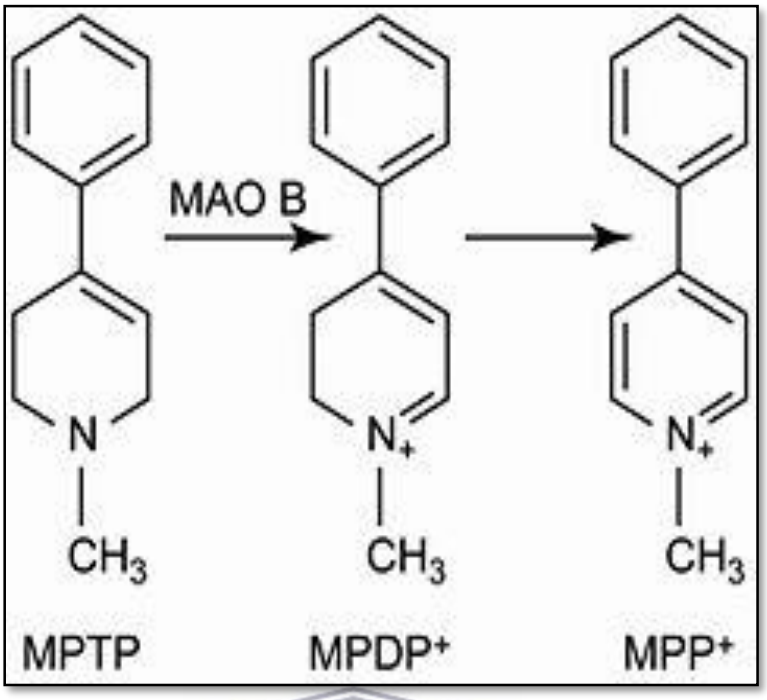
parkinsonian syndrome among the drug abusers. After the discovery of MPTP, there was an upsurge in research aimed at determining its effect on the SNpc (Sahgal et al., 1984, Burns et al., 1985).



Source: https://www.drugtimes.org/designer-drugs/meperidine-and-its-analogues.html

Figure 1.3: Chemical structures of MPTP, meperidine and MPPP

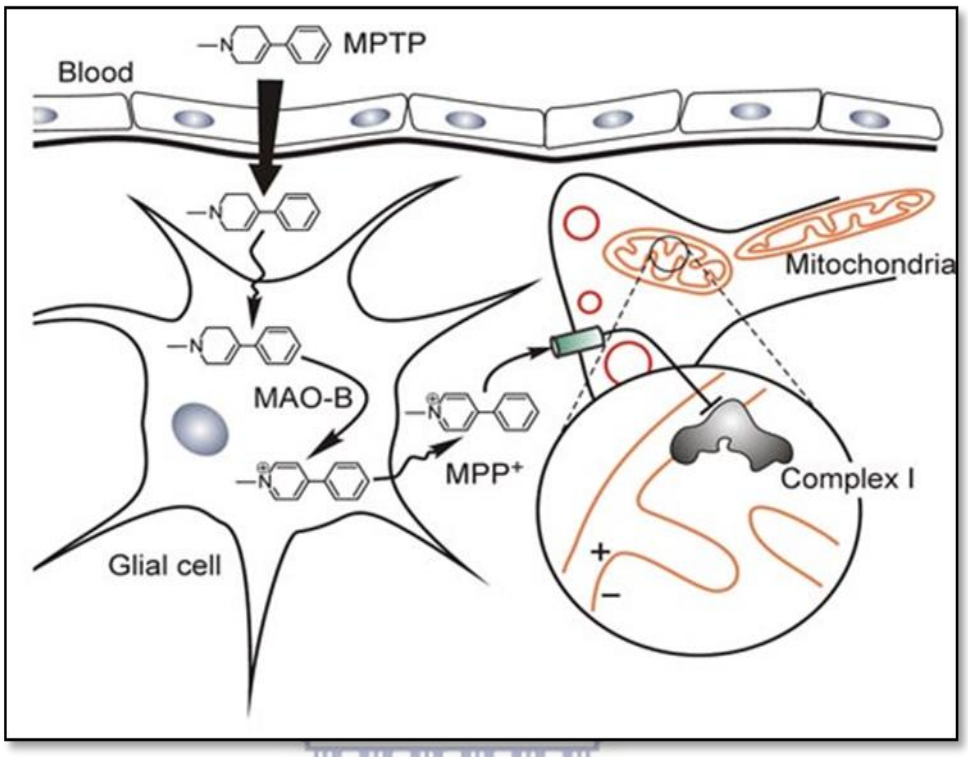
A second scientific discovery showed that although MPTP as a lipophilic compound readily enters into the brain, it was not toxic until it is converted to its toxic metabolite, MPP^+ (Figure 1.4) (Langston et al., 1984). This observation was further confirmed by Markey and colleagues (Markey et al., 1984).



Source: https://bsd.neuroinf.jp/w/images/thumb/9/95/MPTP_Fig2.jpg/240px-MPTP_Fig2.jpg

Figure 1.4: Chemical structures of MPTP and MPP^+

Thereafter, it was found that this biotransformation was mediated by monoamine oxidase-B (MAO-B) (Chiba et al., 1984, Heikkila et al., 1984b, Castagnoli Jr et al., 1985) expressed in astrocytes (Figure 1.5). MPP^+ is then released from astrocytes via the organic cation transporter-3 (OCT3) (Cui et al., 2009) and is taken up by the presynaptic dopamine transporter on dopaminergic nerve terminals. Inhibition of complex 1 occurs when the MPP^+ not sequestered into vesicles by the vesicular monoamine transporter is taken up by the mitochondria, thereby disrupting oxidative phosphorylation (OXPHOS) (Goldman, 2014). Consequently, this disruption reduces adenosine triphosphate (ATP) synthesis and stresses mitochondria by generating free radicals to cause membrane disruption and lipid peroxidation, thus creating a feedback that results in cell death and bioenergetic failure (Przedborski and Jackson-Lewis, 1998).



Source:http://www.qub.ac.uk/schools/SchoolofBiologicalSciences/Connect/AcademicStaff/DrAGalkin/Bioenergetics/AllaroundBioenergetics/

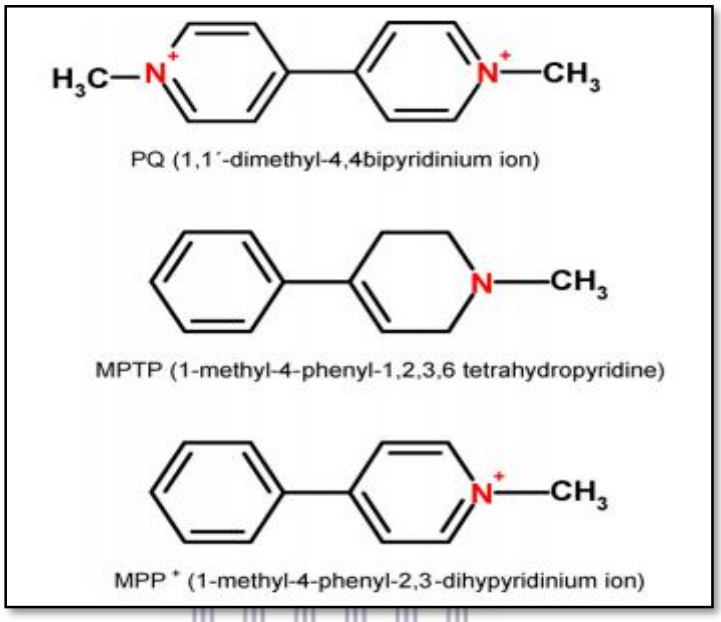
Figure 1.5: Metabolism of MPP^+ in neurons

In emphasizing the importance of the MPTP/MPP^+ experimental model of PD, dysfunction of the mitochondria is a prominent characteristic similar to that seen in conditions of idiopathic PD.

1.4.2.1.2. Pesticides

Over the years, the consequences of environmental chemical exposures have been a significant area of interest, with substantial evidence linking pesticides and herbicides to PD pathogenesis. For example, numerous epidemiological studies suggest that PD is predominantly prevalent in rural agricultural areas (Wirdefeldt et al., 2011, Van Maele-Fabry et al., 2012). In a systematic review and meta-analysis study by Van Maele-Fabry and colleagues, farmers for 10-20 years were 17-16% more likely to have PD than non-farmers and a 28% increased risk was projected between work-related exposure to pesticides and PD (Van Maele-Fabry et al., 2012, Greener,

2013). Interestingly, this association seems biologically conceivable due to the structural similarity of MPTP (Figure 1.6) to some herbicides and pesticides, including paraquat (Wirdefeldt et al., 2011).



Source: (Dinis-Oliveira et al., 2006)

Figure 1.6: Structural similarity of MPP^+ to paraquat

Paraquat (1,1 -dimethyl-4,4 -bipyridinium dichloride), a quaternary nitrogen herbicide structurally comparable to MPP^+ and commercially available since 1962, is a commonly used pesticide (Goldman, 2014). Paraquat crosses the blood-brain barrier (BBB) via the neutral amino acid transporter (McCormack and Di Monte, 2003) and is then taken up into nigral dopaminergic terminals by the dopamine transporter after it is converted to $paraquat^+$ (Rappold et al., 2011). Paraquat produces large amounts of reactive oxygen species (ROS) through redox cycling and generation of superoxide (O^-_2) radicals (McCormack et al., 2005). In animal models, it impairs mitochondrial function, increases expression and aggregation of α-Syn, increases lipid peroxidation, selectively kills nigral dopaminergic neurons and decreases glutathione (GSH) levels (McCormack et al., 2002, Kuter et al., 2010). It is believed that its

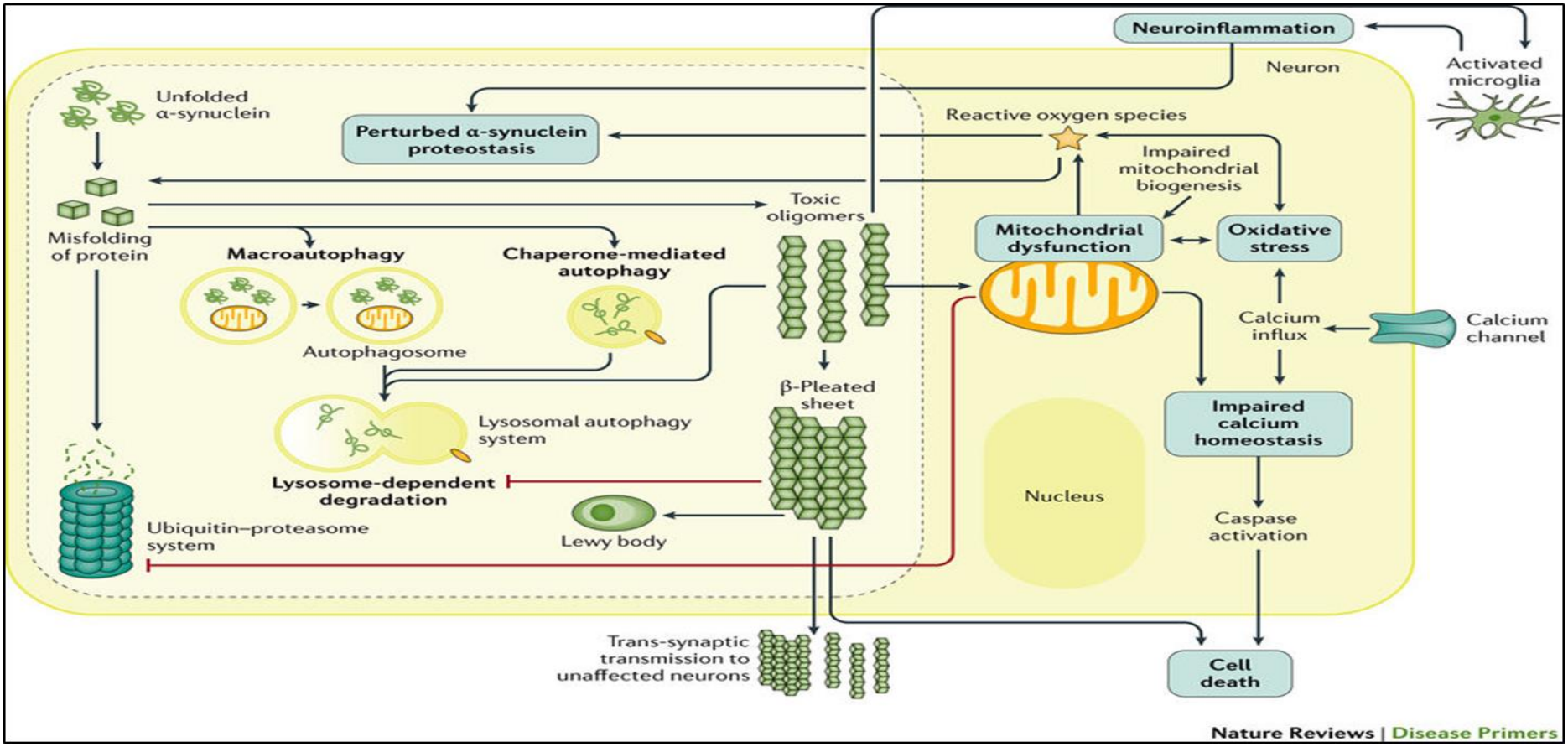
relative selectivity for nigral dopaminergic neurons is partly due to the increased susceptibility of these neurons to oxidative stress.

Other classes of pesticides identified to hinder dopaminergic activity include maneb, a dithiocarbamate fungicide associated with neuronal damages in agricultural workers (Thiruchelvam et al., 2000). Similarly, rotenone is a mitochondrial toxin that impairs complex I of the electron transport chain (ETC) - the same site inhibited by MPP^+ (Sherer et al., 2007). It is commonly used as an insecticide in agriculture and household pet products (Goldman, 2014). Rotenone containing plants are used routinely by local people to catch fish and is presently used as a pesticide to exterminate invasive fish species in lakes (Goldman, 2014). In animal models, rotenone impairs mitochondrial function, generates ROS, reduces ATP production, impairs proteasome function and activates microglia (Betarbet et al., 2006). Although its toxicity is largely due to inhibition of mitochondrial complex I, other primary effects such as microtubule destabilization have been suggested (Choi et al., 2008). Like MPTP, rotenone causes an accumulation of cytoplasmic α-Syn and selectively degenerates nigral dopaminergic neurons. Also, it induces a parkinsonian syndrome that includes rigidity, bradykinesia, tremor as well as some of the non-motor signs seen in PD (Cannon et al., 2009). Epidemiologic data on rotenone exposure in humans are sparse; however, Tanner and colleagues found a significant 2.5-fold increased risk of PD in individuals who utilize rotenone in their activities (Tanner et al., 2011).

1.5. MECHANISMS AND PATHWAYS INVOLVED IN PARKINSON'S DISEASE

Overwhelming evidence from studies on toxin models of PD and gene-functional studies suggest some essential mechanisms involved in the pathogenesis of PD (Poewe et al., 2017). One hypothesis is that abnormal protein folding and aggregation contribute to dopaminergic neuronal death while another indicates that the primary mechanism is the dysfunction of the mitochondria alongside oxidative stress (Dauer and Przedborski, 2003, Poewe et al., 2017).

Some of the identified PD-linked genes code for proteins that partake in molecular pathways that trigger neuropathology when disturbed (Poewe et al., 2017). These pathways include oxidative stress, α-Syn proteostasis, calcium homeostasis, mitochondrial function and neuroinflammation (Figure 1.7) (Poewe et al., 2017). In the following sections, these aspects will be emphasized to clarify and comprehend the pathways that may be implicated in the pathogenesis of PD.



Source: (Poewe et al., 2017)

Figure 1.7: Central molecular pathways and interactions involved in PD pathogenesis

1.5.1. α-Synuclein proteostasis

One of the most researched amyloid-forming proteins is α-Syn and its aggregation is often associated with PD pathogenesis (Roodveldt et al., 2009). The presence of mutations and multiplications of *SNCA* (the gene encoding α-Syn, Table 1.1) strongly supports the concept that α-Syn is a crucial factor in PD (Vekrellis et al., 2011, Nalls et al., 2014). Genome-wide association studies (GWAS) revealed a single-nucleotide polymorphism linked with the *SNCA* locus associated with an increase in expression levels of α-Syn (Vekrellis et al., 2011, Nalls et al., 2014). In another study, post-mortem samples and human neurons of PD patients supported the idea that a risk variant linked to PD in a non-coding distal enhancer element of *SNCA* is coupled to an increase in α-Syn expression (Soldner et al., 2016)

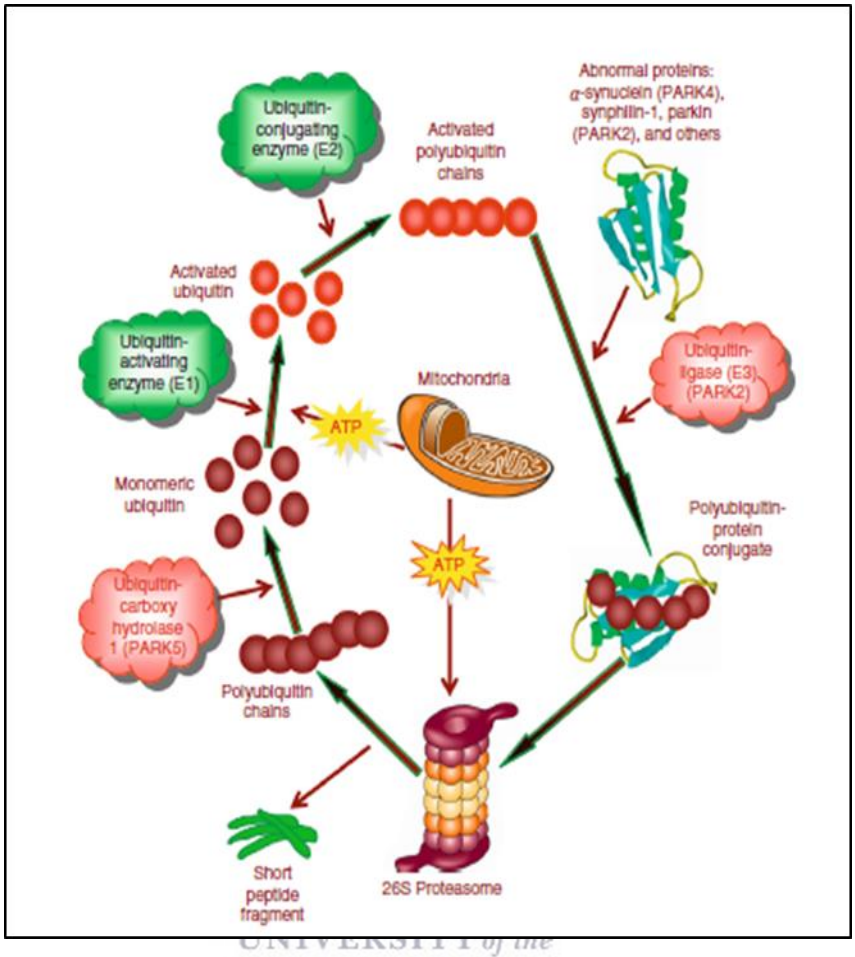
While the common neuronal function of α-Syn is not entirely understood, it is known to occur in the cytosol and probably in mitochondria and the nucleus. It is believed to have a function in mitochondrial and synaptic vesicle dynamics, intracellular trafficking and may also possess a chaperone activity (Vekrellis et al., 2011, Wales et al., 2013, Burré, 2015). α-Syn becomes toxic during a process in which soluble α-Syn monomers gradually unite to produce large insoluble α-Syn fibrils (Kim and Lee, 2008, Melki, 2015). The primary causes of α-Syn accumulation and aggregation include (i) excessive generation of the protein, (ii) presence of mutations that promote its misfolding and oligomerization, and (iii) deficiencies in molecular pathways responsible for the degradation of misfolded α-Syn (Poewe et al., 2017). Also, a progressive, age-related decline in proteolytic defense mechanisms in the ageing brain may have a crucial role in α-Syn accumulation (Xilouri et al., 2013, Kaushik and Cuervo, 2015).

Several reports have proposed that α-Syn can self-propagate (Recasens and Dehay, 2014). Usually, little aggregates are disposed-of by the protein degradation pathways; however, these aggregates can mount up over-time and self-propagate, thereby leading to PD advancement (Prusiner, 2012, Xu and Pu, 2016). Other reports also demonstrates the spreading of α-Syn from

cell to cell and from region to region in a process that promotes PD pathogenesis (Desplats et al., 2009, Hansen et al., 2011, Lelan et al., 2011, Luk et al., 2012a, Luk et al., 2012b, Aulić et al., 2014, Ulusoy et al., 2015).

1.5.2. Ubiquitin-proteasome system (UPS)

The UPS is an intracellular protein degradation system that regulates protein turnover within a cell (Figure 1.8) (Hershko and Ciechanover, 1992). The UPS degrades misfolded and damaged proteins and is thus involved in such conditions as neurodegenerative disorders and cancer (Heinemeyer et al., 1991, Schwartz and Ciechanover, 1999). Given the damaging implications of unregulated protein degradation, the UPS uses a class of enzymes to covalently link ubiquitin polypeptide chains to proteins, and targets those proteins as substrates for the proteasome, thereby enabling targeted or selective degradation (McNaught et al., 2001, Giasson and Lee, 2003). The UPS is managed by three enzymes – namely an E1 ubiquitin-activating enzyme, E2 ubiquitin-conjugating enzymes, and E3 ubiquitin-ligating enzymes. Initially, the E1 enzymes initiate an ATP-dependent process that results in a highly reactive ubiquitin thiol ester being transferred to E2 enzymes. The E3 class of enzymes identify, bind degradation-marked proteins and catalyze the transfer of ubiquitin chains from the E2 to lysine residues on protein substrates, thus serving as an indicator for proteasome-moderated degradation (Cook and Petrucelli, 2009).



Source: (Shadrina et al., 2010)

Figure 1.8: The ubiquitin–proteasome system

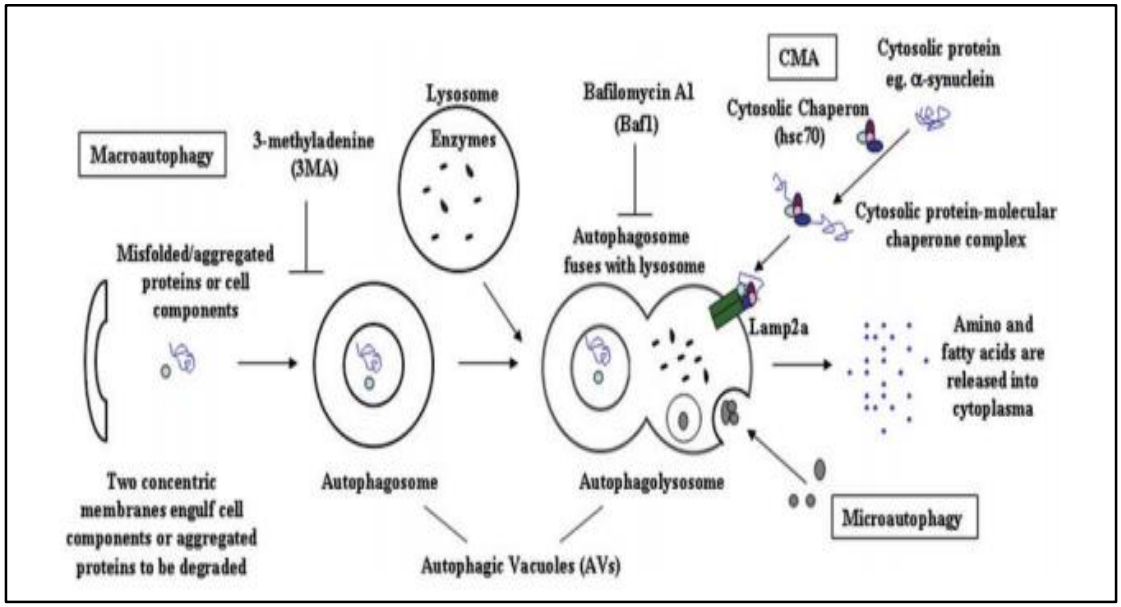
The association between UPS dysfunction and PD has been supported by some studies indicating decreased proteasome activity after exposure to PD-linked toxins (Wang et al., 2005, Betarbet et al., 2006, Wang et al., 2006). For instance, decreased proteasome activity was observed in the midbrain of rats treated with rotenone (Betarbet et al., 2006). Interestingly, utilization of osmotic minipumps to deliver MPTP to mice caused the formation of α-Syn and ubiquitin-positive inclusions, reduced proteolytic and motor activity (Fornai et al., 2005). Remarkably, when experiments were simulated in α-Syn-lacking mice, the effects were alleviated (Fornai et al., 2005), thus suggesting that α-Syn aggravates the harmful effects of

MPTP on UPS function. These findings indicate that α-Syn and UPS dysfunction is implicated in PD (Cook and Petrucelli, 2009).

1.5.3. Lysosomal Autophagy System (LAS).

Autophagy is a cellular catabolic process wherein cytosolic constituents (proteins and protein aggregates) and defunct organelles are conveyed to the lysosome for degradation (Rivero-Ríos et al., 2016). The LAS is separated into macroautophagy (henceforth called autophagy, Figure 1.9), chaperone-mediated autophagy (CMA) and microautophagy (Cuervo et al., 2004, Levine and Klionsky, 2004).

Autophagy involves the development of double-membrane autophagosomes (Pan et al., 2008). Fusion of the autophagosome with lysosomes results in the formation of autophagolysosomes, with both collectively known as autophagic vacuoles (key features of autophagy) (Takeuchi et al., 2005). Furthermore, when the breakdown of the inner membrane structure of the autophagolysosome occurs, vacuolar components are processed and recycled to yield needed cellular energy and amino acids (Figure 1.9).



Source: (Pan et al., 2008)

Figure 1.9: Lysosomal autophagy-system (LAS) in mammalian cells

Microautophagy occurs in a process whereby proteins are removed from the lysosomal membrane in a slow, steady and non-stop manner even under resting conditions whereas CMA is a process that involves the binding of hsc70, a molecular chaperone, to the lysosomal membrane receptor for degradation in the lysosomes (Crotzer and Blum, 2005). It is believed that CMA occurs temporarily as a secondary response following the activation of autophagy (Pan et al., 2008).

The dysfunction of the LAS may occur in several ways, for example, through the defect in autophagosome or autophagolysosome formation, deficiency of lysosomal enzymes or a malfunction of hsc70, individually or concurrently resulting in the accumulation of aberrant proteins leading to cell death (Pan et al., 2008). Consequently, in conditions associated with accumulation of aberrant proteins, autophagy is an extremely vital process that helps to facilitate their elimination before they become toxic (Webb et al., 2003, Ravikumar et al., 2006, Rubinsztein et al., 2007).

1.5.3.1. Autophagy and Parkinson's Disease Pathogenesis

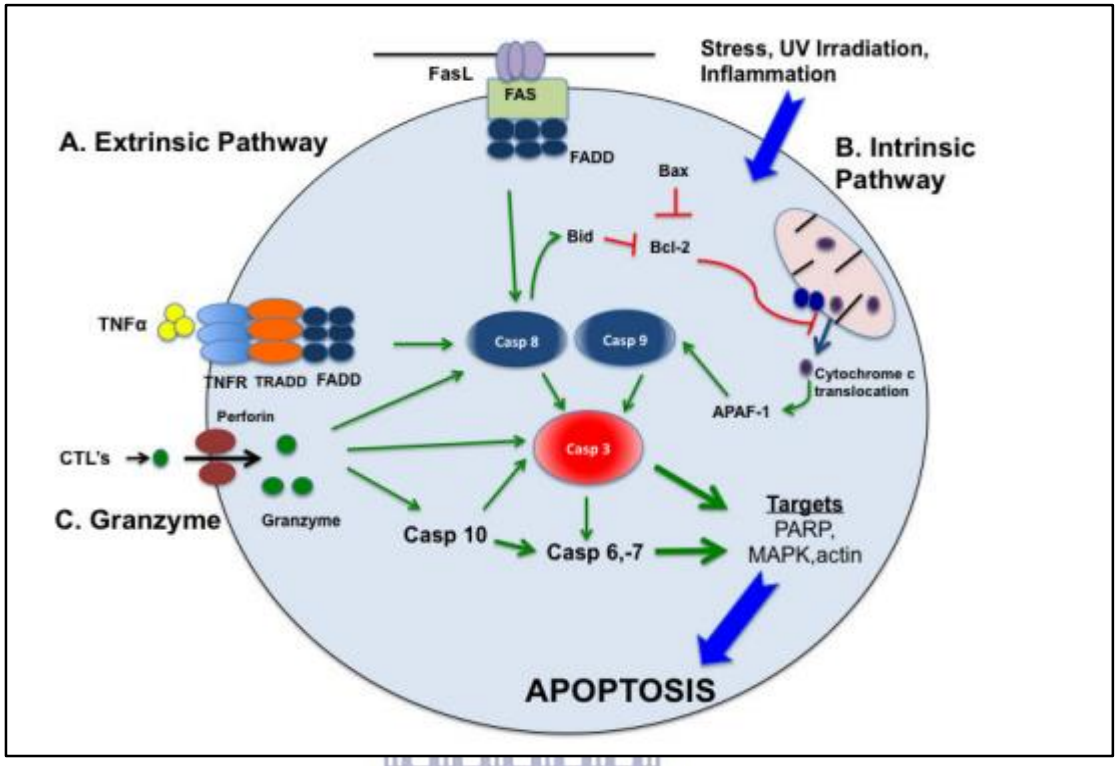
Earlier reports suggest that many PD neurotoxins influence the autophagy pathway. For instance, in a study by Zhu and co-workers, MPP^+ elicited increased autophagy in SH-SY5Y cells (Zhu et al., 2007). Similarly, the SNpc of mice treated with MPTP exhibited autophagic cell death (Meredith et al., 2009) and further treatment with rapamycin rescued dopaminergic neurons and ameliorated cell loss following MPTP treatment (Liu et al., 2013a). Another toxin 6-hydroxydopamine (6-OHDA) induced autophagy in rat nigral neurons (Dagda et al., 2008), and was demonstrated by the accumulation of autophagic vacuoles, activation of lysosomes and enrichment of LC3 in dopaminergic neurons, thus indicating that autophagy contributes to neuronal death (Li et al., 2011). In a different study, paraquat-induced an accumulation of autophagic vacuoles in the cytoplasm of SH-SY5Y cells. It was further revealed that inhibition

of autophagy accelerated apoptotic cell death and therefore indicated a relationship between autophagy and apoptotic cell death (González-Polo et al., 2007).

1.5.3.2. Programmed Cell Death (PCD)

The mechanisms of PCD are multifaceted and involves a cascade of molecular events (Figure 1.10) divided into the intrinsic or mitochondrial pathway and extrinsic or death receptor pathway (Elmore, 2007). Previous reports suggest a connection between both pathways and that molecules in these pathways impact one another (Igney and Krammer, 2002). Additionally, the perforin/granzyme pathway involves a perforin/granzyme-dependent killing of the cell, thus inducing PCD through granzyme A or B. The granzyme B, death receptor and mitochondrial pathways congregate on the execution pathway which is initiated by the cleavage of caspase-3 (Elmore, 2007). The granzyme A pathway begins a caspase-independent cell death pathway via DNA damage (Martinvalet et al., 2005).

Apoptosis is moderated through caspases which initiate a proteolytic cascade when triggered, thereby resulting in the death of the cell (Halonen, 2015). Caspases are divided into initiator and effector caspases from approximately 14 known caspases. The extrinsic, intrinsic or granzyme pathway activates caspase 8, 9 and 10 (initiator caspases), congregates on caspase 3 and further triggers caspase 6 and 7 (effector caspases), thus leading to apoptosis (Halonen, 2015). Also, the effector caspases activate poly-ADP-ribose polymerase (PARP) (Halonen, 2015). Preliminary morphological studies in human PD brains, animal and cell culture PD models show that several apoptotic markers of cell death are active in the disease (Dodel et al., 1998, Dodel et al., 1999, Eberhardt et al., 2000, Coelln et al., 2001).



Source: (Halonen, 2015). Apoptosis is induced through three pathways A (death receptor/extrinsic), B (intrinsic or mitochondrial) and C (granzyme). Pathway A is activated by binding of cell membrane receptors of Fas, TNF or TNFR to its appropriate ligand of FasL or TNFα to attract FADD and TRADD. FADD can bind and cleave caspase 8, but TRADD, unable to do so, binds to FADD before cleaving caspase 8. The cleavage of FADD/TRADD to caspase 8 then initiates caspase 3. Pathway B is controlled by Bcl-2 and is initiated by alterations to the mitochondrial membrane potential (MMP) through conditions like inflammation, stress and toxicity. These alterations to MMP sets off a process that translocates cytochrome c to the cytoplasm from the mitochondria. Cytochrome c binds to APAF-1 in a process that cleaves caspase 9 to form an apoptosome and then initiates caspase 3. Pathway C is activated by CTLs or perforin which produces openings in marked cell membrane, thus allowing granzymes into the cytoplasm to trigger caspase 10 which then initiates caspase 3. *Abbreviations*: Apoptosis protease activating factor 1 (APAF-1); Cytotoxic T lymphocytes (CTLs); Fas-associated death domain (FADD); TNF receptor-associated death domain (TRADD); Tumor necrosis factor (TNF); Tumor necrosis factor family receptor (TNFR).

Figure 1.10: Signaling pathways of Apoptosis

1.5.4. Unfolded protein response (UPR)/Endoplasmic reticulum (ER) stress pathway

The ER is regarded as the largest organelle in the cell with multiple functions such as protein, steroid and phospholipid synthesis, calcium storage and carbohydrate metabolism (Hebert et al., 2005, Clapham, 2007, Fagone and Jackowski, 2009, Braakman and Hebert, 2013, Reid and Nicchitta, 2015). In the ER, chaperones such as 78 kDa glucose-regulated protein (GRP78),

also known as Binding immunoglobulin protein (BiP) or Heat shock 70 kDa protein 5 (HSPA5) and other stress sensor proteins are needed to maintain quality control of proteins to ensure proper handling and to prevent aggregation of misfolded/unfolded proteins (Faitova et al., 2006). Thus, when there is a disturbance in function, oxidative damage, disruption of glucose or calcium homeostasis, the unfolded/misfolded proteins exceeds the folding capacity of the ER and causes a form of stress commonly known as ER stress (Ron and Walter, 2007, Freeman and Mallucci, 2016). The induction of ER stress and the consequent aggregation of misfolded or unfolded proteins have been implicated in PD pathogenesis (Omura et al., 2013, Tsujii et al., 2015).

The ER stress pathway or unfolded protein response (UPR) is known to handle growing quantities of aberrant proteins in the ER (Levy et al., 2009). This response program is tasked with the reduction of misfolded/abnormal proteins through various mechanisms (Figure 1.11). Firstly, GRP78/BiP disassociates from the ER stress sensors – namely, protein kinase RNA-like endoplasmic reticulum kinase (PERK), activating transcription factor 6 (ATF6) and inositol-requiring enzyme 1 (IRE1) to initiate the ER stress response. After that, autophosphorylation and activation of PERK facilitate the phosphorylation of eukaryotic translation initiation factor 2a (eIF2a) to inhibit further protein synthesis and translation (Shi et al., 1998, Harding et al., 1999, Rutkowski and Kaufman, 2004). ATF6 is cleaved in the Golgi after translocation from the ER and then migrates into the nucleus to upregulate ER chaperones such as GRP78/BiP and 94kDa glucose-regulated protein (GRP94) which enhances the folding capacity of the ER (Wang et al., 2009b). Also, IRE1 is involved in endoribonuclease activity and activates X-box binding protein 1 (XBP-1) to promote ER-associated degradation (Yoshida et al., 2001, Calfon et al., 2002, Lee et al., 2003).

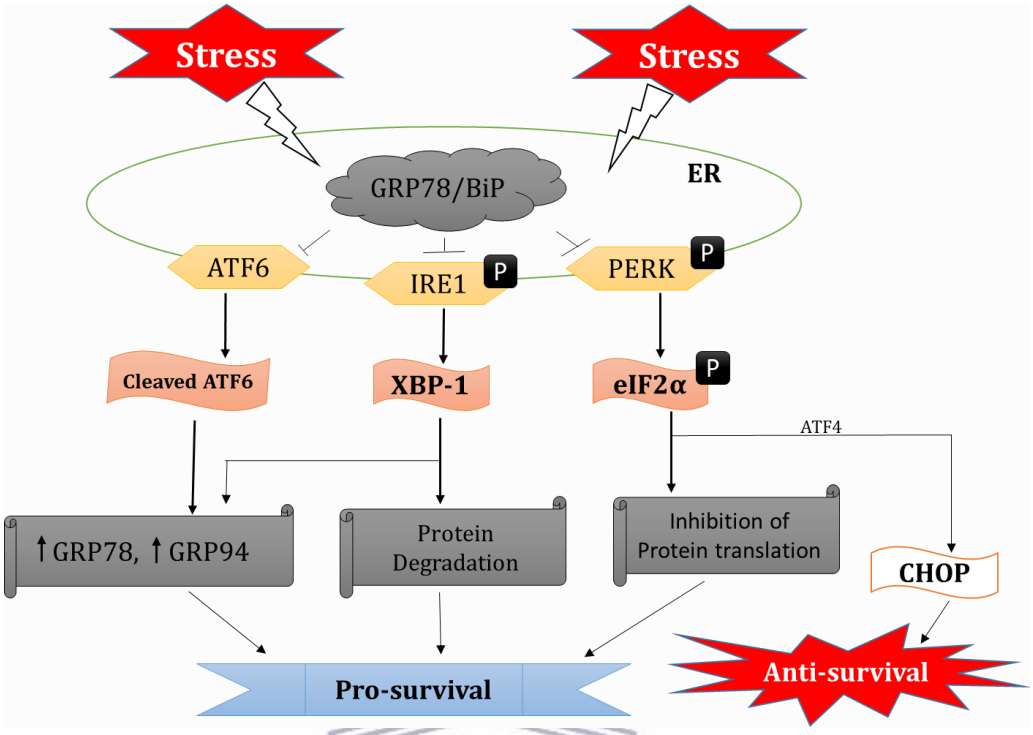


Figure 1.11: Schematic diagram showing the regulation of ER stress signaling pathways

The extent and degree of ER stress and UPR activation may determine if the ER stress response is either anti or pro-survival (Figure 1.12). Certain aspects of the ER stress response such as increased expression of chaperones would appear to be advantageous by lessening the burden of misfolded proteins (Dong et al., 2005, Ahn and Jeon, 2006) while other ER stress responses may be advantageous for a limited amount of time, thus leading to degeneration if sustained. Sustained activation of the UPR under stress would lead to apoptosis via the activation of ER-specific caspases, activation of c-Jun amino-terminal kinase (JNK) and apoptosis signal-regulating kinase 1 (ASK1), induction of CCAAT-enhancer-binding protein homologous protein (CHOP) as well as the activation of p53 upregulated modulator of apoptosis (PUMA), BAX and NOXA (Li et al., 2006).

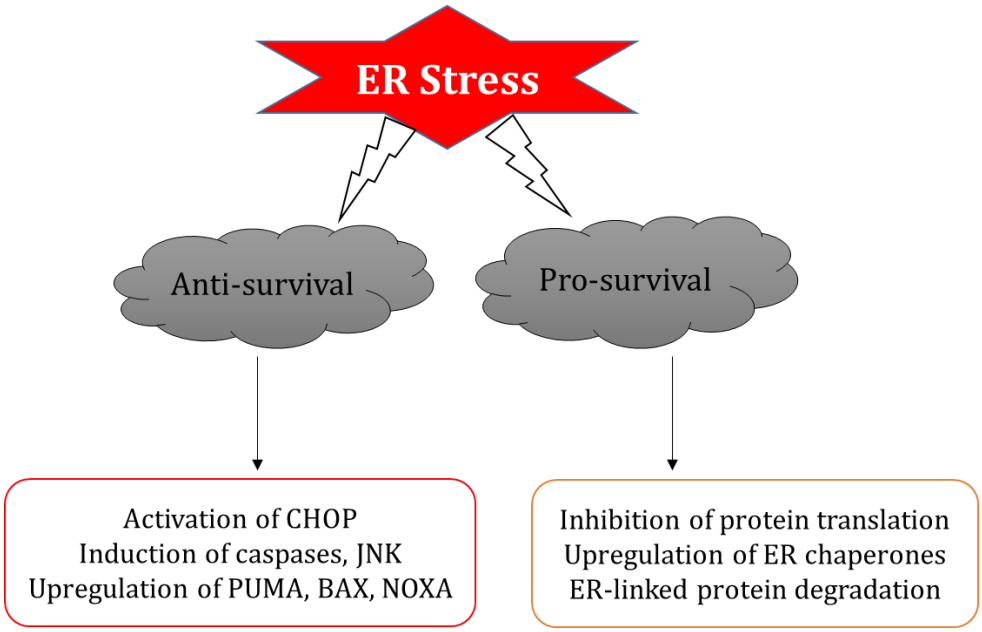


Figure 1.12: Schematic diagram showing cellular response to ER stress

1.5.4.1. GRP78/BiP expression in Parkinson's Disease

GRP78/BiP is a key chaperone essential for proper functioning of the ER and in various cellular processes (Hendershot, 2004, Lee, 2005, Ni and Lee, 2007). Most notably is its dual role of regulating protein folding and the initiation of UPR signaling in the ER (Gorbatyuk and Gorbatyuk, 2013). In PD, there are inconsistent reports on the expression and localization of GRP78/BiP in various experimental models. For instance, treatment of MN9D cells with MPP^+ resulted in a reduction of GRP78/BiP expression while treatment of SH-SY5Y cells with 6-OHDA increased its expression (Holtz and O'Malley, 2003b, Chen et al., 2004). In a PD model using MPP^+-treated rabbits, Ghribi and colleagues revealed the translocation of GRP78/BiP to the nucleus and cytosol from the ER as well as a significant decrease in TH-positive cells in the SNpc (Ghribi et al., 2003). In a different study, Shimoke and co-workers demonstrated an increase in the expression of GRP78/BiP after exposure to tunicamycin; however, they observed no increase in the expression of GRP78/BiP in PC12 cells after treatment with MPTP for 24 hours (Shimoke et al., 2003). Duan and Mattson utilized the MPTP-treated mouse model

of PD to demonstrate that the upregulation of GRP78/BiP by 2-deoxy-D-glucose significantly prevented loss of dopamine neurons (Duan and Mattson, 1999).

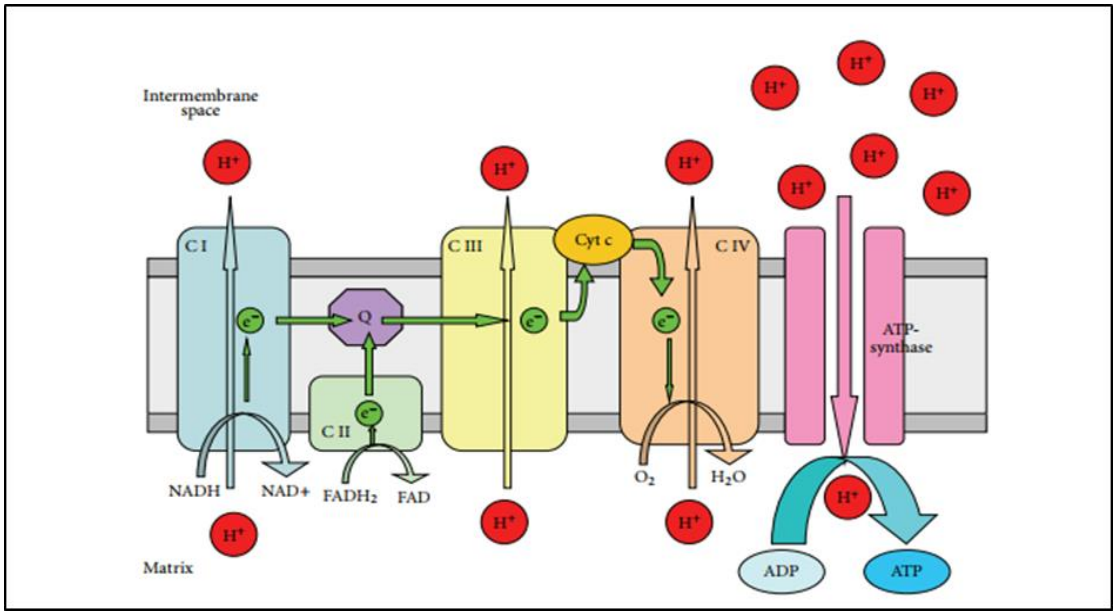
In PD patients, GRP78/BiP was reported to be more expressed in the cingulate gyrus and parietal cortex when compared to healthy controls (Baek et al., 2016). The upregulation of GRP78/BiP in the cingulate gyrus was linked to an increase in α-synuclein expression, thus providing a basis for suggestions that activation of GRP78/BiP attenuates α-synuclein toxicity. This observation is confirmed by a report demonstrating that the knockdown of GRP78/BiP aggravates the toxicity of α-synuclein in rats (Salganik et al., 2015) and in another study showing that miRNA-induced reduction of GRP78/BiP enhanced cell death induced by rotenone (Jiang et al., 2016). Other studies have established that the upregulation of GRP78/BiP suppresses α-synuclein aggregation and toxicity in PD models (Gorbatyuk et al., 2012, Jiang et al., 2014). For example, Gorbatyuk and colleagues in a rat model of PD demonstrated that whereas the accumulation of α-synuclein induces the expression of apoptosis-regulating ATF4, the upregulation of GRP78/BiP attenuates α-synuclein toxicity by regulating ER stress signaling pathways (Gorbatyuk et al., 2012).

LRRK2 is the most significant gene mutated in PD (Cabral-Miranda and Hetz, 2017). Its pathogenesis has been associated with ER stress as it partly localizes in the ER in dopaminergic neurons of individuals with PD (Vitte et al., 2010). Reports show that the neuroprotective activity of *LRRK2* against 6-OHDA or α-synuclein induced neurodegeneration in the nematode, *C.elegans* is attributed to the activity of GRP78/BiP via signaling through the p38 pathway (Harding et al., 1999, Yuan et al., 2011). In confirmation of these reports, Samann and colleagues reported that *LRRK2* mutant *C.elegans* were highly vulnerable to ER stress and developed spontaneous neurodegeneration (Sämann et al., 2009, Varma and Sen, 2015). Also, ageing is the greatest risk factor for PD (Driver et al., 2009, Collier et al., 2011), and various age-related changes in cellular structure and function are observed in PD patients. To corroborate these observations, studies reveal that ageing results in a significant reduction in

the activity and expression of GRP78/BiP in the brain of old versus young rodents (Erickson et al., 2006, Gavilan et al., 2006, Naidoo, 2009). From the aforementioned, GRP78/BiP is undoubtedly an essential component of the UPR and proper regulation of GRP78/BiP could prove valuable in identifying new treatment options in PD.

1.5.5. Mitochondrial Dysfunction

The mitochondria produces energy in a process known as OXPHOS (Anderson et al., 1981, Frey and Mannella, 2000). They also regulate cell death through apoptosis and calcium equilibrium as well as controlling cell division and growth (Keane et al., 2011). Structurally, their double lipid bilayer is made up of a phospholipid outer membrane and an inner membrane surrounding an intra-compartmental matrix. The significant components of the OXPHOS are found in the space between the outer and inner membranes (Frey and Mannella, 2000). Mitochondria are uniquely suited to have their DNA (mtDNA) situated near the ETC, thus making it prone to harm produced by free radicals during OXPHOS (Chen and Butow, 2005). The ETC is made up of five complexes and includes an ATP-synthase that produces ATP energy (Figure 1.13) in a process that involves (i) electron transport between complexes, and (ii) proton (H^+) movements from the matrix to the intermembrane space, thus producing an H^+ concentration gradient used by ATP-synthase to generate ATP (Keane et al., 2011).



Source: (Keane et al., 2011)

Figure 1.13: Diagram showing the mitochondrial ETC involved in OXPHOS

During OXPHOS, electrons leak from CI (Takeshige and Minakami, 1979) and CIII (Beyer, 1992) and react with O_2 to form O^{-}_2. Usually, O^{-}_2 production occurs at reasonably reduced levels (Lass et al., 1997) and is subsequently eliminated by mitochondrial antioxidants such as manganese superoxide dismutase (MnSOD) in a process that converts O^{-}_2 to H_2O_2 and again to H_2O by GSH. A dysfunction in this process leading to an increase in·O^{-}_2 generation is believed to be a major cause of cell death in PD (Schapira et al., 1990b, Sherer et al., 2002). Oxidative stress-induced dysfunction in the mitochondria is known to accelerate neuronal damage and death in PD (Keane et al., 2011). Reports show increased oxidative damage markers in PD brains; they include protein damage (Floor and Wetzel, 1998, Good et al., 1998), lipid peroxidation (Dexter et al., 1989) and oxidative DNA damage (Dexter et al., 1986, Dexter et al., 1989), thus establishing oxidative stress as a key event in PD (see section 2.2 & 2.3.2).

1.5.5.1. Involvement of calcium in mitochondrial dysfunction associated with Parkinson's Disease

The possibility that Ca^{2+} dysregulation participates in the pathogenesis of PD has been frequently reported (Albin and Greenamyre, 1992, Greene and Greenamyre, 1996, Surmeier et al., 2012a). Excitotoxicity occurs when neuronal cell membrane depolarization enables a reduction in the magnesium blockade of N-Methyl-D-aspartate (NMDA) receptors in addition to an activation of the NMDA receptors by glutamate, thus leading to a build-up of intracellular Ca^{2+}. This accumulation of Ca^{2+} causes neurotoxicity by two primary mechanisms (Keane et al., 2011). Firstly, Ca^{2+} increases intracellular NO through the activation of nitric oxide synthase (NOS) which react with$\cdot O_2$ to form peroxynitrites ($ONOO^-$) (Dawson and Dawson, 1996). These $ONOO^-$ causes cellular death by similar mechanisms to ROS. Also, aside from $ONOO^-$, cell injury can be caused by NO through nitrosylation of various proteins. Secondly, Ca^{2+} accumulates in the mitochondria to increase ROS generation and reduce mitochondrial membrane potential (MMP) and ATP production, thus leading to toxicity in dopaminergic neurons and contributing to oxidative damage (Nicholls and Budd, 1998). In a previous study, Sherer and coworkers demonstrated excitotoxic cell death in PD through the inhibition of complex I in SH-SY5Y cells which led to a disturbance of MMP and an amplified vulnerability to Ca^{2+} influx (Sherer et al., 2001). Also, Sheehan and colleagues reported that mitochondria in PD patients demonstrated reduced sequestration of Ca^{2+} which suggested impairment of Ca^{2+} balance in PD (Sheehan et al., 1997). Furthermore, increased cytosolic Ca^{2+} due to mitochondrial dysfunction activates calpains and increases toxic α-Syn, thus suggesting additional links to PD (Esteves et al., 2010). This discovery is buttressed by the protective activity of calpain-inhibition in an MPTP model of PD (Crocker et al., 2003).

Similarly, Ca^{2+} is linked to an increased vulnerability to cell death which is specific to dopaminergic neurons in the SNpc. These neurons have unique features of self-generating pacemaker activity (Grace and Bunney, 1983), mediated by Cav1.3, a rare L-type Ca^{2+} channel

(Striessnig et al., 2006). This unique feature means that the Cav1.3 channels are open for a more extended period than other Ca^{2+} channels in different neurons, thus causing higher ATP expenditure in cells propelling Ca^{2+} across sharp concentration gradients in membranes (Surmeier et al., 2010). Higher activity of the ETC due to increased ATP demand increases ROS production and aggravates any mitochondrial dysfunction, thereby making dopaminergic neurons of the SNpc more prone to cell death (Keane et al., 2011).

1.5.6. Neuroinflammation

Several postmortems, brain imaging and fluid biomarker studies have shown that neuroinflammation is implicated in PD (Moehle and West, 2015). The neuroinflammatory mechanisms which contribute to neuronal degeneration include microglial activation, astrogliosis, and lymphocytic infiltration. McGeer and colleagues provided the first evidence for a role of neuroinflammation in PD when they presented large numbers of human leukocyte antigen DR (HLA-DR)- positive reactive microglia in the SN of patients with PD (McGeer et al., 1988). Furthermore, GWAS indicate that genes such as *LRRK2* often code proteins that are found in immune cells and involved in immune control (Nalls et al., 2014, Schapansky et al., 2014, Ma et al., 2016).

More evidence from PD patients and experimental models suggest that neuroinflammation can promote α-Syn misfolding and aggregation (Gao et al., 2008). For example, in postmortem brain samples, several cytokines such as TNFα, IL-1β, IL-6 and IFN-g were elevated in the SNpc (Boka et al., 1994, Mogi et al., 1994, Mogi et al., 2007). Also, inflammatory enzymes such as iNOS, COX-1 and 2 were increased in PD brains (Knott et al., 2000). Microarray studies confirmed increased inflammation-related genes such as *HLA-C* and cytotoxic T lymphocyte antigen 4 (*CTLA-4*) in postmortem brains of PD patients (Moran et al., 2007, Moran and Graeber, 2008, Simunovic et al., 2008).

1.6. DOPAMINERGIC SH-SY5Y CELLS AS A MODEL FOR PARKINSON'S DISEASE STUDY

Suitable models for investigating the pathobiological mechanisms of PD are necessary in the search for novel pharmacological targets and disease-modifying therapies (Dawson et al., 2010, Morgan et al., 2010, Obeso et al., 2010). Although cellular models are far from being able to reproduce the complexity of PD, the development of a stable and reliable cellular model that mimics the dopaminergic cell death in the SNpc is necessary to provide initial valuable insights for validation in animal models and⁄or PD patients (Blandini and Armentero, 2012, Ravid and Ferrer, 2012).

In this regard, the human neuroblastoma SH-SY5Y cell line has been widely used in the field of neurodegeneration, mostly to generate various toxin-induced PD models (Lopes et al., 2010, Xie et al., 2010, Korecka et al., 2013). This cell line, originally a subclone from the SK-N-SH cell line, is utilized as an *in-vitro* model to replicate diminished dopamine homeostasis. These cells express tyrosine hydroxylase (TH) and display adequate dopamine-β-hydroxylase (DßH) activity, which is particular for dopaminergic neurons (Ross et al., 1983). Also, SH-SY5Y cells exhibit properties of stem cells and express DAT which is responsible for the control of dopamine equilibrium via precise uptake and sequestration of dopamine (Takahashi et al., 1994). DAT is the access point for 6-OHDA and MPP^+ into neurons, consequently making SH-SY5Y cells an appropriate candidate for studies involving 6-OHDA and MPP^+.

A variety of agents induce differentiation in SH-SY5Y cells to make these cells possess more biochemical, morphological and ultrastructural similarity to neurons. These agents include retinoic acid (RA) (Påhlman et al., 1984, Korecka et al., 2013), 12-O-tetradecanoyl phorbol-13-acetate (TPA) (Påhlman et al., 1981, Påhlman et al., 1983), brain-derived neurotrophic factor (Spinelli et al., 1982), dibutyryl cyclic AMP (Kume et al., 2008) and staurosporine (Jalava et al., 1992). Phenotypic changes in differentiated SH-SY5Y cells are observed based on the differentiation conditions. For example, a 6-day period of RA (10 μM for three days)

treatment, followed by TPA (150 nM for three days) administration causes the cells to express markers such as DAT, VMAT2, TH, dopamine D2 and D3 receptors. Alternatively, a differentiation procedure utilizing RA alone demonstrates high expression of VMAT2 but a marginal expression of the markers stated earlier (Presgraves et al., 2003).

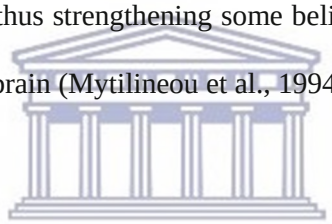
Studied pathways illustrate the effects of RA-induced differentiation of neurons. RA triggers the ataxia telangiectasia mutated (ATM) kinase as well as the PI3K/Akt signaling pathway (López-Carballo et al., 2002) leading to phosphorylation of ATM-dependent cAMP response element binding (CREB) whereas cells fail to differentiate when these pathways are blocked by RNAi inhibitors (Fernandes et al., 2007). Also, there are observed changes in vulnerability between RA-differentiated and undifferentiated SH-SY5Y cells when treated with neurotoxins. For instance, exposure of cells to MPP^+ (1 mM) or 6-OHDA (25 μM) for 24 hours revealed significant changes in toxicity, viability, apoptosis and oxidative stress response, with the undifferentiated cells being more vulnerable to MPP^+ and 6-OHDA. From the findings, the authors suggested that undifferentiated SH-SY5Y cells may be a superior model for evaluating neurotoxicity (Cheung et al., 2009).

1.7. PRIMARY SKIN FIBROBLASTS AS A MODEL FOR PARKINSON'S DISEASE STUDY

Skin disorders are well documented as a non-motor symptom in PD but are often overlooked (Skorvanek and Bhatia, 2017). Krestin provided the first evidence of skin disorders in PD and several other reports have further corroborated this evidence (Krestin, 1927, Gregory and Miller, 2015, Ravn et al., 2017). Some of these disorders include seborrheic dermatitis, malignant melanoma, rosacea hyperhidrosis and bullous pemphigoid (Arsenijevic et al., 2014, Huang et al., 2015, Lyon et al., 2016).

Derivation of primary skin fibroblasts to study various mechanisms in PD has become an appealing approach. Earlier beliefs on an exclusive regard for the dopaminergic nigrostriatal

pathway and bias against fibroblasts have been outdated by data on sporadic PD patients (Auburger et al., 2012). Skin fibroblasts can be obtained from 2mm punch skin biopsies, resulting in a cell culture combination of keratinocytes and primary fibroblasts at the commencement of the culturing process, with subsequent purity of culture fibroblasts only attained in the third passage (Auburger et al., 2012). Over the years, researchers have deliberated and doubted the validity of results obtained in fibroblasts, questioning their ability to emulate the pathophysiological changes associated with dopaminergic neurons in the brain. However, there are very old observations that fibroblasts revealed decreased complex I activity, ATP production, MMP and higher lipid peroxidation. These observations were comparable to those seen in PD affected brain tissue and were among initial findings showing bioenergetic deficits characteristic of PD, thus strengthening some beliefs that PD is not limited to loss of dopamine neurons in the midbrain (Mytilineou et al., 1994, Wiedemann et al., 1999, del Hoyo et al., 2010).



The principal benefits of using skin fibroblasts as PD models include the robustness and availability of these cells. Others include its ability to mirror collective cell damage reflective of the patient's age, demonstrate *PARK* genes at essential levels, undergo cell to cell contacts comparable to neurons, maintain precise environmental and aging history of PD patients, respond to genetic manipulation and be comparable with mouse mutant-embryonal fibroblasts and finally ability to undergo reprogramming to stem cells (Auburger et al., 2012).

Interestingly, reports show that gene expression profiles in fibroblasts with down-regulated PINK1 have provided better insights into the function of this protein (Da Costa et al., 2009, Ren et al., 2010). Also, a bioenergetic deficiency was reported in *parkin*-mutant fibroblasts. Increased mitochondrial susceptibility to DNA damage, increased activity of p53 and alterations in the MAP kinase pathway have been observed in studies using fibroblasts (Ren et al., 2009, Rothfuss et al., 2009, Grünewald et al., 2010). In 2008, Piccolo and co-workers assessed mitochondrial respiratory function in a *PINK1*-mutant fibroblast and observed reduced

respiratory activity, increased generation of ROS and a reduction in cytochrome c (Piccoli et al., 2008). This detection of differential expression patterns of genes/proteins in PD patients and their controls offer significant knowledge into the mechanisms of PD and makes a strong case for these fibroblasts to be used in studies that can aid the discovery of novel therapeutic agents in PD.

1.8. TREATMENT OPTIONS

The primary treatment for symptomatic patients and most effective pharmacologic agent for PD is Levodopa (Miyasaki et al., 2002, Goetz et al., 2005) which has been reported to be the most effective at controlling rigidity and bradykinesia (Miyasaki et al., 2002); however, postural reflex, gait disturbance and speech are less likely to respond. Levodopa is combined with carbidopa because carbidopa blocks dopa decarboxylase thereby preventing peripheral conversion of levodopa to dopamine. Additionally, its combination with levodopa reduces the peripheral adverse effects of dopamine (e.g., nausea, hypotension) and increases cerebral levodopa bioavailability.

Treatment with monoamine oxidase-B (MAO-B) inhibitors, amantadine (Symmetrel), or anticholinergics may modestly improve mild symptoms; nevertheless, most patients need a dopamine agonist or levodopa (Rao et al., 2006). Furthermore, advances in brain imaging and neurosurgical techniques have highlighted surgical treatment for this disorder. In an evidence-based review, it is reported that deep brain stimulation of the subthalamic nucleus effectively improves motor function and reduces dyskinesia and motor fluctuations (Goetz et al., 2005, Pahwa et al., 2006).

1.8.1. Natural therapies

Over the past century, the development of synthetic drugs has transformed the health care sector in various parts of the world, however, in developing countries, a significant portion of the

populace still depend on herbal medicines or its derivatives for their primary health care (W.H.O, 2005). The practice of traditional and western medicine co-exist in South Africa (Stafford et al., 2008) and the majority of the low-income population utilize the overburdened public healthcare facilities (Morris, 2001, Eastman, 2005) and in most cases, concurrently with the relatively accessible and affordable traditional medicines (Marais et al., 2015, Audet et al., 2017). Recently, the misleading reports that 80% of South Africans use traditional medicines for their primary healthcare needs have been controverted (Nxumalo et al., 2011, Oyebode et al., 2016), although it is common knowledge that the practice of traditional medicine remains active especially among rural communities in South Africa. Also, in China, up to 40% of all health care delivered depend on traditional medicine while more than 90% of general hospitals in China have units for traditional medicine (W.H.O, 2005).

Currently, herbal or natural therapies are becoming indispensable in the prevention and treatment of such diseases as neurodegenerative, cardiovascular and cancer (Kincheloe, 1997). The utilization of traditional medicine is not only restricted to developing countries, as evidenced by the amplified interest for natural remedies in industrialized countries. For example, according to a 2007 survey in the United States showed that about 38% of adults and 12% of children used some form of traditional medicine (Ernst et al., 2005, Barnes et al., 2008).

Herbs and plants can be processed and consumed in different forms such as whole herb, essential oils, salves, teas, syrup, ointments, rubs, capsules and tablets containing powdered form of a raw herb or its dried extract. Plants, rich in a variety of compounds such as phenols, tannins and flavonoids. (Hartmann, 2007, Jenke-Kodama et al., 2008), possess numerous antioxidant properties. These properties are important for researches investigating pharmacologically active compounds in plants, thus making them useful starting materials for the synthesis of novel drugs (Li and Vederas, 2009).

Due to the increasing prevalence of PD, there is pressing need for improved treatment in order to alleviate the social and financial burden of this disease. Some medicinal plants are reportedly used for the treatment of PD but the challenge of crossing the blood-brain barrier makes some of these sources of medicinal molecules ineffective for the treatment of PD. Before now, the main trigger for the dopaminergic neuronal loss observed in PD was largely unknown and has contributed to the poor understanding of the pathogenesis of PD. Today, the search for plant-derived compounds capable of stopping neuronal cell death is a worthwhile effort and research geared towards testing and validating identified plant-derived compounds using various PD models should be encouraged. The next chapter will review available scientific literature on a plant-derived compound (rutin) and its use in various neurodegenerative disease models.

CHAPTER 2

RUTIN AS A POTENT ANTIOXIDANT: IMPLICATIONS FOR NEURODEGENERATIVE DISORDERS

2.1 INTRODUCTION

Neurodegenerative diseases (NDs) are regarded as an age-related group of chronic and untreatable conditions which constitutes a major threat to human health (Gitler et al., 2017). They are becoming increasingly prevalent, due to a significant increase in the size of elderly populations worldwide (Hindle, 2010). NDs represents the fourth highest source of disease burden in high-income countries, in terms of economic cost for society (W.H.O., 2004). NDs are characterized by the gradual and progressive loss of neurons and diverse clinical features such as memory and cognitive impairments and others affecting a person's ability to move, speak and breathe (Canter, 1963, Aarsland et al., 2009, Kovacs, 2014). Some overlapping pathways recognized in the pathogenicity of NDs include free radical formation and oxidative stress, protein misfolding and aggregation, metal dyshomeostasis, phosphorylation impairment and mitochondrial dysfunction. (Sheikh et al., 2012) (Figure 2.1).

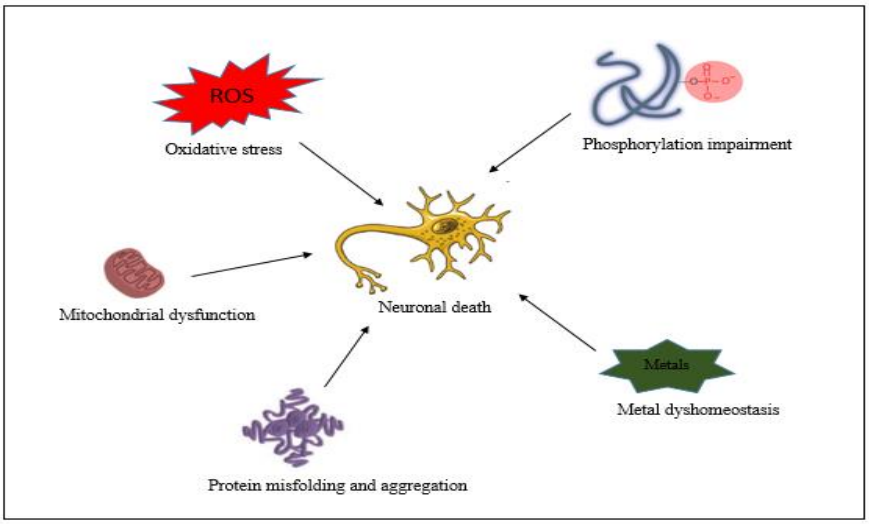


Figure 2.1: Various processes shown to be dysregulated in neurodegenerative disorders

Oxidative stress has been shown by many studies to be a crucial player in the development and progression of NDs (Chen et al., 2012). Oxidative stress is defined as the disturbance in balance between pro-oxidant and antioxidant levels, and results from an imbalance between the production of reactive oxygen species (ROS) and the biological system's ability to detoxify the reactive intermediates (Chen et al., 2012). ROS play important roles in mediating cellular activities (Uttara et al., 2009, Kim et al., 2015); however due to their reactivity, high amounts of ROS can cause cell death or oxidative stress (Liu et al., 2017). While it is still unclear whether ROS is the triggering factor for NDs, they are likely to aggravate disease progression through oxidative damage and effects on mitochondria.

In view of the important roles of oxidative stress in NDs, the manipulation of ROS levels may be an encouraging treatment option to delay neurodegeneration and attenuate associated symptoms. Presently, there is no potent treatment for NDs, and the available drugs are mainly focused on symptoms though with many adverse effects and limited ability to prevent disease progression (Cirmi et al., 2016). Accordingly, medicinal plants such as *Hypericum perforatum* possessing antioxidant properties have been studied for their potential to attenuate neurodegenerative symptoms (Khalifa, 2005, Kraus et al., 2007, Kiasalari et al., 2016, Oliveira et al., 2016). For instance, previous reports show that extracts of *H. perforatum* significantly attenuated oxidative stress by reducing lipid peroxidation (Silva et al., 2004), reducing oxidation of the mitochondrial lipid membrane (Silva et al., 2008), preserving the activities of antioxidant enzymes (Sanchez-Reus et al., 2007) and consequently preventing neurotoxicity in experimental models of NDs. As a result of these findings amongst others, Sanchez and co-workers proposed standardized extracts of *H. perforatum* as a possible treatment for elderly patients showing signs of NDs associated with elevated oxidative stress (Sanchez-Reus et al., 2007). Although reports show that treatments involving *H. perforatum* are generally safe, minor adverse effects have been reported; they include dizziness, allergic reactions, restlessness, gastrointestinal symptoms, dryness of the mouth and lethargy (Ernst et al., 1998, Barnes et al., 2001, Greeson et al., 2001).

Similarly, there is currently an increase in the usage of natural compounds/products as potential neuroprotective agents. Examples include, curcumin, bilobalide, chitosan and apigenin, all known to have potent protective effects on neurons (Sano et al., 1997, Zhou and Zhu, 2000, Choi et al., 2010a, Khodagholi et al., 2010, Janhom and Dharmasaroja, 2015, Van der Merwe et al., 2017). Recently, bioflavonoids have found use in the healthcare system owing to their wide range of biological activities, low cost and significantly high safety margins (Sharma et al., 2013). Rutin (3, 3', 4', 5, 7-pentahydroxyflavone-3-rhamnoglucoside, Figure 2.2) also called sophorin, rutoside and quercetin-3-rutinoside is a polyphenolic bioflavonoid, largely extracted from natural sources such as oranges, lemons, grapes, limes, berries and peaches (Kreft et al., 1999, Huang et al., 2012). Rutin is a vital nutritional component of plants (Harborne, 1986) and its name originates from the plant *Ruta graveolens*, which also contains rutin (Ganeshpurkar and Saluja, 2017) . Chemically it is a glycoside comprising of flavonolic aglycone quercetin along with disaccharide rutinose (Ganeshpurkar and Saluja, 2017). Some studies suggest that rutin has a potential protective role in NDs due to its beneficial effects as a potent antioxidant (Park et al., 2014, Yu et al., 2015). Hence, this review presents an outline of the scientific literature regarding the potential neuroprotective role of rutin in the NDs.

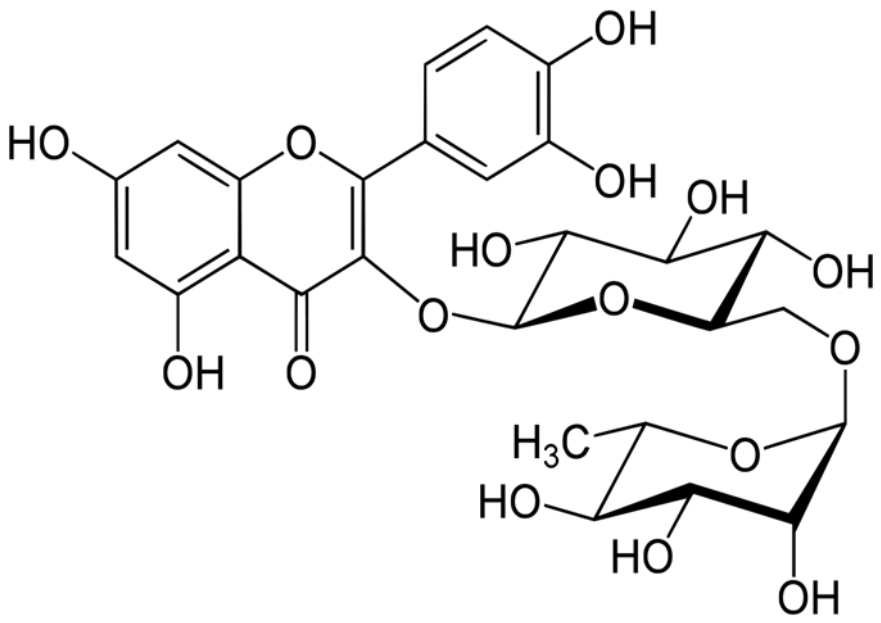


Figure 2.2: Diagram showing the chemical structure of rutin

2.2. OXIDATIVE STRESS AND REACTIVE OXYGEN SPECIES

Oxygen is essential for all multicellular life but in excess it is potentially hazardous. ROS is formed when cells exposed to oxygen continuously generate oxygen free radicals. Endogenous free radicals are generated from inflammation, mental stress, immune cell activation, excessive exercise, infection, ischemia, cancer and aging while exogenous free radicals are produced from air and water pollution, radiation, alcohol, cooking (smoked meat, used oil, fat), heavy or transition metals, cigarette smoke and industrial solvents (Young and Woodside, 2001, Valko et al., 2005, Valko et al., 2006). The main source of endogenous ROS production is the mitochondria but it can also occur in other organelles (Balaban et al., 2005). ROS include free radicals (superoxide, $^{\bullet}O^{-}_{2}$), hydroxyl radical ($^{\bullet}OH$), or non-radicals (hydrogen peroxide, H_2O_2). $^{\bullet}O^{-}_{2}$ is proposed to play a crucial role in ROS production and $^{\bullet}OH$ is recognized as the most reactive ROS that are primarily liable for the toxic effects of ROS (Bolisetty and Jaimes, 2013).

Cellular levels of ROS may be decreased through the defence mechanisms of small-molecule antioxidants and antioxidant enzymes (Gandhi and Abramov, 2012). $^{\bullet}O^{-}_{2}$ is reduced by superoxide dismutases (SOD) into the more stable form of H_2O_2. H_2O_2 may produce highly reactive hydroxyl radicals $^{\bullet}OH$ and can be reduced to H_2O and O_2 by catalase (CAT), glutathione peroxidase (GPx), and other peroxidases (Patten et al., 2010, Song and Zou, 2015). The cellular antioxidant glutathione (GSH) is involved in two types of reactions; First of all, in its reduced form, GSH non-enzymatically reacts with $^{\bullet}O^{-}_{2}$ and $^{\bullet}OH$ for the elimination of ROS (Dringen et al., 2000, Gandhi and Abramov, 2012). Next, GSH serves as the electron contributor for the reduction of peroxides in the GPx reaction (Dringen, 2000). When GSH reacts with ROS, it is oxidized (GSSG) and produces glutathione disulfide (the last product of GPx reactions). GSH can be further restored from glutathione disulfide by the reaction with glutathione reductase through a transfer of electrons from NADPH to glutathione disulfide (Dringen and Hirrlinger, 2003). Numerous studies have stated that GSH is involved in impeding apoptotic cell death and DNA damage in cells following oxidative stress (Abramov et al., 2007, Presnell et al., 2013). Hence, cellular antioxidants and antioxidant enzymes work together to

prevent the accumulation of damaging ROS in the cell. Dysregulation of their functions is an indication of altered oxidative states, which may contribute to cell death.

The harmful effects of ROS include damage of DNA or RNA, oxidation of amino acids in proteins, oxidative deactivation of particular enzymes by oxidation of co-factors and oxidations of polyunsaturated fatty acids in lipids (lipid peroxidation). The uninterrupted attack of protein by ROS forms protein carbonyls and nitrites, such that monitoring of their levels provides an additional measure of the effect of oxidative stress (Francisco et al., 2010). Lipid peroxidation results in the generation of lipid peroxidation products such as malondialdehyde (MDA) and thiobarbituric acid reactive substances (TBARS) (Gaweł et al., 2004). Assay of TBARS measures MDA present in the sample and MDA generated from lipid hydroperoxides. An increase in free radicals is directly proportional to overproduction of MDA and is therefore a commonly used marker of oxidative stress and antioxidant status (Gaweł et al., 2004).

2.3. LINK BETWEEN OXIDATIVE STRESS AND NEURODEGENERATIVE DISORDERS.

The pathogenesis of NDs is a complex interplay between genetic and non-genetic factors (Ramassamy, 2006). Generally, non-genetic/sporadic forms represent the majority of these cases. There are a number of NDs but for the purposes of this review we will focus on Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD) and Human Prion diseases (PrDs) (Cirmi et al., 2016, Gitler et al., 2017).

2.3.1. Alzheimer's Disease

AD is the most common ND and it primarily affects middle- to old-aged individuals, nearly one in four individuals over the age of 85 (Kalaria et al., 2008). AD has various etiological factors including genetic and environmental factors (Kalaria et al., 2008, Feng and Wang, 2012). It is characterized by neuronal loss and atrophy in the neocortex, hippocampus, amygdala, and basal forebrain (Pennanen et al., 2004, Devanand et al., 2007). Its pathophysiological hallmarks include depositions in the forms of senile plaques, extracellular

β-amyloid (Aβ) protein and intracellular deposits of the microtubule linked protein tau as neurofibrillary tangles in the AD brains leading to dementia (Glenner and Wong, 1984).

A common pathological feature in AD is the oxidation of nucleic acids, proteins and lipids in neurons (Praticò, 2008). ROS interacts with polyunsaturated fatty acids in the neurons, leading to high levels of lipid peroxidation (Chen and Zhong, 2014). Increased levels of oxidative stress biomarkers (carbonyls, MDA and 3-nitrotyrosine) in the blood (Butterfield and Kanski, 2001, Beal, 2002) and changes in the activities of antioxidant enzymes (SOD and CAT) reflect oxidative stress in the brain (Marcus et al., 1998, Torres et al., 2011).

The underlying mechanisms (Figure 2.3) proposed for the initiation of oxidative stress in AD include Aβ accumulation (Yan et al., 2013, Zhao and Zhao, 2013), hyperphosphorylated tau (Stamer et al., 2002, Dias-Santagata et al., 2007), inflammation (Candore et al., 2010, Lee et al., 2010b), mitochondrial dysfunction (Federico et al., 2012, Yan et al., 2013) and metal accumulation (Ayton et al., 2013, Greenough et al., 2013).

To date, there is no treatment that can cure AD, but there are available symptomatic drug treatments consisting mostly of cholinesterase inhibitors such as donepezil, rivastigmine and galantamine (Hong-Qi et al., 2012). Others include memantine (Chohan and Iqbal, 2006, Peskind et al., 2006), a N-methyl-D-aspartate receptor antagonist approved by the U.S. Food and Drug Administration (FDA) and a combination of memantine with donepezil (Matsuzono et al., 2015).

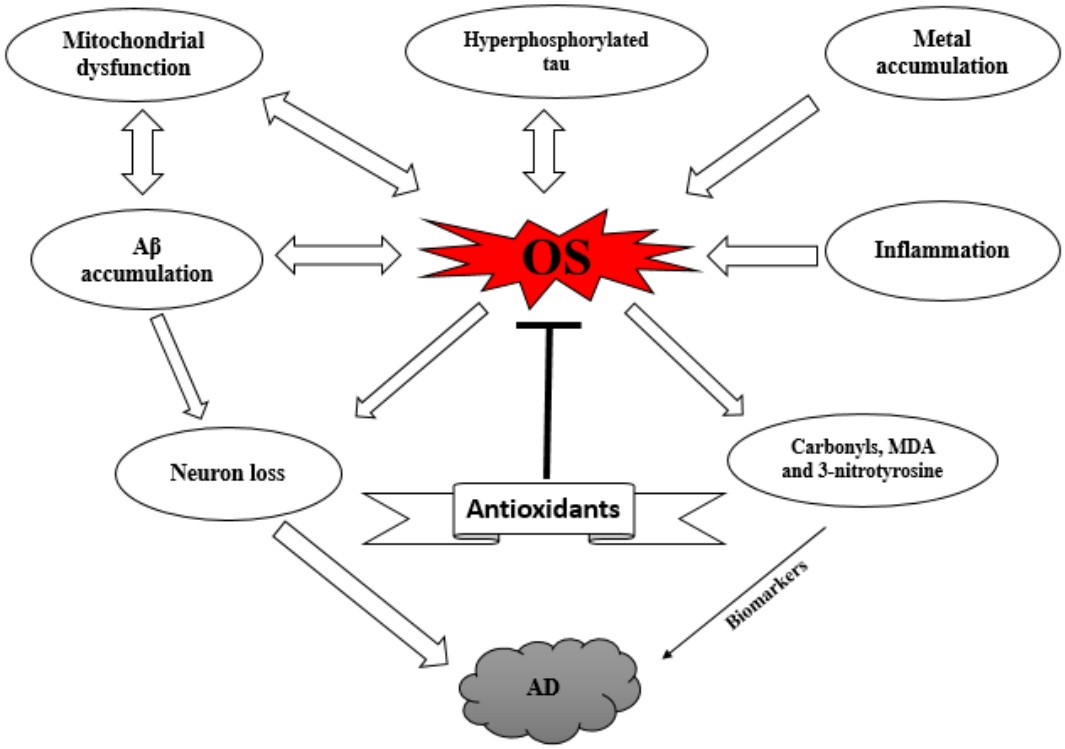


Figure 2.3: Schematic diagram showing the role of oxidative stress (OS) in Alzheimer's disease

2.3.2. Parkinson's Disease

PD is characterized by chronic degeneration of dopaminergic neurons in the substantia nigra pars compacta of the midbrain (Mazzio et al., 2011). This in turn results in the depletion of dopamine neurotransmitter production, which leads to motor deficits such as symptomatic rigidity, bradykinesia, postural instability and resting tremor (Sharma et al., 2016). The cause of dopaminergic neuronal cell death in PD remains unidentified, but several factors such as oxidative stress may contribute to this degeneration and have been closely linked to other sections of neurodegenerative processes, such as α-synuclein, inflammation and cell death (Jenner, 2003, Gao et al., 2008, Hirsch and Hunot, 2009, Khan et al., 2012).

Oxidative stress is believed to be a fundamental mechanism leading to cellular dysfunction in both idiopathic and familial forms of PD. An increase in protein oxidation has been detected in the substantia nigra of PD patients compared to healthy individuals (Ihara et al., 1999). Accordingly, the substantia nigra of PD patients reveal decreased levels of GSH and higher

levels of oxidized proteins, DNA and lipids (Cadet and Brannock, 1998, Manoharan et al., 2016). The accumulation of lipid peroxidation by-products has been reported in the serum and cerebral spinal fluid of PD patients while higher levels of MDA and TBARS have been reported in the substantia nigra and stratum of PD brains (Yoritaka et al., 1996, Floor and Wetzel, 1998, Faucheux et al., 2003).

Various mechanisms for the generation of ROS in PD include mitochondrial dysfunction, metabolism of dopamine, iron, aging, calcium and neuroinflammation (Dias et al., 2013). PD causing genes such as *SNCA*, *DJ-1*, *LRRK2*, *PINK1*, and *PARK2* also affect in complex ways leading to aggravation of ROS production and vulnerability to oxidative stress (Dias et al., 2013). In addition, homeostatic processes such as mitophagy and the ubiquitin-proteasome system are affected by oxidative stress (Dias et al., 2013). The interaction amongst these numerous mechanisms are thought to contribute to neurodegeneration in PD (Figure 2.4). For treatment strategies, see section 1.8.

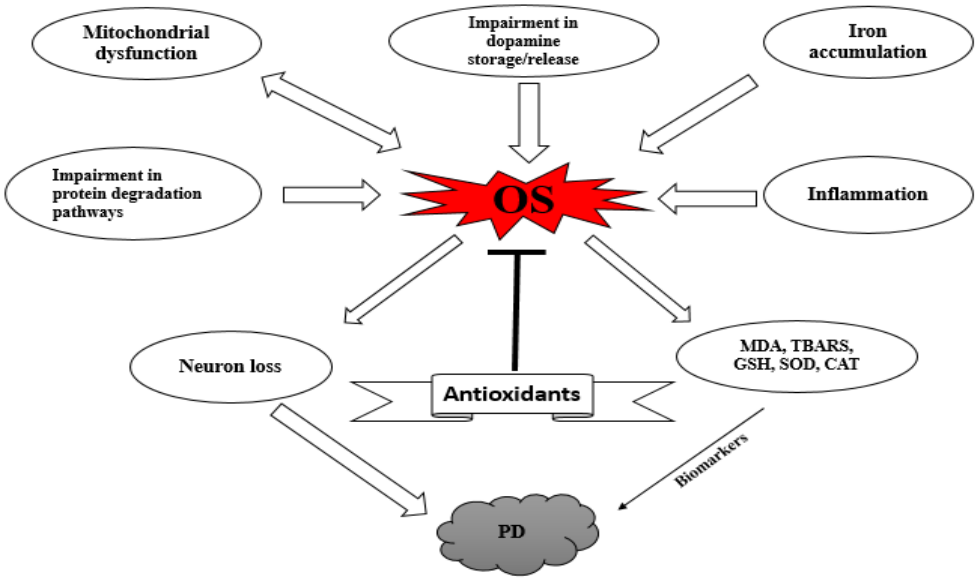


Figure 2.4: Schematic diagram showing the role of oxidative stress in Parkinson's disease

2.3.3. Huntington's Disease

HD is characterized by motor, cognitive, behavioural dysfunction (Adam and Jankovic, 2008) and demonstrates an autosomal dominant mode of inheritance (Pidgeon and Rickards, 2013). It is characterised by a remarkable specificity of neuronal loss and the regions most affected are the striatum, where there is usually 50-60% loss of cross-sectional area from the caudate nucleus and the putamen in advanced stages of the disease (Rubinsztein, 2003). HD is linked with a triad of symptoms which includes cognitive deterioration, movement disorders and psychiatric disturbances (Rubinsztein, 2003). These signs begin subtly, most frequently between the ages of 35 to 50, but the age of onset can differ from early childhood until old age. The disease is relentlessly progressive and is deemed to be fatal 15–20 years after the onset of symptoms (Rubinsztein, 2003). Classical features of HD are disturbances of motor function which include chorea (unintentional brief movements that tends to flow between body regions), and progressive deficiency of co-ordination of voluntary movements (Folstein et al., 1983, Brandt et al., 1984, Rubinsztein, 2003, Frank, 2014).

Convincing data supports a critical role for oxidative stress in the pathogenesis of HD (Chen et al., 2007, Johri and Beal, 2012, Rotblat et al., 2014) (Figure 2.5). Mutant huntingtin proteins (MTPs) serve as the source of ROS, owing to a substantial amount of oxidized proteins in partially purified MTP aggregates (Rotblat et al., 2014). It is proposed that elevated oxidative stress is a major mechanism in the late stages of HD pathogenesis. (Johri and Beal, 2012). Another mechanism involved in ROS mediated HD pathogenesis is the impairment of the electron transport chain and mitochondrial dysfunction (Sayre et al., 2007, Trushina and McMurray, 2007). Defects in oxidative phosphorylation has been detected in the brain tissues of HD patients (Lee et al., 2012) and enhanced lipid peroxidation accompanied by reduced GSH content has been reported in patients with severe symptoms of HD (Chen et al., 2007, Klepac et al., 2007, Túnez et al., 2011). Substantial oxidative DNA damage has also been reported in HD mouse models (Browne et al., 1997, Goula et al., 2009).

There are no existing treatments to alter the course of HD, but symptomatic therapies and education are effective tools used by clinicians in addressing patients and families affected by HD. Several drugs and surgical procedures have been assessed in HD for their effectiveness in subduing chorea. These include dopamine-depleting agents, agonists and antagonists, deep brain stimulation, benzodiazepines, fetal cell transplantation, acetylcholinesterase inhibitors, glutamate antagonists, anti-seizure prescriptions, lithium and cannabinoids (Bagchi, 1983, Armstrong and Miyasaki, 2012, Pidgeon and Rickards, 2013). Tetrabenazine is the only FDA approved drug for HD designated for the treatment of chorea linked with HD (Shen et al., 2013, Kaur et al., 2016). Other promising drugs shown in controlled trials to considerably lessen chorea in HD patients include amantadine (Lucetti et al., 2002), olanzapine (Dipple, 1999, Bonelli et al., 2002), quetiapine (Bonelli and Niederwieser, 2002, Alpay and Koroshetz, 2006) and aripiprazole (Brusa et al., 2009, Ciammola et al., 2009).

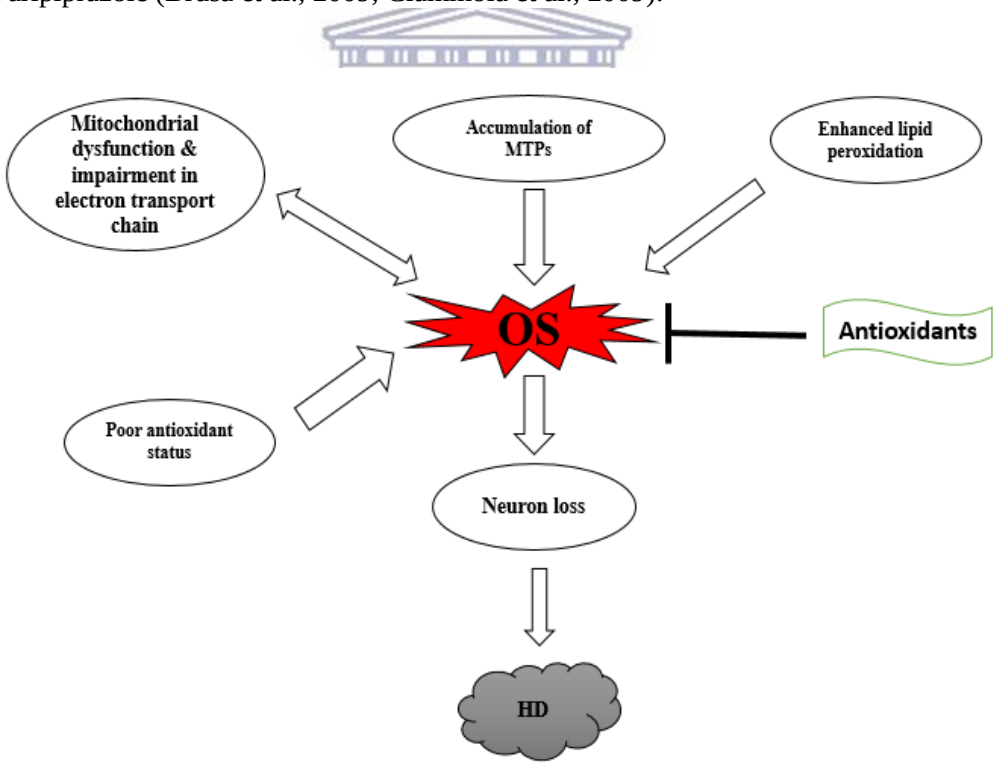


Figure 2.5: Schematic diagram showing the involvement of oxidative stress in Huntington's disease

2.3.4. Human Prion diseases

PrDs are related to a variety of clinical presentations and have attracted vast research awareness for many years not only due to their distinctive composition and properties but also because of their effect on public health (Prusiner, 1998, Collinge, 2001, Collinge and Clarke, 2007). Examples of PrDs include Gerstmann Sträussler-Scheinker syndrome, Creutzfeldt-Jakob disease (CJD), kuru and fatal familial insomnia while animal PrDs include scrapie and bovine spongiform encephalopathy (Brown, 2005).

According to the 'protein-only' hypothesis (Griffith, 1967, Wadsworth and Collinge, 2011), host-encoded cellular prion protein (PrP^{C}) is converted to a different form often designated as scrapie PrP^{Sc} (Prusiner, 1998, Collinge, 2001, Collinge and Clarke, 2007, Surewicz and Apostol, 2011). It is widely regarded as the infectious agent which can duplicate itself with high conformity by enlisting endogenous PrP^{C}, and that the modification between these isoforms lies strictly in its state of aggregation and its monomer conformation (Collinge, 2001, Prusiner, 2012). Microscopic examination of the brains of patients with PrDs typically shows characteristic histopathologic alterations, comprising of neuronal degeneration and vacuolation, which gives the cerebral grey matter a spongiform appearance, and a reactive increase of astroglial cells (Budka et al., 1995, Wadsworth and Collinge, 2011).

Various lines of evidence have recognized markers of oxidative stress in the brains of rodents with prion disease (Basu et al., 2007, Westergard et al., 2007) (Figure 2.6). Immunohistochemical studies in the brains of scrapie-infected mice have revealed the presence of lipid oxidation markers, nitrotyrosine (a marker of peroxynitrite production) and heme-oxygenase-1 (an enzyme leading to the development of antioxidant molecules), suggesting that oxidative stress might be one mechanism of neuronal loss (Guan et al., 1996, Guentchev et al., 2000). There are also indications for mitochondrial damage induced by oxidative stress in cells from brains of scrapie-infected mice and hamsters (Choi et al., 1998, Lee et al., 1999).

Furthermore, a study from Kim and colleagues suggested that iron induced oxidative stress might be a key mechanism of neuronal loss in scrapie (Kim et al., 2000).

Unfortunately, there is presently no effective treatment or disease modifying therapy for PrDs. The search for treatments is primarily hindered by inadequate understanding of prion disease pathogenesis. However, identified drugs which show some effectiveness in treating prion diseases in *in-vitro* and *in-vivo* systems include quinacrine and pentosan polysulfate (Trevitt and Collinge, 2006). These compounds have been used as compassionate therapy in CJD patients, however, no therapeutic value was observed (Haik et al., 2004, Whittle et al., 2006). Other treatment options attempted for PrDs that have had limited success include immunotherapy and vaccination (Li et al., 2010a).

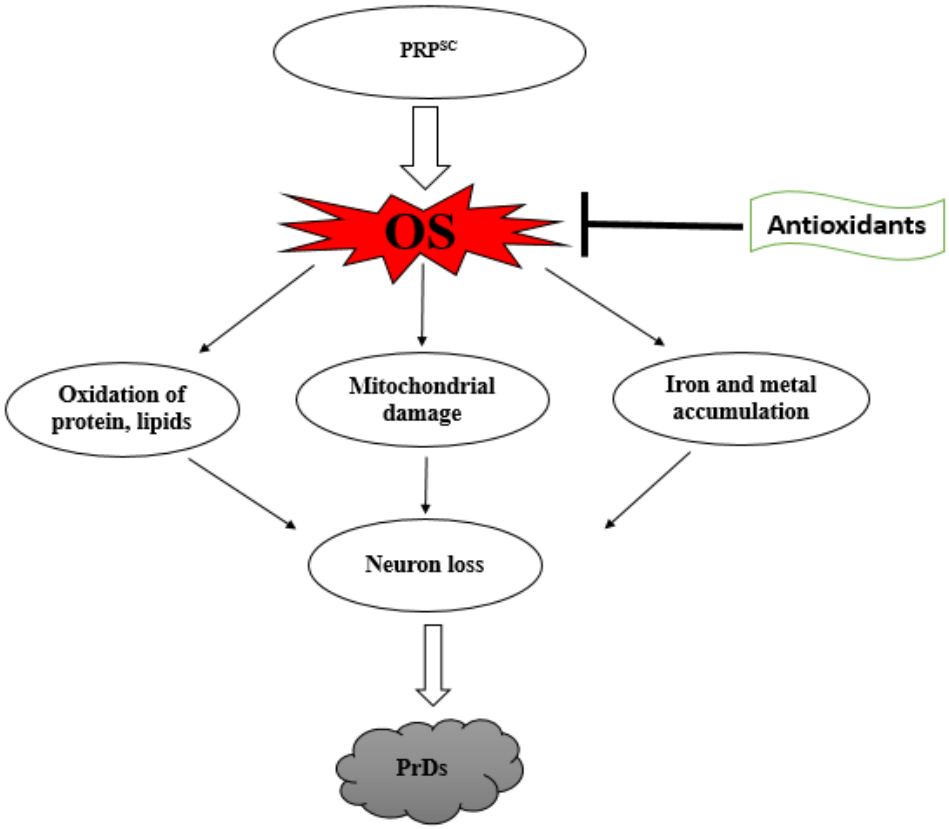


Figure 2.6: Schematic diagram showing the involvement of oxidative stress in Prion diseases

2.4. RUTIN

Rutin is a yellow crystalline powder that is more soluble in alcohol and alkaline solutions than in water (Couch et al., 1946, Habtemariam, 2016). Its synthesis in higher plants is achieved through the 3-O-glycosylation and rhamnosylation of quercetin and isoquercetin respectively (Suzuki et al., 2015). The acidic hydrolysis of rutin in plants is achieved by the breakage of the α-L-rhamnoside bond and β-D-glucoside bond to produce quercetin and isoquercetin as major products (Wang et al., 2011). Studies on the bioavailability of rutin showed that rutin was less effective and less rapidly absorbed than quercetin and its glycosides (Manach et al., 2005). In a bid to increase the solubility and oral bioavailability of rutin, various delivery formulations have been produced (Miyake et al., 2000, Mauludin et al., 2009). Reports also indicate that the bioavailability of rutin differs significantly depending on food conditions (Tamura et al., 2007, Ou-Yang et al., 2013), which accordingly alters the metabolism of quercetin and its glycoside forms (Budzynska et al., 2017). Furthermore, the pharmacokinetics of rutin differs considerably in various *in-vivo* models (Gohlke et al., 2013, Maciej et al., 2015).

Rutin has been shown to have an extensive array of pharmacological applications due to its numerous properties including antioxidant, anti-inflammatory, cardiovascular, neuroprotective, anti-diabetic and anticancer activities (Perk et al., 2014b, Al-Dhabi et al., 2015). Particularly, it has been suggested that its neuroprotective activity is linked to its 4-Oxo group and the 2,3 double bond in the C ring of the rutin structure (Khan et al., 2009). Over the years, various mechanisms have been found to be responsible for its antioxidant activities in both *in-vitro* and *in-vivo* models. Firstly, it was reported that its chemical structure can directly scavenge ROS (Hanasaki et al., 2005). Secondly, it increases the production of GSH and cellular oxidative defence systems are believed to be up-regulated by an increased expression of numerous antioxidant enzymes such as CAT and SOD (Ahmed and Zaki, 2009, Al-Enazi, 2014, Kandemir et al., 2015). Thirdly, rutin inhibits xanthine oxidase which is involved in generating ROS (Kostić et al., 2015). From the aforementioned, the optimism generated by the therapeutic potential of rutin in many health conditions in which oxidative stress is an underlying cause, is

understandable (Magalingam et al., 2013, Al-Enazi, 2014, Park et al., 2014, Wang et al., 2015b). The rest of this review will summarize the main findings of the neuroprotective effects of rutin in various experimental models of NDs. The various *in-vitro* and *in-vivo* studies are summarized in Table 2.1 and Table 2.2, respectively.

2.5. STUDIES OF RUTIN IN ALZHEIMER'S DISEASE

2.5.1. Toxins used to generate models of Alzheimer's Disease

Several lines of evidence indicate that Aβ peptides are the key factors in AD pathogenesis (Joachim and Selkoe, 1992, Cerpa et al., 2008, Simpson et al., 2010). Aβ peptide, produced from amyloid precursor protein (APP) is a very important part of amyloid plaques, and has been described to be neurotoxic (Kihara et al., 2001). It is hypothesized that an anomaly in the proteolytic processing of the APP leads to an increase in the generation of Aβ peptides (such as $A\beta_{40-42}$ and $A\beta_{25-35}$) which in turn leads to the build-up of Aβ, a key event in the pathogenesis of AD (Jiménez-Aliaga et al., 2011, Domínguez-Prieto et al., 2017). Aβ may also induce oxidative stress by causing mitochondria dysfunction which results in increased ROS and decreased levels of antioxidants such as GSH and the activity of antioxidant enzymes such as SOD, GPx and CAT (Wang et al., 2012). Aβ induced ROS production is believed to aid Aβ production and accumulation, thereby quickening the progression of AD (Kern and Behl, 2009, Lee et al., 2010b). Additionally, Aβ induces nitric oxide (NO) generation by up-regulating the expression of nitric oxide synthase (iNOS) (Akama et al., 1998, Wang et al., 2004) which plays a fundamental role in the series of events leading to cell death (Huang et al., 2011).

2.5.2. *In-vitro* studies

Aβ accumulation is a key feature of AD and rutin has been shown to decrease and reverse $A\beta_{25-35}$ fibril formation *in-vitro* indicating that its action might be connected to their free-radical scavenger activity and might subdue neurotoxicity (Jiménez-Aliaga et al., 2011). Furthermore, in a different study (Wang et al., 2012), rutin acted as a multifunctional agent by inhibiting Aβ aggregation and cytotoxicity, prevented mitochondrial damage, reduced production of MDA,

ROS, NO, GSSG, iNOS and proinflammatory cytokines, and increased CAT, SOD, GSH, and GPx, levels. Yu and colleagues demonstrated the ability of rutin to inhibit amylin-induced neurocytotoxicity and enhance antioxidant enzyme activities in the SH-SY5Y cells (Yu et al., 2015). Treatment of human neuroblastoma SH-SY5Y cells with rutin loaded nanoparticles conferred protective effects on Aβ-induced cytotoxicity, decreased levels of NO and ROS (Hu et al., 2015). In a related activity, rutin modulated the generation of pro-inflammatory cytokines by reducing TNF-α and interleukin (IL)-1β generation in $A\beta_{40-42}$ treated BV-2 cells (Wang et al., 2012). Da Silva and colleagues established that rutin treatment was not toxic to microglial cells and induced a dose-dependent increase in microglial proliferation, decreasing the mRNA levels of *TNF*, *IL1b, IL6 and iNOS*; reduced production of IL6, TNF, and NO; increased production of the M2 regulatory cytokine IL10 and arginase; significantly inhibited the LPS-induced expression of *PTGS2, IL18* and *TGFβ* (Da Silva et al., 2017).

2.5.3. *In-vivo* studies

Several studies have utilized animal models as a pre-clinical tool to evaluate the neuroprotective potential of bioactive compounds such as edaravone and vitamin D3 in AD (Zhou et al., 2013, Mohamed et al., 2015). In a study, Xu and colleagues (Xu et al., 2014a) showed that following oral administration of rutin at a daily dose of 100 mg/kg for six weeks, rutin attenuated memory deficits in APPswe/PS1dE9 transgenic mice, reduced oligomeric Aβ level as well as downregulated microgliosis and astrocytosis, and reduced IL-1 and IL-6 levels in the brain. In an interesting and similar study by Hu and colleagues, intravenous administration of Congo red/Rutin magnetic nanoparticles (MNPs) resulted in rescue of memory deficits and amelioration of neurologic changes in the brains of APPswe/PS1dE9 transgenic mice (Hu et al., 2015). Cheng and co-workers showed that rutin and exercise enhanced high fat diet induced cognitive defects in mice (Cheng et al., 2016). Rutin's ability to alleviate impaired cognition and memory in $A\beta_{25-35}$ induced mouse model of AD was demonstrated by Choi and colleagues in 2015 (Choi et al., 2015).

Most recently, Ramalingayya and colleagues (Ramalingayya et al., 2017) demonstrated that pretreatment with rutin inhibited doxorubicin (DOX)-induced ROS generation, increased DOX-induced reduction of CAT, GSH and SOD levels in wistar rats. Other findings include prevention of DOX-induced cell cycle and morphological changes, reduction of DOX-induced apoptosis, prevention of DOX-induced episodic like memory deficit, prevention of rise in TNF-α levels and reversal of myelosuppressive effect of DOX (Ramalingayya et al., 2017). In a similar AD study by Ramalingayya and co-workers (Ramalingayya et al., 2016), rutin dose-dependently improved recognition and discriminative indices in time-induced long-term as well as scopolamine-induced short-term episodic memory deficit AD models without disturbing locomotor activity. Moghbelinejad and colleagues demonstrated that rutin significantly increased extracellular signal regulated protein kinase 1 (ERK1), cAMP response element-binding protein (CREB) and brain-derived neurotrophic factor (BDNF) gene expression in the hippocampus of rats. Studies show that the mitogen-activated protein kinase

(MAPK) cascade that includes ERK1/2 and CREB is involved in neural plasticity and survival (Spencer, 2008). Long lasting changes in synaptic plasticity and memory are the resultant effects arising from the activation the MAPK cascade (Spencer, 2008). BDNF affects the survival and function of neurons in the CNS and is essential for normal synaptic connection formation during growth and for learning and memory in adults (Thomas and Davies, 2005). They also found rutin to significantly increase memory retrieval while significantly lowering MDA levels in the hippocampus (Moghbelinejad et al., 2014).

In a different type of AD model, Javed and co-workers showed that rutin significantly reduced intracerebroventricular-streptozotocin (ICV-STZ)-induced increase in TBARS, poly ADP-ribosyl polymerase and nitrite in the hippocampus of rats. Rutin also significantly increased levels of GSH, GPx, glutathione reductase (GR) and CAT (Javed et al., 2012). Furthermore, rutin also significantly improved cognitive deficits, attenuating STZ-induced inflammation by decreasing the expression of interleukin-8 (IL-8), glial fibrillary acidic protein (GFAP), cyclooxygenase-2 (COX-2), nuclear factor-kB, inducible iNOS, and reduced histological abnormalities in the hippocampus (Javed et al., 2012). In a different model of AD using zebrafish, Richetti and co-workers were able to show that rutin did not affect zebrafish general locomotor activity and prevented scopolamine-induced amnesia (Richetti et al., 2011).

The various studies highlighted in this section demonstrates the neuroprotective capability of rutin in ameliorating the adverse effects of neurodegeneration as well as cognitive impairments associated with AD in various animal models.

2.6. STUDIES OF RUTIN IN PARKINSON'S DISEASE

2.6.1. Toxins used to generate models of Parkinson's Disease

Over the years, neurotoxins used to induce dopaminergic neurodegeneration include 6-hydroxydopamine (6-OHDA), 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP), 1, 1 -dimethyl-4, 4 –bipyridinium (paraquat) and rotenone (Bové et al., 2005, Hisahara and Shimohama, 2010). Seemingly, all of these toxins provoke the formation of ROS. 6-OHDA is

known to be taken up by dopaminergic neurons through the dopamine transporter (Schober, 2004, Bové et al., 2005). In the neurons, oxidized molecules of 6-OHDA produces free radicals that hinders mitochondrial complex I and produces $^{\bullet}O^{-}_{2}$ and $^{\bullet}OH$ which becomes toxic to dopaminergic neurons and induces microglial activation. Rotenone and MPTP are known for their ease of use in animals and their similar ability to potently inhibit complex I. After its systemic administration, MPTP swiftly crosses the blood brain barrier (Hisahara and Shimohama, 2010).

Once in the brain, MPTP is converted in the astrocytes by monoamine oxidase B (MAO-B) to 1-methyl-4-phenylpyridinium (MPP^{+}) and is thereafter released into the extracellular space (Nicklas et al., 1985, Przedborski and Vila, 2003, Hisahara and Shimohama, 2010). Once inside dopaminergic neurons, MPP^{+} accumulates in mitochondria and impairs mitochondrial respiration by impeding complex I in the electron transport chain, which induces the production of ROS (Nicklas et al., 1985, Ramsay and Singer, 1986). Rotenone is also very lipophilic and is circulated evenly throughout the brain after crossing the BBB (Uversky, 2004, Bové et al., 2005). Paraquat, an herbicide, has a very close structural similarity to MPP^{+} and has been proposed to be a risk factor for PD (Di Monte et al., 1986). A neurobehavioral syndrome characterized by reduced ambulatory activity, a decline in striatal dopamine nerve terminal density and a significant decrease in substantia nigra dopaminergic neurons have all been associated and linked to effects from systemic administration of paraquat (Brooks et al., 1999). Experimental evidence show that paraquat crosses the BBB to cause damage of the dopamine neurons in the substantia nigra, like MPP^{+} (Brooks et al., 1999). In addition, sustained exposure to paraquat results in a marked accrual of α-synuclein-like aggregates in neurons of the substantia nigra pars compacta in mice (Manning-Bog et al., 2002).

2.6.2. *In-vitro* studies

PD has been modelled *in-vitro* through the specific neurotoxic effect of the 6-OHDA on dopaminergic neurons. Neurotoxicity triggered by 6-OHDA was attenuated by rutin treatment

in PC12 cells where a significant dose-dependent cytoprotective activity was detected in rutin pretreated cells (Magalingam et al., 2013). Rutin activated antioxidant enzymes including SOD, CAT, GPx and GSH when compared to cells incubated with 6-OHDA alone in conjunction with a significantly reduced lipid peroxidation activity (Magalingam et al., 2013, 2016). In 2015, Magalingam and co-workers reported that pretreatment with rutin in PC12 cells downregulated the mRNA expression of PD linked genes (*PARK2*, *UCHL1*, *DJ-1*) and pro-apoptotic (*Casp3* and *Casp7*) genes which were upregulated in the 6-OHDA-treated PC12 cells (Magalingam et al., 2015). The study showed that rutin up-regulated the *TH* gene which is essential in dopamine biosynthesis and further up regulated the ion transport and anti-apoptotic genes (*NSF* and *Opa1*) (Magalingam et al., 2015).

In a different model of PD, rutin pretreatment prevented rotenone induced loss of SH-SY5Y cells, inhibited rotenone-induced ROS formation and suppressed elevation of calcium (Park et al., 2014). Rutin attenuated rotenone-induced reduction of mitochondrial membrane potential, activation of JNK and p38 MAPK pathways, reversed changes of Bcl-2 and Bax levels and inhibited apoptosis and caspase-9/3 activation (Park et al., 2014).

2.6.3. *In-vivo* studies

In one of the very few and earliest studies documenting the neuroprotective effects of rutin in *in-vivo* models, oral administration of rutin significantly protected against 6-OHDA induced increase in rotations, deficits in locomotor activity and motor coordination in male wistar rats (Khan et al., 2012). Immunohistochemical and histopathological findings in the substantia nigra showed that rutin protected neurons from toxic effects of 6-OHDA (Khan et al., 2012). In a different model of PD, Sharma and co-workers (Sharma et al., 2016) showed that rutin played an important role in attenuating behavioural, biochemical and histological parameters after haloperidol administration in rats and further confirmed the protective effects of rutin.

These *in-vivo* and *in-vitro* studies exhibit the potential of rutin as a neuroprotector and suggest a role for this compound in the prevention and reversal of degenerative diseases such as PD.

2.7. STUDIES OF RUTIN IN HUNTINGTON'S DISEASE

2.7.1. Toxins used to generate models of Huntington's Disease

Animal models of HD have provided understanding into disease pathology and previous studies of HD used toxin-induced models to study excitotoxicity-induced cell death and mitochondrial impairment, both mechanisms of HD degeneration. These models, based on quinolinic acid (QA) and 3-nitropropionic acid (3-NP) are still often used in HD studies (Ramaswamy et al., 2007). QA is experimentally administered straight to the striatum because it is incapable of crossing the BBB (Foster et al., 1984). Its key features include striatal neurodegeneration in rats (Bordelon et al., 1997, Ribeiro et al., 2006), mice (McLin et al., 2006), and primates (Kendall et al., 2000, Emerich et al., 2006) in a strikingly similar pattern to that seen in human HD. Its advantages as a HD model includes its ease of use in more complex animals, its influences on cognitive function, numerous resemblances between pathology observed in the HD brain and its mode of cell death mimics the mechanism of neuronal death seen in HD brains (Beal et al., 1991, Ferrante et al., 1993, Roberts et al., 1993). 3-NP is known to irreversibly inhibit the mitochondrial enzyme succinate dehydrogenase (Alston et al., 1977, Coles et al., 1979). Its major advantage is that it mimics cell death seen in the HD brain through a combination of apoptosis and necrosis (Ramaswamy et al., 2007). Instantly after administration of 3-NP, there is a surge of necrotic cell death followed by gradual apoptosis (Pang and Geddes, 1997). 3-NP crosses the blood-brain barrier and can be administered systemically to mice, rats, and nonhuman primates (Ramaswamy et al., 2007).

2.7.2. *In-vivo* studies

In a pioneering work on HD with rutin in 3-NP treated rats, Suganya and Sumathi reported that oral administration of rutin (25 mg/kg and 50 mg/kg) significantly decreased protein oxidation and improved endogenous antioxidant defense system. Furthermore rutin improved 3-NP induced behavioral alterations and restored the activities of mitochondrial complex enzymes (I, II, IV, V) when compared to the 3-NP induced group (Suganya and Sumathi, 2014).

In 2016**,** Suganya and Sumathi again reported that oral administration of rutin (25mg/kg body weight) to wistar rats increased the levels of non-enzymatic antioxidants (vitamin C and E) when compared to a reduction in the 3-NP induced group. In addition, rutin protected against 3-NP induced reduction in motor activities, muscle coordination and activities of adenosine triphosphatases (ATPases) (Suganya and Sumathi, 2016).

Most recently, Suganya and Sumathi showed that rutin restored 3-NP induced reduction of body weight, locomotor activities, memory and antioxidants levels. They further stated that rutin ameliorated 3-NP induced striatal damage by reducing levels of lipid peroxides, nitrite, GFAP and activity of acetylcholine esterase (Suganya and Sumathi, 2017).

Although these few *in-vivo* studies offer concrete evidence for the therapeutic potential of rutin, there exists a critical need to further elucidate and provide more evidence for the therapeutic potential of rutin in *in-vitro* models of HD.

2.8. STUDIES OF RUTIN IN HUMAN PRION DISEASES

2.8.1. Toxins used to generate models of Human Prion diseases

The prion protein peptide 106–126 (PrP (106–126)) has frequently been used as a model system to study prion-induced neurodegeneration (Della-Bianca et al., 2001, Pérez et al., 2003). This peptide induces neurotoxicity in neuronal cells owing to its amyloidogenic properties both *in-vivo* and *in-vitro* (Ettaiche et al., 2000). One of the major advantage of PrP (106–126) is that it is comparable to PrP^{Sc} in numerous respects and at the same time is more soluble and easy to deploy for cell culture experiements (Gu et al., 2002). PrP (106–126) is rich in β-sheet structure, increases the membrane micro-viscosity of neurons and astrocytes (Diomede et al., 1996) and forms aggregates that are protenase K-resistant and detergent-insoluble (Brown, 2000, Ettaiche et al., 2000, Rymer and Good, 2000). PrP (106–126) weakens liposomes and induces liposome fusion (Dupiereux et al., 2005).

2.8.2. *In-vitro* studies

In a pioneering study (Na et al., 2014), the authors studied the neurotoxicity of PrP (106–126) in the HT22 hippocampal cell line and assessed the neuronal protection provided by rutin against the toxic effects of PrP(106–126). Rutin treatment blocked PrP (106–126)-mediated increases in ROS production, NO release and delayed the decrease of neurotrophic factors that resulted from PrP accumulation. In addition, rutin mitigated PrP (106–126)-associated mitochondrial apoptotic events by hindering mitochondrial permeability transition, caspase-3 activity and blocking expression of the apoptotic signals (Bax and PARP) in conjunction with a significantly reduced expression of the death receptor Fas and its ligand Fas-L (Na et al., 2014).

There are currently no *in-vivo* studies on the therapeutic potential of rutin in PrP models. Consequently, there is a dire need to further elucidate and provide more evidence for the therapeutic potential of rutin in more *in-vitro* and *in-vivo* models of PrD.

Table 2.1: Summary of the protective effects of rutin in *in-vitro* models of neurodegeneration

Toxin used in cellular model	Disorder	Key Findings	Reference
$A\beta_{25-35}$ treated SH-SY5Y neuroblastoma cells and $A\beta_{25-35}$ treated APP695-transfected SH-SY5Y (APPswe) cells.	AD	↓ Aβ fibrils, ↓ β-secretase enzyme (BACE), ↓ ROS, ↑ GSH, ↓ lipid peroxidation.	(Jiménez-Aliaga et al., 2011)
Aβ42 treated SH-SY5Y and BV-2 cells.	AD	↓ ROS, ↓ NO, ↓ GSSG, ↓ MDA, ↓ iNOS, ↓ MMP, ↑ GSH/GSSG ratio, ↑ SOD, CAT and GPx, ↓ TNF-a, ↓ IL-1β.	(Wang et al., 2012)
Amylin treated SH-SY5Y neuroblastoma cells.	AD	↑ cell viability, ↓ ROS, ↓ NO, ↓ GSSG, ↓ MDA and ↓ TNF-α and ↓ IL-1β, ↑ GSH/GSSG ratio, ↑ SOD, ↑ CAT, ↑ GPx, ↓ iNOS.	(Yu et al., 2015)
6-OHDA treated PC-12 cells.	PD	↑ cell viability, ↑ 6-OHDA induced reduction in SOD, CAT, GPx, GSH, ↓ lipid peroxidation.	(Magalingam et al., 2013)
6-OHDA treated PC-12 cells.	PD	↑ 6-OHDA induced reduction in SOD, CAT, GPx, GSH. ↓ lipid peroxidation, ↓ MDA	(Magalingam et al., 2016)
6-OHDA treated PC-12 cells.	PD	↓ Park2, ↓ UCHL1, ↓ DJ-1, ↓ Casp3, and ↓ Casp7, ↑ TH ↑ NSF ↑ Opa1.	(Magalingam et al., 2015)
Prion Peptide-treated HT22 cells.	PrD	↓ ROS, ↓ NO, ↓ apoptosis, ↓ Fas, ↓ Fas-L.	(Na et al., 2014)

6-OHDA: 6-hydroxydopamine; CAT: Catalase; Fas L: Fas ligand; GPx: glutathione peroxidase; GSH: Reduced Glutathione; GSSG: glutathione disulphide; IL 10; Interleukin 10, IL 6; Interleukin 6, IL 8; Interleukin 8, IL-1β; Interleukin 1beta, iNOS; Inducible nitric oxide synthase, MDA; Malondialdehyde, MMP; Mitochondrial membrane potential, NSF; N-ethylmaleimide-sensitive factor, Opa1; optic atrophy 1, ROS; Reactive oxygen species, SOD; Superoxide dismutase, TH; Tyrosine hydroxylase; TNF-α; Tumor necrosis factor-α.

Table 2.2: Summary of the protective effects of rutin in *in-vivo* models of neurodegeneration.

Toxin used in animal model	Disorder	Key Findings	Reference
Doxorubicin (DOX) treated neuroblastoma cells (IMR32) and doxorubicin induced cognitive dysfunction in Wistar rats.	AD	↓apoptosis, ↓ROS, ↓episodic memory deficit, ↓TNF-α, ↑DOX-induced reduction of catalase, GSH, and SOD	(Ramalingayya et al., 2017)
Microglial cells obtained from the cortex of Wistar newborn rats.	AD	↓TNF, ↓IL1b, ↓IL6, ↓iNOS, ↑IL10, ↑arginase, ↓PTGS2, ↓IL18,↓*TGFβ*.	(Da Silva et al., 2017)
**APPswe/PS1dE9 transgenic mice	AD	↑memory, ↑SOD, ↑GSH/GSSG ratio, ↓GSSG, ↓MDA. ↓IL-1, ↓IL-6.	(Xu et al., 2014a)
High Fat diet induced obese (DIO) cognitively impaired C57BL/6J mice	AD	↓cognitive defects.	(Cheng et al., 2016)
Scopolamine treated Wistar rats	AD	↑ Recognition, ↑discriminative indices	(Ramalingayya et al., 2016)
$A\beta_{25-35}$-infused mouse model	AD	↓ Impaired cognition, ↑memory, ↓NO, ↓lipid peroxidation.	(Choi et al., 2015)
Beta-amyloid induced neurotoxic rats	AD	↑ERK1, ↑CREB, ↑ BDNF, ↑memory retrieval, ↓MDA.	(Moghbelinejad et al., 2014)
Intracerebroventricular-streptozotocin (ICV-STZ)–infused rats	AD	↓TBARS, ↓nitrite level,↓poly ADP-ribosyl polymerase, ↑GSH,↓lipid peroxidation, ↓cognitive deficits, ↓COX-2, ↓GFAP, ↓IL-8, ↓iNOS, ↓NF-kB	(Javed et al., 2012)

Scopolamine-induced zebrafish	AD	↓ Amnesia.	(Richetti et al., 2011)
Intrastriatal injection of 6-OHDA in rats	PD	↓ 6-OHDA induced increase in rotations, ↓deficits in locomotor activity, ↓motor coordination, ↑antioxidant levels, ↑DA, ↑dopaminergic D2 receptors	(Khan et al., 2012)
Haloperidol treated rats	PD	↓catalepsy,↓Akinesia,↑locomotor activity, ↑GSH, ↑SOD, ↓TBARS	(Sharma et al., 2016)
3-Nitropropionic (3-NP) acid treated rats	HD	Improved 3-NP induced behavioral alterations; Restored activities of mitochondrial complex enzymes.	(Suganya and Sumathi, 2014)
3-Nitropropionic (3-NP) acid treated rats	HD	Restored biochemical, behavioral and cellular alterations	(Suganya and Sumathi, 2016)
3-Nitropropionic (3-NP) acid treated rats	HD	↑Body weight, ↑locomotor activities, ↑memory, ↑antioxidants levels. ↓lipid peroxides, ↓ nitrite, ↓GFAP, ↓AchE.	(Suganya and Sumathi, 2017)

** Rutin loaded magnetic nanoparticles was used in this experiment;
6-OHDA: 6-hydroxydopamine; AchE: Acetylcholine esterase; BDNF: Brain-derived neurotrophic factor; CAT: Catalase; CREB: cAMP response element binding protein; DA: Dopamine; Doxorubicin: DOX; ERK1: extracellular signal–regulated kinase 1; GFAP : Glial fibrillary acidic protein; GPx: glutathione peroxidase; GSH: Reduced Glutathione; GSSG: glutathione disulphide; IL10: Interleukin 10; IL6: Interleukin 6; IL8: Interleukin 8; IL-1b: Interleukin 1beta; iNOS: Inducible nitric oxide synthase; MDA, Malondialdehyde; MMP: Mitochondrial membrane potential; NF-kB: Nuclear factor-kappaB; NSF: N-ethylmaleimide-sensitive factor; PTGS2: Prostaglandin-endoperoxide synthase 2; ROS: Reactive oxygen species; SOD: Superoxide dismutase; TBARS: Thiobarbituric acid reactive substances; TGFb: transforming growth factor beta; TH: Tyrosine hydroxylase; TNF-α: Tumor necrosis factor-α.

2.9. THE PRESENT STUDY

Neurotoxins such as 6-OHDA, rotenone, paraquat and MPTP/MPP^+ are widely used to investigate the molecular pathways involved in PD pathogenesis. Extensive and far reaching studies of various PD models have shown many important signaling pathways and pathological features critical to dopaminergic neurodegeneration. Furthermore, novel protective strategies for PD may be designed by targeting specific molecules or intracellular signaling cascades that participate in PD pathogenesis. Accordingly, many *in-vitro* and *in-vivo* studies have been designed to evaluate the possibility of antioxidants attenuating dopaminergic neuronal death (Ramassamy, 2006).

This study therefore investigated the neuroprotective activity of rutin in MPP^+ treated dopaminergic human SH-SY5Y neuroblastoma cell line and primary dermal fibroblasts with PD linked mutations. Although previous studies have shown that rutin could attenuate 6-OHDA neurotoxicity in PC12 cells, its mechanism of action and molecular pathways in MPP^+ treated SH-SY5Y cells and fibroblasts have not been investigated. Consequently, for the first time, this research project investigated the protective activity of rutin in cellular models of PD, thus revealing possible molecular pathways and mechanisms of action.

2.10. AIM OF THE STUDY

The present study aimed to investigate the protective effects of rutin in MPP^+ treated dopaminergic human SH-SY5Y neuroblastoma cell line and fibroblasts with PD linked mutations.

2.11. OBJECTIVES OF THE STUDY

. The following research objectives directed this study:

1. To optimize the establishment of an *in-vitro* model of PD.
2. To screen for the cytotoxic effects of MPP^+ and rutin in SH-SY5Y cells.
3. To determine cell viability of SH-SY5Y cells following MPP^+ treatment in the presence and absence of rutin using MTT, Trypan blue, CCK-8 and Apotox-glo assays.

4. To determine the mechanisms of action of rutin treatment using assays for ROS, NO and oxidative stress markers (CAT, SOD and GSH).
5. To determine the changes in intracellular Ca^{2+} in SH-SY5Y cells following treatment with rutin and then MPP^+ using the fluo-4 direct Ca^{2+} assay kit.
6. To evaluate apoptosis in SH-SY5Y cells following treatment with rutin and then MPP^+ using flow cytometry (Annexin V/FITC), hoechst staining, caspase 3/7 activation (assay kit) and caspase 9 activity (ApoTarget™ caspase-9 protease assay kit).
7. To evaluate protein expression levels in in SH-SY5Y cells following treatment with rutin and then MPP^+ using western blot markers for apoptosis (cleaved PARP, cytochrome c and full-length caspase 3), autophagy (LC3-II and p62), ER stress (BiP and CHOP), cell signaling (p-Akt, NF-κB and p-AMPKα), DNA damage (γH2AX) and inflammation (COX-2).
8. To further evaluate autophagy and ultrastructural changes in cellular morphology using transmission electron microscopy.
9. To determine MMP in SH-SY5Y cells following treatment with rutin and then MPP^+ using JC-1 and rhodamine123 fluorescent dyes.
10. To screen for the toxicity of MPP^+ and determine cell viability in fibroblasts following treatment with rutin and then MPP^+ using MTT assays.
11. To evaluate bioenergetic profiles of mitochondrial respiration such as basal respiration, ATP-coupled respiration, maximal respiration and spare respiratory capacity as well as levels of glycolytic respiration in SH-SY5Y and fibroblasts following treatment with rutin and MPP^+ respectively.

2.12. HYPOTHESIS

A previous study showed that rutin could protect against 6-OHDA-triggered neurotoxicity in PC12 cells (Magalingam et al., 2013). Inspired by these findings, the overall hypothesis of this study was that rutin may also be protective against MPP^+-induced dopaminergic cell death in SH-SY5Y cells and further prove its potentials as a neuroprotective agent in PD. Additionally, we hypothesized that rutin could replicate its protective activity in a different model of PD using fibroblasts with PD linked mutations and their matched controls. Results from this study would provide the first research evidence on the protective activity of rutin in MPP^+-treated SH-SY5Y cells and fibroblasts.

CHAPTER 3

RESEARCH METHODOLOGY

3.0. OUTLINE OF CHAPTER

This chapter (sections A and B) describes the experimental design and research methodology chosen for this study. Section A summarizes the materials and methods used such as chemicals required, compounds tested and the maintenance of the parental SH-SY5Y (American Type Culture Collection / ATCC® CRL2271™) neuroblastoma cell line. Section B summarizes the culturing of dermal fibroblasts and methods used in evaluating the bioenergetic profiles of mitochondrial respiration in SH-SY5Y cells and fibroblasts. The statistical methods used for data analysis are also described.

SECTION A: NEUROBLASTOMA

3.1. CULTURE AND MAINTENANCE SH-SY5Y NEUROBLASTOMA CELLS

SH-SY5Y neuroblastoma cells initially purchased from the American Type Culture Collection (ATCC, Rockville, MA), was kindly provided by Dr AM Serafin (Division of Radiobiology, Stellenbosch University, South Africa). All tissue culture operations were carried out in a model NU-5510E NuAire DHD autoflow automatic CO_2 air-jacketed incubator and an AireGard NU-201-430E horizontal laminar airflow tabletop workstation that provides a HEPA filtered clean work area (NuAire). Dishes were appropriately labelled and the worktable was adequately cleaned with ethanol to guarantee no contamination. SH-SY5Y cells were maintained in Dulbecco's modified Eagle medium (DMEM) (Life Technologies, USA), supplemented with 1% penicillin-streptomycin (Lonza, USA) and 10% Fetal bovine serum (FBS) (Life Technologies, USA), at 37°C, 5% CO_2 in a humidified atmosphere. For every two days, culture media was replaced and cells were cultured in Poly-L-lysine-coated 100-mm dishes.

3.1.1. Trypsinization and freezing of cells.

Confluent cells were trypsinized and transferred to another dish for regrowth or to a culture plate for an experiment. Adherent cells that had attained 80-90% confluency were washed in 1X phosphate buffered saline (PBS) (Lonza, USA), brought into suspension by trypsinizing with 1 ml 1X trypsin-versene (EDTA) mixture (Lonza, USA) and placed in the incubator for 2-3 minutes to allow for the detachment of cells. Dishes were removed from the incubator and returned to the laminar flow cabinet. Media was introduced to deactivate trypsin action, detached cells were aspirated and transferred to a 15 ml centrifuge tube for centrifugation at 3000 rpm for 5 minutes to pellet cells. After centrifugation and discarding of the supernatant, cell pellets were resuspended in 3 ml media to ensure even mixing, after which 1ml was transferred to 100-mm dishes containing 9 ml of complete growth media to maintain stock cultures. Documentation of passage numbers was done, and cells below passage 15 were utilized. After maintenance of stock cultures, a volume of 1.35 ml from the 3 ml of cell suspension and 0.15 ml of DMSO were aliquoted into 2 ml cryotubes and placed in storage boxes. Thereafter these were temporarily stored at -80°C for 48 hours and finally transferred to the liquid nitrogen storage facility.

3.1.2. Cell Counting and Viability Testing

Counting of cells and viability testing was done using the TC20™ automated cell counter (Bio-Rad). The automated cell counter is programmed to use an optical method to count the cells in fluid samples enclosed in a cell counting slide or chamber. Consequently, cell viability was evaluated by mixing 10 µl of cell suspension with 0.4% trypan blue stain in a 1:1 ratio (v/v). This mixture was placed in a counting slide chamber and inserted into the TC20™ automated cell counter. The total number of cells counted together with the number of viable cells is thereafter displayed on the screen of the cell counter in 30 sec. The number of cells required for a particular experiment was then calculated in relation to the results of viable cells displayed on the automated cell counter.

For experiments involving screening for the cytotoxic effects of MPP^+ (St. Louis, MO, USA) and screening for the cytotoxicity/neuroprotective activities of rutin (St. Louis, MO, USA), cells were seeded into 96-well tissue culture plates and treated at approximately 60% confluency.

3.2. CYTOTOXICITY SCREENING

To ensure that SH-SY5Y cells were exposed to the right concentration of MPP^+ and rutin for the appropriate time period, cytotoxicity screening and dose-response curves were performed using the MTT assay. A fresh 50 mM stock concentration of MPP^+ was prepared in un-supplemented DMEM and dilutions were made up in culture media for final concentrations ranges between 200 µM - 2000 µM. For rutin, a fresh 50 mM stock concentration was prepared in dimethyl sulfoxide (DMSO, Life Technologies, USA) and dilutions were made up in culture media for final concentrations ranges between 5 µM - 200 µM. Some of these concentrations were modified from previous studies using rutin or MPP^+ in different cells (Pettifer et al., 2007, Magalingam et al., 2013, Wang et al., 2014a).

3.2.1. MTT Assay

SH-SY5Y cells were cultured in 96-well plates with a total of $5x10^4$ cells seeded per ml and left to attach overnight. Thereafter, cells were treated with concentration ranges of MPP^+ (section 3.2), and a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, St. Louis, MO, USA) assay was performed to evaluate cell viability after 24 and 48 hours. The objective for MPP^+ was to induce stress in the cells so that only approximately 40 -60% of the SH-SY5Y cells remained viable. A 5 mg/ml solution of MTT was made in PBS (see appendix I), covered in foil and vortexed until it was properly dissolved, after which 20 µl of the MTT solution was introduced to each well and cells incubated for 4 hours under standard incubation conditions. Thereafter, media was removed and 100 µl of DMSO was added to each well to dissolve the formazan crystals. Absorbance was read at a wavelength of 560 nm using a GloMax™ Multiscan plate reader (Promega, USA).

Similarly, the MTT assay was repeated in SH-SY5Y cells treated with concentration ranges of rutin (section 3.2). After the cytotoxicity screening, the exposure time of the cells to rutin before treatment with MPP^+ was determined. SH-SY5Y cells were pretreated with 25 µM, 50 µM and 100 µM concentrations of rutin for 1 hour, 2 hours, 4 hours and 6 hours before the addition of MPP^+ at a concentration of 1000 µM. Based on the results (section 4.3), it was decided that for SH-SY5Y cells, pretreatment with rutin would be for 4 hours before the addition of MPP^+ (for a total of 48 hours).

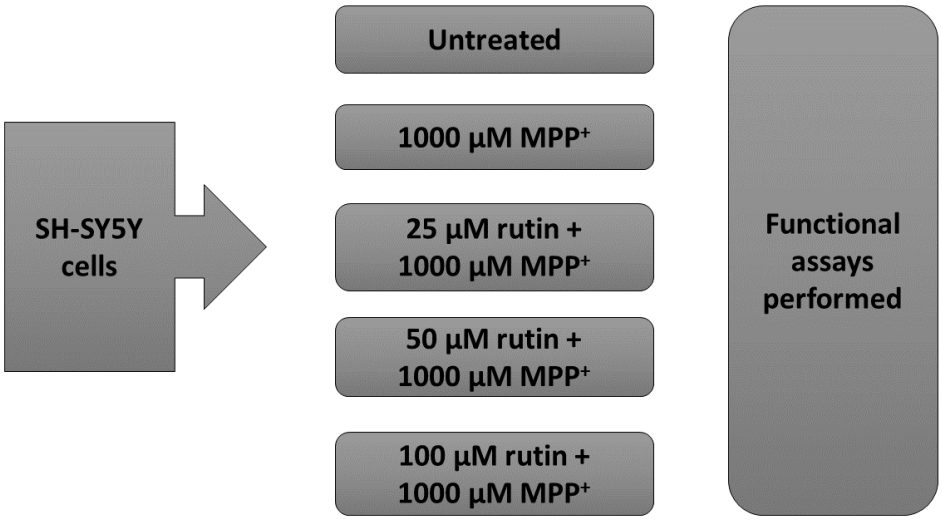


Figure 3.1: Diagram showing the protocol used in this study

3.3. MORPHOLOGICAL EVALUATION OF CELLS

SH-SY5Y cells were cultured in 60-mm dishes and allowed to attach for 24 hours. Cells were treated as described earlier (Figure 3.1), and cell morphology studies was done using a ZEISS Primo Vert (Germany) light microscope.

3.4. CELL VIABILITY AND TOXICITY

SH-SY5Y cells were seeded into 96-well plates and allowed to attach for 24 hours. After treatment with rutin and then MPP^+, an MTT assay (section 3.2.1) was performed and statistical

analysis (Section 3.16) was utilized to determine differences between treated and untreated cells.

3.4.1. Trypan Blue Dye Exclusion Assay

The trypan blue dye (St. Louis, MO, USA) exclusion assay is often used to quantify the population of live cells present in a cell suspension as live cells with intact membranes exclude the dye while dead cells with damaged membranes permit entry of the dye and cell staining (Denizot and Lang, 1986, Altman et al., 1993). Cells were seeded at a density of 1.1 x 10^5 /ml in 60-mm dishes and were allowed to attach for 24 hours under standard incubation conditions. Thereafter, supernatants were discarded, and cells were treated as described earlier (Figure 3.1). After treatment, adherent cells detached by trypsinization were centrifuged using Bio-Rad tabletop centrifuge at 3000 rpm for 5 minutes. The supernatant was discarded, and cell pellets were resuspended in 1 ml of fresh media, thereafter 10 µl of cell suspension was added to an equal volume of 0.4% trypan blue dye and loaded into BioRad TC20™ cell counter. Readings were automatically generated from the machine and calculations were obtained based on the formula below;

$$\% \text{ Viability} = \frac{\text{Live Cells}}{\text{Total Cells}} \times 100\%$$

$$\% \text{ Toxicity} = \frac{\text{Dead Cells}}{\text{Total Cells}} \times 100\%$$

3.4.2. Cell Counting Kit-8 (CCK-8) Assay

The Cell Counting Kit-8 (CCK-8; St. Louis, MO, USA) permits precise assays by employing extremely water-soluble tetrazolium salt WST-8 to produce a water-soluble formazan dye, thus allowing sensitive colorimetric assays for the determination of viable cells. Briefly, cells were seeded at a density of 5 x 10^4 cells/ml and were allowed to attach for 24 hours under standard incubation conditions. After rutin and MPP^+ treatment as described (Figure 3.1), 20 µl of CCK-

8 solution was added to each well of the plate and incubated for 4 hours. The optical density (OD) was obtained at 450 nm using a GloMax™ Multiscan plate reader. The mean blank-corrected absorbance (MBCA) was derived from the following equation:

$$MBCA = \frac{1}{4}\sum_{i=1}^{4}(A_i - A_0)$$

where A_i represents the absorbance reading of well i and A_0 is the absorbance reading of the blank well (inoculated cells without test compound=untreated controls with variable molar concentrations of vehicle approximating final concentrations present in the test wells).

3.4.3. ApoTox-Glo™ Assay

The ApoTox-Glo™ Triplex Assay (Promega, USA) is a three-way assay that assesses viability, cytotoxicity and caspase activation actions within an assay well. Briefly, cells were seeded at a density of 5.0 x 10^4 /ml in white-walled 96 well plates and were allowed to attach for 24 hours under standard incubation conditions. Thereafter, supernatants were discarded and cells were treated as described earlier (Figure 3.1). After treatment, experiments were performed according to manufacturer's instructions. Briefly, 20 µl of Viability/Cytotoxicity reagent containing both GF-AFC Substrate and bis-AAF-R110 substrate were added to all wells and then mixed by orbital shaking (300–500 rpm for ~30 seconds). Thereafter, it was incubated for 30 minutes at 37°C and fluorescence was measured using the GloMax™ Multiscan plate reader at the wavelength sets of 490Ex/510-570Em. (Cytotoxicity). Unfortunately, the filter set for 400Ex/505Em (viability) was unavailable and couldn't measure fluorescence intensity for cell viability.

3.5. FLUO-4 DIRECT CALCIUM ASSAY

Fluo-4 Direct Calcium Assay Kit (Life Technologies, USA) is a cutting-edge fluorescent Ca^{2+} indicator (assay kit bulletin). SH-SY5Y cells were seeded at a density of 5.0 x 10^4 /ml in white

walled 96-well plates and were allowed to attach for 24 hours under standard incubation conditions. After treatment of cells with rutin and MPP^+, determination of intracellular Ca^{2+} was performed according to manufacturer's instructions. Briefly, an equal volume of 2X Fluo-4 direct calcium reagent loading solution was added directly into wells containing cells in culture media. Thereafter, plates containing cells were incubated for 30 minutes each at 37°C and room temperature respectively. Fluorescence intensity measured using the GloMax™ Multiscan plate reader at the wavelength sets of 490Ex/510-570Em.

3.6. EVALUATION OF ROS PRODUCTION

The 2′,7′-Dichlorofluorescin diacetate (DCFH-DA, St. Louis, MO, USA) was utilized to evaluate intracellular ROS production. SH-SY5Y cells were seeded at a density of 5.0 x 10^4 /ml and were allowed to attach for 24 hours under standard incubation conditions. Thereafter, supernatants were discarded, and cells were treated as described earlier (Figure 3.1). Measurement of intracellular ROS generation was performed according to a previously described procedure (Wang and Xu, 2005) with slight modifications. After treatment, cells were incubated with 25 µM DCFH-DA at 37°C for 1 hour and washed twice with PBS, with fluorescence intensity of DCF measured using the GloMax™ Multiscan plate reader at the wavelength sets of 490Ex/510-570Em.

As a complementary measurement of ROS, cells were evaluated using flow cytometry. SH-SY5Y cells were seeded at a density of 1.2 x 10^5 /ml in 100-mm dishes and were allowed to attach for 24 hours under standard incubation conditions. After treatment with rutin and MPP^+, adherent cells dislodged with a cell scraper were spun using Bio-Rad table top centrifuge at 3000 rpm for 5 minutes. The supernatants were discarded, cell pellets were re-suspended in 2 ml DMEM containing 25 µM DCFH-DA and incubated at 37°C for 1 hour. Thereafter, cells were centrifuged for 5 minutes at 3000 rpm and pellets were re-suspended in 500 µl PBS. Cells were acquired at 10000 events and analyzed on an Accuri flow cytometer (BD Biosciences Pharmingen, San Diego, CA, USA).

3.7. EVALUATION OF INTRACELLULAR NITRIC OXIDE PRODUCTION

The Griess reagent (Life Technologies, USA) was utilised in this study to evaluate the production of nitric oxide (NO). Briefly, cells were seeded at a density of 5.0 x 10^4 cells/ml and were allowed to attach for 24 hours under standard incubation conditions, after which supernatants were discarded, and cells were treated as described earlier (Figure 3.1). The supernatants were collected and mixed with an equivalent volume of Griess reagent (Life Technologies, USA) and incubated for 30 minutes at room temperature. The absorbance was measured at 560 nm using the GloMax™ Multiscan plate reader.

3.8. EVALUATION OF ANTIOXIDANT ENZYMES

3.8.1. Measurement of Superoxide Dismutase (SOD)

The SOD colorimetric activity kit (Life Technologies, USA) is designed to measure all types of SOD activity (i.e., Cu/Zn, Mn, and Fe superoxide dismutases) in a variety of samples. This assay measures the activity of SOD in plasma, tissue lysates, serum, cell lysates, and erythrocyte lysates (assay kit bulletin). SH-SY5Y cells were seeded at a density of 1.2 x 10^5/ml in 100-mm dishes and were allowed to attach for 24 hours under standard incubation conditions. After treatment (Figure 3.1), cells were rinsed with PBS and adherent cells were dislodged with a cell scraper. The cells were transferred to a tube on ice, pelleted at 3000 rpm for 5 minutes and experiments were performed according to manufacturer's instructions. Briefly, supernatants were discarded and pellets were re-suspended in 40 µl of ice-cold PBS and transferred to a microcentrifuge tube on ice. Cells were sonicated, centrifuged at 1,500 × g for 10 minutes at 4°C and supernatants were collected immediately for analysis. 10 µl of standards and diluted samples, 50 µl of 1X Substrate and 25 µl of 1X xanthine oxidase were added to each well of a 96 well half area plate and then incubated for 20 minutes at room temperature. Absorbance was read using a Polarstar Omega plate reader (BMG Labtech, USA) at 450 nm and a curve-fitting software generated the standard curve using a four parameter algorithm.

3.8.2. Measurement of Catalase

The catalase colorimetric activity kit (Life Technologies, USA) is designed to measure catalase activity in cell lysates. Catalase, a highly conserved enzyme expressed in all mammalian tissues, catalyzes the hydrolysis of H_2O_2 into H_2O and O_2, to prevent the potential harmful effects of excessive levels of H_2O_2 (Nagata et al., 1999, Agati et al., 2012). SH-SY5Y cells were seeded at a density of 1.2 x 10^5 /ml in 100-mm dishes and were allowed to attach for 24 hours under standard incubation conditions. After treatment (Figure 3.1), cells were rinsed with PBS and adherent cells were dislodged with a cell scraper. The cells were transferred to a tube on ice, pelleted at 3000 rpm for 5 minutes and experiments were performed according to manufacturer's instructions. Briefly, pellets were sonicated in 1 ml of cold assay buffer per 100 mg of cells. It was centrifuged at 10,000 x *g* for 15 minutes at 4°C and supernatants were collected immediately for analysis. 25 µl of standards, 25 µl of diluted samples and 25µl of hydrogen peroxide reagent was added into each well and incubated for 30 minutes at room temperature. Thereafter, 25 µl of substrate and 25 µl of 1X HRP solution was added to each well and further incubated for 15 minutes at room temperature. Absorbance was read using a Polarstar Omega plate reader at 560 nm and a curve-fitting software generated the standard curve using a four parameter algorithm.

3.8.3. Measurement of Glutathione (GSH)

The GSH colorimetric kit (Life Technologies, USA) measures GSH and uses a colorimetric substrate that reacts with the free thiol group on GSH to produce a highly colored product. GSH plays a key role in many biological processes, including proteins and DNA synthesis, amino acid transport and oxidative stress protection (assay kit bulletin). SH-SY5Y cells were seeded at a density of 1.2 x 10^5 /ml in 100-mm dishes and were allowed to attach for 24 hours under standard incubation conditions. After treatment, cells were rinsed with PBS and adherent cells were dislodged with a cell scraper. The cells were then transferred to a tube on ice, pelleted in ice cold PBS at 3000 rpm for 5 minutes and experiments were performed according to manufacturer's instructions. Briefly, pellets were re-suspended in ice cold 5% SSA (1 g of

aqueous 5-sulfo-salicyclic acid dehydrate to 20 ml of water) at 1.2 x 10^6 cells per 100 µl. Cells were lysed by sonication, incubated for 10 minutes at 4°C, centrifuged at 14000 rpm for 10 minutes at 4°C and supernatants were thereafter collected for analysis. 50 µl of standards and diluted samples, 25 µl of colorimetric detection reagent and 25 µl of reaction mixture were added to appropriate wells and incubated for 20 minutes at room temperature. Absorbance was read using a Polarstar Omega plate reader at 405 nm and a curve-fitting software generated the standard curve using a four parameter algorithm.

3.9. ASSAY-BASED EVALUATION OF APOPTOSIS

3.9.1. Estimation of Caspase 3/7 Activation

After fluorescence reading from the cytotoxicity experiment using the ApoTox-glo assay kit (Section 3.4.3), evaluation of caspase 3/7 activation was performed according to manufacturer's instructions. Briefly, 100 µl of caspase 3/7 reagent was added to all wells and was further mixed by orbital shaking (300–500 rpm for ~30 seconds). It was then incubated for 30 minutes at 37°C and luminescence was measured using the GloMax™ Multiscan plate reader.

3.9.2. Estimation of Caspase 9 Activity

The ApoTarget™ Caspase-9 Protease Assay (Life Technologies, USA) measures caspase-9 proteolytic activity in lysates of mammalian cells. SH-SY5Y cells were seeded at a density of 1.2 x 10^5 /ml in 100-mm dishes and were allowed to attach for 24 hours under standard incubation conditions. After treatment (Figure 3.1), cells were rinsed with PBS and adherent cells were dislodged with a cell scrapper. The cells were transferred to a tube on ice, pelleted in ice cold PBS at 3000 rpm for 5 minutes, re-suspended in 50 µl of chilled cell lysis buffer and incubated on ice for 10 minutes. Experiments were then performed according to manufacturer's instructions. Briefly, cells were centrifuged at 10000 x g for 1 minute in a micro centrifuge, supernatants were transferred to a fresh tube on ice and thereafter 50 µl of samples, 50 µl of 2X reaction buffer containing DTT and 5 µl of the 4 mM LEHD-pNA substrate (200 µM final concentration) were added to samples and incubated at 37°C for 2 hours. Samples were kept in

the dark during incubation and absorbance was read using a Polarstar Omega plate reader at 405 nm.

3.9.3. Quantification of apoptosis using FITC Annexin V/Dead Cell Apoptosis Kit

The FITC Annexin V/Dead cell apoptosis kit (Life Technologies, USA) provides a quick and suitable assay for apoptosis. Once cells are stained with FITC Annexin V and PI, apoptotic and dead cells fluoresce in green and red while viable cells do not fluoresce. SH-SY5Y cells were seeded at a density of 1.2 x 10^5 /ml in 100mm dishes and were allowed to attach for 24 hours under standard incubation conditions. After treatment (Figure 3.1), experiments were performed according to the manufacturer's instructions. Briefly, cells were rinsed with PBS and adherent cells were dislodged with a cell scrapper. The cells were transferred to a tube on ice, pelleted in ice cold PBS at 3000 rpm for 5 minutes and re-suspended in 1X annexin-binding buffer. Cell density was determined and diluted in 1X annexin-binding buffer to 1 × 10^6 cells/ml, thereby preparing a sufficient volume to have 100 µl per assay. Thereafter, 5 µl of FITC annexin V and 1 µl of the 100 µg/ml PI working solution was added to each 100 µl of cell suspension and further incubated at room temperature for 15 minutes, after which 400 µl of 1X annexin-binding buffer was added. Cells were kept on ice and analyzed using a BD FACS Calibur flow cytometer (Becton Dickinson, Franklin Lakes, New Jersey, USA) measuring fluorescence emission at 530 nm (FL1) and >575 nm (FL3).

3.9.4. Hoechst Staining

Hoechst 33342 nucleic acid stain (Life Technologies, USA) is a standard counterstain that produces blue fluorescence when bound to double-stranded DNA and is commonly used to demonstrate apoptotic cells (assay kit bulletin). SH-SY5Y cells were seeded at a density of 1.1 x 10^5 /ml in 60-mm dishes and were allowed to attach for 24 hours under standard incubation conditions. Thereafter, supernatants were discarded and the cells were treated as described earlier (Figure 3.1). Experiments were thereafter performed according to manufacturer's

instructions. Briefly, adherent cells were dislodged with a cell scrapper, transferred to a tube on ice, pelleted in ice cold PBS at 3000 rpm for 5 minutes and re-suspended in 400 µl PBS. Subsequently, 100 µl of Hoechst 33258 staining solution (1:5000) was added to the same tube and incubated at 37°C for 30 minutes with protection from light. Thereafter, 10 µl of cells in the staining solution was placed on a glass slide, covered with a cover slip and viewed using a Zeiss Axio-plan 2 fluorescent microscope (Zeiss, Germany).

3.10. WESTERN BLOTTING

Western blots were used to evaluate the effects of rutin pretreatment in MPP^+ treated SH-SY5Y cells. SH-SY5Y cells were seeded at a density of 1.2 x 10^5 /ml in 100-mm dishes and were allowed to attach for 24 hours under standard incubation conditions. Following the appropriate treatment regime, whole cell lysates were extracted in passive lysis buffer (see Appendix I) and protein concentrations quantified with a Bradford protein assay using a Bradford protein reagent (see Appendix I). Equal protein amounts of cell lysates (15-40 µg) were mixed with the 4X Laemmli Sample Buffer dye (3:1 ratio, BioRad, USA), and denatured for 5 minutes at 95°C in the GeneE Thermal Cycler (Techne Inc., USA). Samples and 7 µl of protein ladder (Spectra™ Broad Range Multicolor, Thermo Scientific, USA) were electrophoresed on 4-20% Mini-Protean® SDS-polyacrylamide gel electrophoresis (SDS-PAGE) gels (Bio-Rad, USA) in 1X SDS running buffer (see Appendix I) at 110 V for 1 hour. This was followed by a transfer to PVDF membranes using the iBlot Gel Transfer Stacks and PVDF Membrane (Novex, Life technologies, USA).

The membranes were blocked in 5% milk-TBST for 1 hour at room temperate while shaking on a Stuart® SSL1 orbital shaker before incubation with the relevant primary antibody at 4°C overnight (Table 3.1-3.2). After washing in TBST, membranes were incubated with appropriate HRP-conjugated secondary antibodies (Table 3.1-3.2) for 1 hour at room temperature with shaking on a Stuart® SSL1 orbital shaker. Membranes were washed in TBST to remove excess secondary antibodies and proteins were detected by incubating membranes in a

chemiluminescent kit (SuperSignal® West Pico, Thermo Scientific, USA) for 5 minutes in a darkroom. Membranes were exposed to autoradiography films (CL-Xposure™, Thermo Scientific, USA) in an autoradiography cassette and exposed films were developed with an automatic autoradiography film processor (Hyperprocessor™, GE Healthcare Ltd., UK).

Membrane stripping or cutting was done in situations where the detection of multiple proteins of interest was necessary using same lysate on a single membrane. After protein detection, the membrane was washed in TBST for 20 minutes and incubated in stripping buffer (see Appendix I) for a total of 30 minutes at 37 °C with shaking on an orbital shaker. Thereafter, membrane was adequately washed, blocked and incubated with the appropriate primary antibody.

.

Table 3.1: Optimized western blot conditions

Antigen	Primary Ab and manufacturer	Membrane Blocking	Primary Ab Dilution Ratio	Primary Ab Diluent	Secondary Ab	Secondary Ab Dilution Ratio	Secondary Ab Diluent	Expected Size (kDa)
Caspase 3	CST9662, Rabbit pAb to caspase 3 (Cell Signalling Technology)	5% milk-TBST	1:1000	5% milk-TBST	DaR	1:5000	5% milk-TBST	36
Cleaved PARP	CST9541S, Rabbit polyclonal Ab to cleaved PARP (Cell Signalling Technology)	5% milk-TBST	1:1000	TBST	DaR	1:5000	5% milk-TBST	89
Cytochrome C	CST11940, Rabbit mAb to cytochrome c (Cell Signalling Technology)	5% milk-TBST	1:1000	5% milk-TBST	DaR	1:5000	5% milk-TBST	14
CHOP	CST2895, Mouse mAb to CHOP (Cell Signalling Technology)	5% milk-TBST	1:1000	5% milk-TBST	DaM	1:5000	5% milk-TBST	27
COX-2	CST12282, Rabbit mAb to Cox2 (Cell Signalling Technology)	5% milk-TBST	1:1000	5% milk-TBST	DaR	1:5000	5% milk-TBST	74
NF-κB	CST8242, Rabbit mAb to NF-κB (Cell Signalling Technology)	5% milk-TBST	1:1000	5% milk-TBST	DaR	1:5000	5% milk-TBST	65
Phospho NF-κB	CST3033, Rabbit mAb to NF-κB (Cell Signalling Technology)	5% milk-TBST	1:1000	5% milk-TBST	DaR	1:5000	5% milk-TBST	65

Manufacturer details: Cell Signaling (Cell Signaling Technology, Cambridge, UK); Abbreviations: Ab, antibody; DaM, donkey anti-mouse; DaR, donkey anti-rabbit; kDa, kiloDalton; mAb, monoclonal antibody; pAb, polyclonal antibody; TBST, tris-buffered saline with 0.1% Tween-20.

Table 3.2: Optimized western blot conditions

Antigen	Primary Ab and manufacturer	Membrane Blocking	Primary Ab Dilution Ratio	Primary Ab Diluent	Secondary Ab	Secondary Ab Dilution Ratio	Secondary Ab Diluent	Expected Size (kDa)
BiP	CST3177, Rabbit mAb to BiP (Cell Signalling Technology)	5% milk-TBST	1:1000	5% milk-TBST	DaR	1:5000	5% milk-TBST	78
Phospho-Histone H2A.X	CST9718, Rabbit mAb to H2A.X (Cell Signalling Technology)	5% milk-TBST	1:1000	5% milk-TBST	DaR	1:5000	5% milk-TBST	15
Phospho-Akt	CST4056, Rabbit mAb to Akt (Cell Signalling Technology)	5% milk-TBST	1:500	5% milk-TBST	DaR	1:5000	5% milk-TBST	60
Phospho-AMPK-α	CST2535, Rabbit mAb to AMPK-α (Cell Signalling Technology)	5% milk-TBST	1:500	5% milk-TBST	DaR	1:5000	5% milk-TBST	62
LC3-II	CST2775S, Rabbit mAb to LC3-II, (Cell Signalling Technology)	5% milk-TBST	1:2000	TBST	DaR	1:3000	TBST	16
SQSTM1/p62	CST5114, Rabbit pAb to SQSTM1/p62	5% milk-TBST	1:2000	TBST	DaR	1:3000	5% milk-TBST	62
GAPDH	FL-335, sc-25778, Rabbit pAb to GAPDH, (Santa Cruz Biotechnology)	5% milk-TBST	1:1000	5% milk-TBST	DaR	1:5000	5% milk-TBST	36

Manufacturer details: Cell Signaling (Cell Signaling Technology, Cambridge, UK); Santa Cruz (Santa Cruz Biotechnology, Dallas, Texas, USA). Abbreviations: Ab, antibody; DaR, donkey anti-rabbit; kDa, kiloDalton; mAb, monoclonal antibody; pAb, polyclonal antibody; TBST, tris-buffered saline with 0.1% Tween-20.

In particular, autophagic proteins are measured after initiation of basal autophagy, as well as after the addition of bafilomycin (BafA1), which inhibits vacuolar ATPase and autophagosome-lysosome fusion. For western blots on autophagy, 100 nM of bafilomycin (BafA1) was added to the culture media 2 hours before the end of treatment. After incubation, the cells were lysed for western blot as described earlier. Image J software (http://imagej.nih.gov/ij/) was employed to quantify western blots by means of densitometric measurements and final value obtained was used for statistical analysis (section 3.16).

3.11. TRANSMISSION ELECTRON MICROSCOPY (TEM)

3.11.1. Fixation of cells and preparation of grids

The ultrastructural morphology of treated SH-SY5Y cells were evaluated using a transmission electron microscope. Briefly, cells were seeded at a density of 1.2 x 10^5 /ml in 100-mm dishes and were allowed to attach for 24 hours under standard incubation conditions. Thereafter, supernatants were discarded and cells were treated as described earlier (Figure 3.1). After treatment, the adherent cells were dislodged with a cell scraper, transferred to a tube on ice and pelleted in ice cold PBS at 3000 rpm for 5 minutes. Subsequently, the pellets were fixed in 500 µl of 2.5% phosphate buffered glutaraldehyde and post-fixed in 1% osmium tetroxide in the same buffer. The Reichert ultramicrotome (SMM Instruments, SA) with a diamond knife (Agar Scientific, SA) was used to cut silver to gold sections on processed fixed samples.

3.11.2. Production of electron micrographs

A Jeol JEM 1011 transmission electron microscope (Advanced Laboratory Solutions, SA) at 80kV was utilized to examine thin sections on copper grids. Electron micrographs were obtained with a Megaview III digital camera fitted onto the microscope and by means of the ITEM software package (Advanced Laboratory Solutions, SA). Magnifications ranging from 1000 nm to 20000 nm were employed to produce electron micrographs.

3.12. EVALUATION OF MITOCHONDRIAL MEMBRANE POTENTIAL (MMP)

MMP is evaluated by staining cells with the JC-1 or rhodamine123 fluorochromes, using flow cytometry. A stock concentration of 5 mg/ml JC-1 (Life Technologies, USA) was dissolved in DMSO and 1 mg/ml rhodamine123 (St. Louis, MO, USA) was dissolved in ethanol and kept at -20°C for subsequent use (see Appendix I).

3.12.1. Staining cells with JC-1

SH-SY5Y cells were seeded at a density of 1.2 x 10^5 /ml and were allowed to attach for 24 hours under standard incubation conditions, followed by rutin and MPP^+ treatment as described earlier. Thereafter, adherent cells dislodged with a cell scraper were spun using Bio-Rad table top centrifuge at 3000 rpm for 5 minutes. Supernatants were discarded and cell pellets were re-suspended in 2 ml DMEM. 4 µl of the diluted JC-1 stock solution (10 µg/ml) was added to the re-suspended cells. Cells were incubated at room temperature for 15 minutes, vortexed, centrifuged for 3 minutes at 1500 rpm and resuspended in 500 µl PBS. Stained cell suspensions were analyzed using a BD FACSCalibur flow cytometer and BD CellQuest PRO software to measure red and green fluorescence, with a total of 10000 events collected for each sample. Fluorescence acquisition, compensation and data analysis was performed according to manufacturer's instructions.

3.12.2. Staining cells with Rhodamine123

Rhodamine123 was used as a complementary measurement of MMP. Briefly, SH-SY5Y cells were seeded at a density of 1.2 x 10^5 /ml and were allowed to attach for 24 hours under standard incubation conditions, followed by rutin and MPP^+ treatment as previously described. Thereafter, adherent cells were dislodged with a cell scraper were spun using Bio-Rad table top centrifuge at 3000 rpm for 5 minutes. Supernatants were discarded and cell pellets were re-suspended in 2 ml DMEM, after which 1 µl of the stock solution was added to the re-suspended cells and further incubated for 15 minutes at room temperature. Thereafter, cells were vortexed,

centrifuged for 3 minutes at 3000 rpm and pellets were resuspended in 500 µl PBS. Stained cell suspensions were analysed using an Accuri flow cytometer (BD Biosciences Pharmingen, San Diego, CA, USA). A total of 10000 events was collected for each sample.

SECTION B: HUMAN PD FIBROBLASTS

3.13. PRIMARY DERMAL FIBROBLAST CELL LINES

In addition to MPP^+-treated SHSY5Y cells, primary dermal fibroblasts and their matched controls were used in this study as an *ex vivo* cell model of PD. These fibroblasts were isolated from explants of skin punch biopsies obtained, with written informed consent, from three patients diagnosed with PD and three age- and sex-matched control individuals without any history of neurological disease (Table 3.3). The three PD fibroblast cells harbored pathogenic mutations in PD-linked genes (homozygous *PARK2* exon 3-4 deletion, *LRRK2* G2019S and *LRRK2* R1441C, respectively). Genotypes of mutation-carrying and wild-type control fibroblasts were previously confirmed by means of DNA sequencing (Haylett et al., 2016). The fibroblasts were cultured in DMEM supplemented with 1% pen-strep and 10% FBS in a 5% CO_2 humidified incubator at 37°C.

Table 3.3: Demographic and genotypic characteristics of dermal fibroblast donors

Disease status	Lab number	Ethnicity	Date of Birth	Gender	Age at PD onset	Gene	Mutation	Age at Biopsy
PD	53.44	Mixed ancestry	09/06/1975	F	27	*PARK 2*	homozygous exon 3-4 deletion	39
PD	68.06	Caucasian	22/11/1939	F	42	*LRRK2*	heterozygous G2019S	75
PD	13.149	Mixed ancestry	20/06/1945	M	62	*LRRK2*	heterozygous R1441C	72
Control	13.122	Mixed ancestry	12/09/1969	F	n/a		n/a, control for 53.44	45
Control	13.071	Mixed ancestry	20/03/1948	F	n/a		n/a, control for 68.06	67
Control	12.741	Mixed ancestry	05/08/1938	M	n/a		n/a, control for 13.149	77

3.14. CELL VIABILITY SCREENING FOR FIBROBLASTS

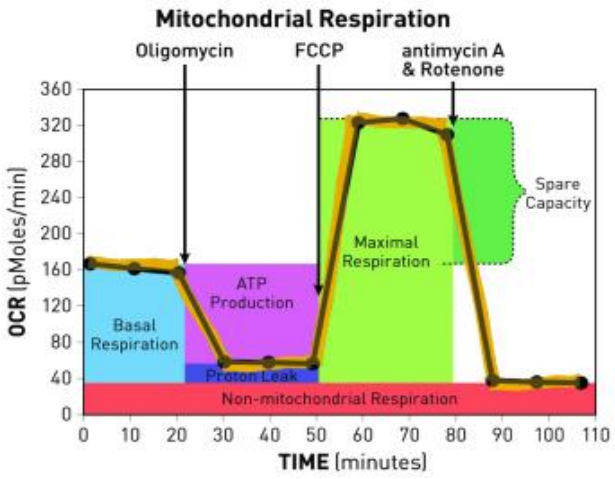
Fibroblasts were seeded at a density of 7.5 x 10^4 cells/ml in 96-well tissue culture plates and were allowed to attach for 24 hours under standard incubation conditions. MTT assays were performed to screen for MPP^+ toxicity and the most active concentration of rutin after treatment with MPP^+. Based on the cell viability results, 1000 µM and 25 µM concentrations of MPP^+ and rutin respectively were selected for subsequent experiments.

Figure 3.2: Diagram showing designed treatment groups for fibroblasts

3.15. EVALUATION OF MITOCHONDRIAL RESPIRATION

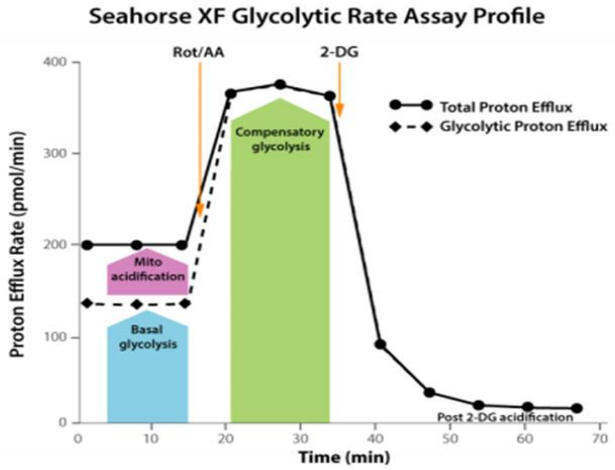
3.15.1. Agilent Seahorse XF cell Mito stress test and glycolytic rate assay

This Mito stress test evaluates mitochondrial function in cells by determining the oxygen consumption rate (OCR) of cells after successive compound injections (Figure 3.3). This glycolytic rate assay evaluates glycolysis in cells and provides precise amounts of glycolytic rates for basal and compensatory glycolysis following mitochondrial inhibition (Figure 3.4).



Source: Agilent Seahorse XF cell Mito stress kit manual

Figure 3.3: Diagram showing Agilent Seahorse XF cell Mito stress



Source: Agilent seahorse XF Glycolytic Rate Assay Kit manual.

Figure 3.4: Diagram showing Agilent Seahorse XF Glycolytic rate assay profile

In order to evaluate the effect of rutin pretreatment on the bioenergetic status of SH-SY5Y cells and fibroblasts treated with MPP^+, the Seahorse extracellular flux (XF^e96) analyzer (Agilent, Santa Clara, USA) was used to perform two different assays, namely, the XF cell Mito stress test and the glycolytic rate assay. The Seahorse XF analyzer is capable of simultaneously measuring pH and oxygen in real-time to assess the extracellular acidification rate (ECAR) and

oxygen consumption rate (OCR), indicators of glycolysis and OXPHOS respectively (Rana et al., 2012).

SH-SY5Y cells and primary dermal fibroblasts, seeded at $3x10^3$ cells/well and $12x10^3$ cells/well respectively, were allowed to adhere to the XF cell culture plate overnight. The following day, cells were pretreated with 25 µM rutin and then treated with 1 mM of MPP^+ for 48 hours (figure 3.5-3.6). After that, all cell culture media was replaced with XF base medium (without phenol red) supplemented with 1 mM sodium pyruvate, 2 mM L-glutamine and 10 mM glucose. The XF base media used for the Glycolytic Rate Assay was further supplemented with 5 mM HEPES. Following the exchange of media, plates were incubated for one hour at 37°C in a non-CO_2 incubator. After that, the plate was loaded into the Seahorse XF^e96 analyzer where standard procedures were followed for the individual assays, as recommended by the manufacturer.

For the Mito Stress Test, basal respiration was initially measured followed by successive injections of 1 µM oligomycin, 0.25 µM (SH-SY5Y cells)/0.75 µM (primary dermal fibroblasts) FCCP and 1 µM rotenone/antimycin A. For the Glycolytic Rate Assay, basal glycolysis was measured followed by two injections, firstly 1 µM rotenone and antimycin A followed by 100 mM 2-deoxyglucose. Plates were normalized to cell number using the CyQUANT Cell Proliferation Assay kit (Thermo Fisher Scientific, Waltham, USA) according to manufacturer instructions. All data were processed using Wave version 2.40 and used to calculate various mitochondrial parameters, including basal respiration, maximum respiration, ATP-coupled respiration and spare respiratory capacity according to established protocols (Brand and Nicholls, 2011).

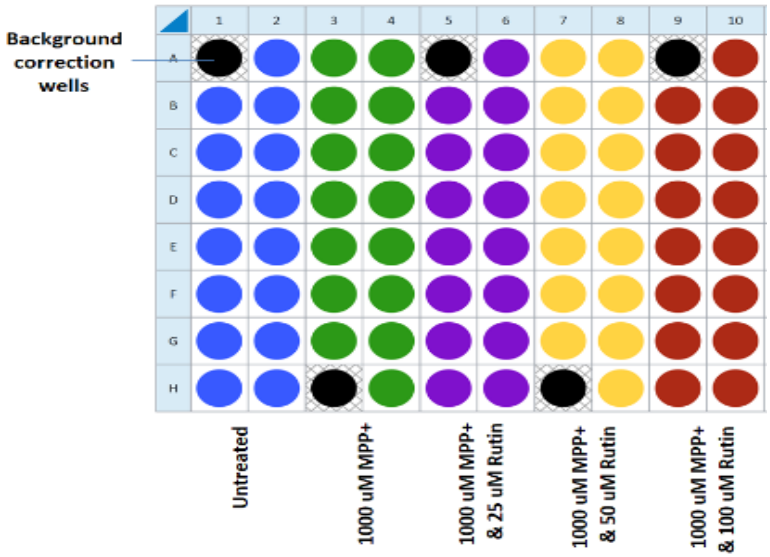


Figure 3.5: Outline of 96-well plate utilized for the XF Analyzer Mito Stress Test (SH-SY5Y)

Figure 3.6: Outline of 96-well plate utilized for the XF Analyzer Mito Stress Test (Fibroblasts)

3.16. STATISTICAL ANALYSIS

GraphPad Prism Software V7 was used for all statistical analyses (www.graphpad.com/scientific-software/prism/). Data are expressed as mean with standard error of mean. One-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons post-hoc test was performed to determine statistical significance ($p<0.05$).

CHAPTER 4

RUTIN PROTECTS SH-SY5Y CELLS FROM TOXIN-INDUCED OXIDATIVE STRESS, DNA DAMAGE AND INFLAMMATION.

4.1. INTRODUCTION

The main characteristic feature of PD is the gradual degeneration of dopaminergic neurons in the SNpc of the midbrain and one of the risk factors linked to the etiology of PD is oxidative stress (Hou et al., 2008). The study of various markers in animal models and PD patients indicate that increased ROS production triggered by increased oxidative damage represents an essential component of toxicity and a possible cause of cell death in PD (Li et al., 2010b). ROS-induced oxidative injury in the brain is demonstrated by an increase in DNA damage, inflammation, protein oxidation and lipid peroxidation in the SNpc. (Jenner, 1998, Dias et al., 2013).

ROS-triggered DNA damage has generated much attention due to the participation of ROS in various pathological conditions such as ageing, cancer and neurodegenerative disorders (Kirkinezos and Moraes, 2001, Petersen et al., 2005b, Waris and Ahsan, 2006). ROS is capable of reacting with multiple portions of the DNA to generate a number of alterations (Jena and Mishra, 2012) such as modification of bases (Jena and Mishra, 2005, 2006, 2007), induction of inter- and intra-strand crosslinks (Bauer and Povirk, 1997, Minko et al., 2008), promotion of DNA– protein crosslinks (Johansen et al., 2005, Perrier et al., 2006, Xu et al., 2008) and creation of strand breaks (Yermilov et al., 1996, Balasubramanian et al., 1998).

Also, ROS production is key to the development of several inflammatory diseases (Mittal et al., 2014). ROS is generated by cells that participate in the host-defense response and is known to enhance endothelial dysfunction through oxidation of vital cellular signaling proteins, thus acting as an inflammation moderator and a signaling molecule (Mittal et al., 2014). The

initiation of nitrosative stress by reactive nitrogen species (RNS) exacerbates the pro-inflammatory liability of ROS. For instance, ROS such as O^{-}_{2} rapidly combines with NO to form RNS such as $ONOO^{-}$ (Beckman, 1996) which further reacts with other compounds to generate more toxic products. In a bid to locate cytosolic targets, ROS traverses the mitochondrial outer membrane and leads to various consequences such as initiation of inflammasomes (Ichimura et al., 2003, Naik and Dixit, 2011), stimulation of redox-vulnerable transcription factors (Chandel et al., 2000, Mansfield et al., 2005, Wang et al., 2010) and activation of pro-inflammatory cytokines (Bulua et al., 2011).

However, antioxidant compounds act as reducing agents that scavenge for free radicals in cells to protect them from degeneration (Uslu et al., 2003). For instance, rutin is a bioflavonoid compound known for its antioxidant activity (Khan et al., 2009). Although previous evidence suggests a link between the antioxidant properties of rutin and neuroprotection, its mechanism of action in an MPP^{+} model of PD is yet to be reported. Thus, this study sought to determine the neuroprotective and antioxidant mechanisms of rutin by investigating the activity of ROS, NO, antioxidant enzymes, DNA damage and inflammation in SH-SY5Y cells treated with MPP^{+}.

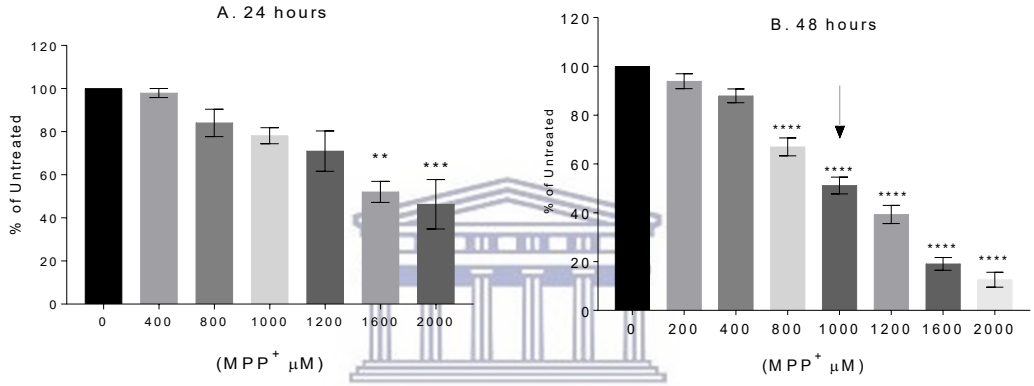
4.2. CYTOTOXICITY SCREENING OF RUTIN AND MPP^{+}

Earlier studies used different concentrations of MPP^{+} or rutin over varying time periods (Magalingam et al., 2013, Janhom and Dharmasaroja, 2015), and to establish a final concentration and exposure time for both MPP^{+} and rutin, MTT assays were utilized (Janhom and Dharmasaroja, 2015).

Based on our already established laboratory seeding density for SH-SY5Y cells, $5x10^{4}$ cells/ml were seeded in a 96 well plate resulting in 60-70% confluency after twenty-four hours. Cell viability of SH-SY5Y cells was evaluated after treating with MPP^{+} for 24 and 48 hours respectively. Treating cells with 1000 µM MPP^{+} for 48 hours was found to consistently and

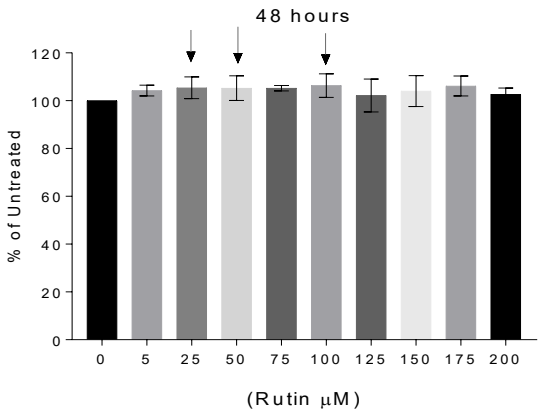
significantly reduce ($p<0.0001$) cell viability to 45-55% when compared to untreated cells, thus validating the selection of this concentration for further experiments (Figure 4.1B).

Similarly, exposure of SH-SY5Y cells to increasing concentrations of rutin (0-200 µM) resulted in little or no effect on cell viability after 48 hours (Figure 4.2). The cytotoxicity screening was important to establish the concentrations of rutin needed for the study and based on the highest absorbance values, three concentrations of 25 µM (105.4 ± 4.52, p=0.9956), 50 µM (105.2 ± 5.155, p=0.9964) and 100 µM (106.3 ± 4.903, p=0.9869) were chosen as appropriate concentrations for future experiments (Figure 4.2).



(A) At 24 hours, dose and time curves showed a significant decrease in cell viability at 1600 µM (52.06 ± 4.878, p=0.0021). (B) At 48 hours, concentrations higher than 800 µM showed a highly significant decline (67.00 ± 3.667, p<0.0001) in viability, however, a concentration of 1000 µM MPP^+ (51.18 ± 3.418, p<0.0001) was used as an appropriate concentration for future experiments. Bars represent the mean ± SEM. ** P<0.005, *** P<0.001 and **** P<0.0001 vs. untreated SH-SY5Y cells.

Figure 4.1: Effect of varying concentrations of MPP^+ in SH-SY5Y cells.

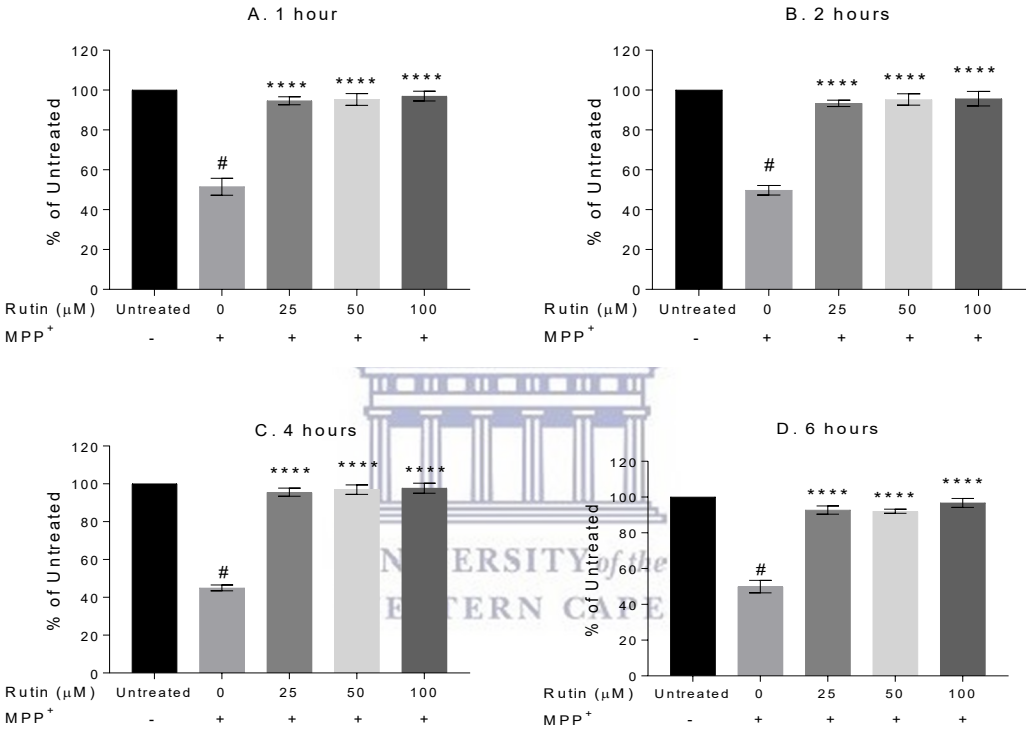


At 48 hours, dose curves indicate no significant reduction in cell viability at different concentrations of rutin. Bars represent the mean ± SEM.

Figure 4.2: Effect of varying concentrations of rutin in SH-SY5Y cells.

4.3. CELL VIABILITY

Pretreatment of SH-SY5Y cells with the optimal concentrations of rutin (25 μM, 50 μM and 100 μM) and then MPP^+ (1000 μM) showed no significant change in cell viability over various time periods (1hr, 2hr, 4hr and 6hr). Based on highest absorbance mean values from the MTT cell viability assays, a pretreatment time of rutin for 4-hours before exposure to MPP^+ was chosen for subsequent experiments (Figure 4.3).

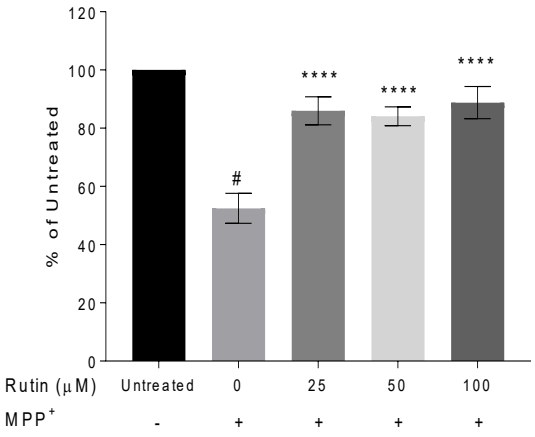


Pretreatment with rutin at different time points showed no significant difference in cell viability. Based on highest absorbance values from the cell viability assay, an optimal time of 4 hours rutin pretreatment at 25 μM (95.59 ± 2.118, $p<0.0001$), 50 μM (96.96 ± 2.514, $p<0.0001$) and 100 μM (97.71 ± 2.658, $p<0.0001$) was chosen for future experiments. Bars represent the mean ± SEM from three independent experiments. $^{\#}P<0.0001$ vs untreated SH-SY5Y cells; ****$P<0.0001$ vs SH-SY5Y cells treated with MPP^+ only.

Figure 4.3: Cell viability in SH-SY5Y cells after pretreatment with rutin and then with MPP^+

To further establish the neuroprotective effects of rutin, cell viability was confirmed using the trypan blue dye exclusion and CCK-8 viability assays. Findings show that whereas treatment with MPP^+ resulted in a significant decrease in cell viability ($p<0.0001$, Figure 4.4),

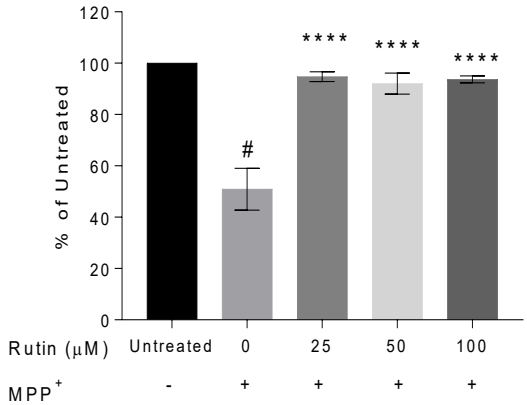
pretreatment of SH-SY5Y cells with rutin significantly increased cell viability at all concentrations ($p<0.0001$).



Bars represent the mean ± SEM from three independent experiments. #P<0.0001 vs untreated SH-SY5Y cells; ****P<0.0001 vs SH-SY5Y cells treated with MPP^+ only.

Figure 4.4: Cell viability in SH-SY5Y cells after pretreatment with rutin and then with MPP^+ (trypan blue dye exclusion assay)

Also, results from the CCK-8 viability assay (Figure 4.5) revealed that treatment of SH-SY5Y cells with MPP^+ resulted in a significant decrease in cell viability ($p<0.0001$), however, a significant increase was observed following pretreatment with rutin at 25 µM p=0.0002); 50 µM (p=0.0003) and 100 µM (p=0.0002) respectively.

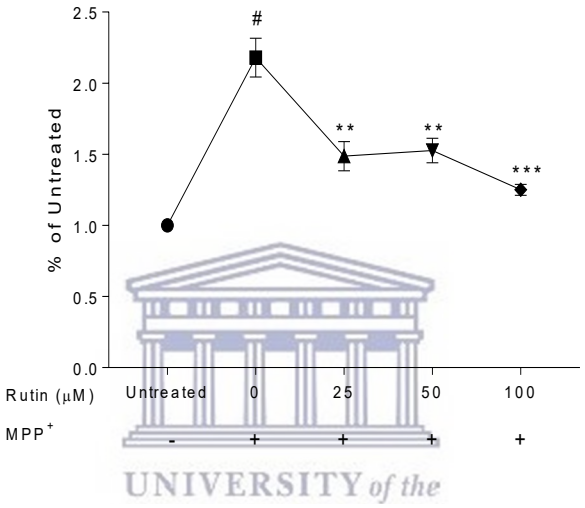


Bars represent the mean ± SEM from three independent experiments. #P<0.0001 vs untreated SH-SY5Y cells; ****P<0.0005 vs SH-SY5Y cells treated with MPP^+ only.

Figure 4.5: Cell viability in SH-SY5Y cells after pretreatment with rutin and then with MPP^+ (CCK-8 assay)

4.4. CELL TOXICITY

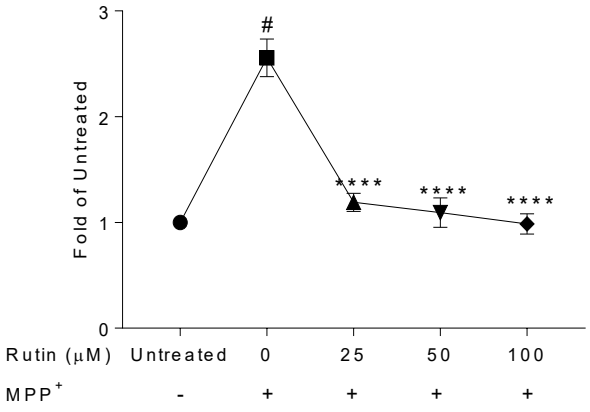
To evaluate the neuroprotective effects of rutin, cytotoxicity in SH-SY5Y cells was investigated using the trypan blue dye exclusion and the ApoTox-glo assay. Findings from the trypan blue dye exclusion assay showed that treatment of SH-SY5Y cells with MPP^+ resulted in a significant increase ($p<0.0001$, Figure 4.6) in cell toxicity; however, pretreatment of SH-SY5Y cells with rutin significantly reduced toxicity at concentrations of 25 µM ($p=0.0016$); 50 µM ($p=0.0025$) and 100 µM ($p=0.0001$) respectively.



Bars represent the mean ± SEM from three independent experiments. #P<0.0001 vs untreated SH-SY5Y cells; **P<0.005 and ***P<0.0005 vs SH-SY5Y cells treated with MPP^+ only.

Figure 4.6: Cell toxicity in SH-SY5Y cells after pretreatment with rutin and then with MPP^+ (trypan blue dye exclusion assay)

Results from the Apotox-Glo assay showed that treatment of SH-SY5Y cells with MPP^+ resulted in a significant increase ($p<0.0001$, Figure 4.7) in cell toxicity; however, all concentrations of rutin significantly decreased cell toxicity in SH-SY5Y cells ($p<0.0001$).

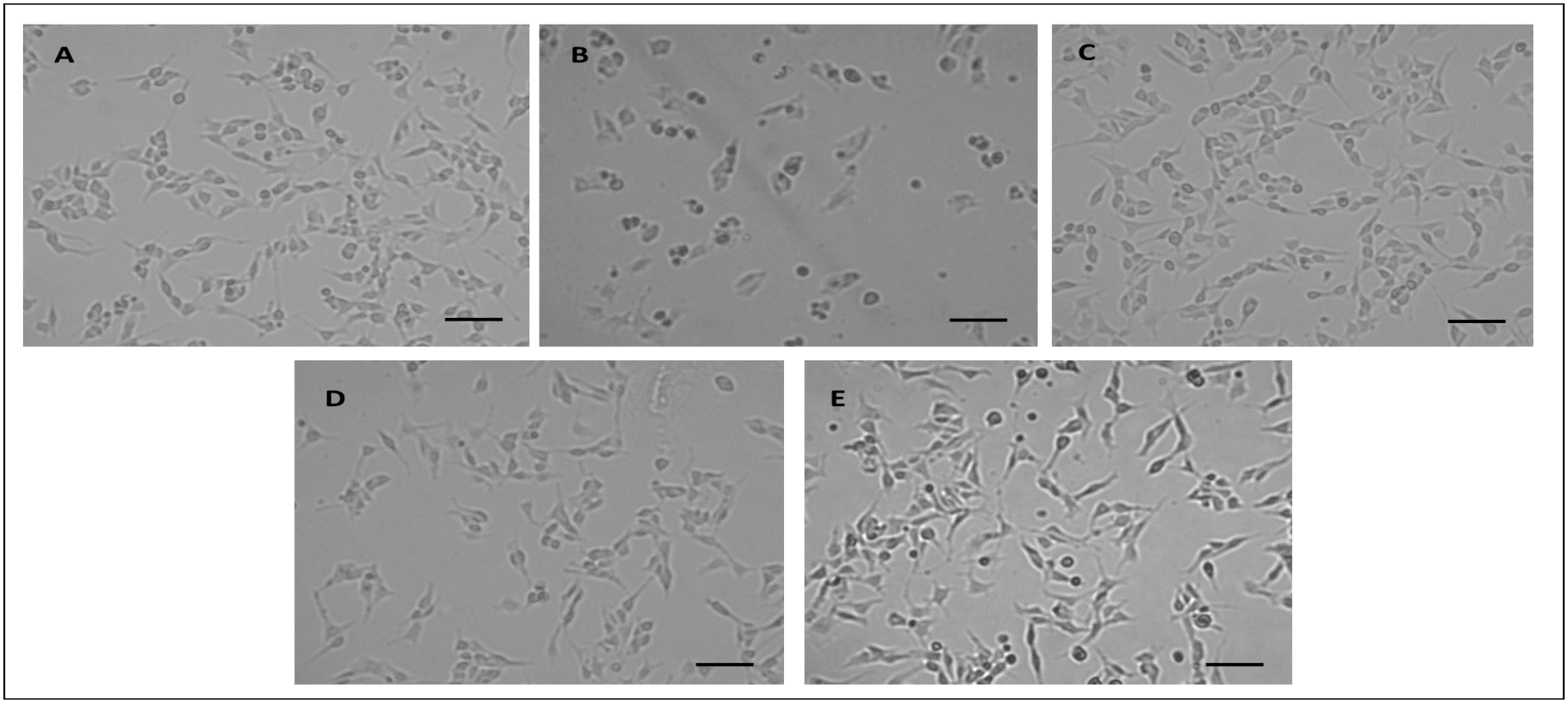


Bars represent the mean ± SEM from three independent experiments. #P<0.0001 vs untreated SH-SY5Y cells; *P<0.0001 vs SH-SY5Y cells treated with MPP^+ only.

Figure 4.7: Cell toxicity in SH-SY5Y cells after pretreatment with rutin and then with MPP^+ (Apotox-Glo assay)

4.5. EFFECT OF RUTIN ON MPP^+-INDUCED CHANGES IN CELLULAR MORPHOLOGY

The morphology of SH-SY5Y cells visualized by a Zeiss light microscope showed that the cell bodies were evenly attached with a regular shape in the untreated SH-SY5Y cells (Figure 4.8A). Shrinkage and detachment were observed in the cells treated with MPP^+ only (Figure 4.8B). Pretreatment with rutin appears to have protected the cells from the morphological damage caused by MPP^+ (Figure 4.8C-E).

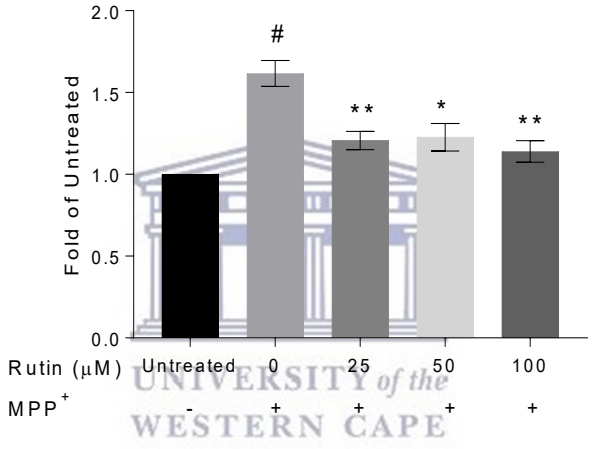


The morphological changes were observed under a Zeiss light microscope: (A) Untreated SH-SY5Y cells (B) SH-SY5Y cells treated with MPP^+ only(C) SH-SY5Y cells pretreated with 25 µM rutin and then treated with MPP^+ (D) SH-SY5Y cells pretreated with 50 µM rutin and then treated with MPP^+ (E) SH-SY5Y cells pretreated with 100 µM rutin and then treated with MPP^+. Scale bar: 5 µm.

Figure 4.8: Effects of rutin on MPP^+ triggered morphological damage

4.6. EFFECT OF RUTIN ON MPP$^+$-INDUCED ROS FORMATION

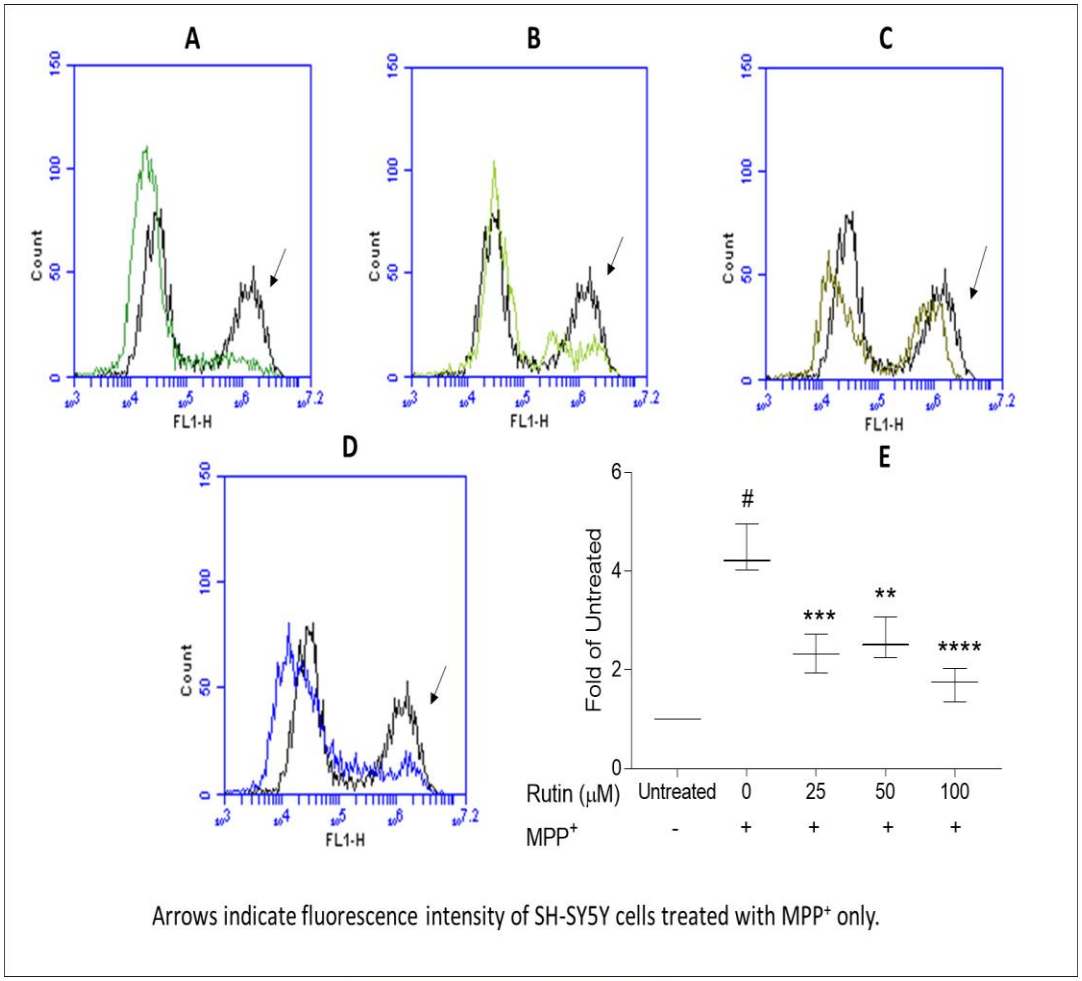
Oxidative stress is known to serve an important role in the pathological process of PD (Katunina et al., 2015). To investigate the underlying protective mechanism of rutin on MPP$^+$-induced neurotoxicity, changes in ROS production in untreated and treated SH-SY5Y cells were determined using the DCFH-DA staining technique. Findings revealed that MPP$^+$ treatment resulted in a significant increase (p=0.0004, Figure 4.9) in ROS formation; however, pretreatment of SH-SY5Y cells with rutin significantly reduced ROS production at concentrations of 25 µM (p=0.0078); 50 µM (p=0.0108) and 100 µM (p=0.0027) respectively.



Bars represent the mean ± SEM from three independent experiments. # P<0.001 vs untreated SH-SY5Y cells; * P<0.05 and ** P< 0.01 vs. SH-SY5Y cells treated with MPP$^+$ only.

Figure 4.9: Effect of rutin on MPP$^+$-induced production of ROS

As a complementary measurement of ROS and to further confirm our earlier results from the plate reader based DCFH-DA assay, flow cytometry was utilized to evaluate the changes in ROS production in treated and untreated SH-SY5Y cells. Treatment with MPP$^+$ was found to result in a significant increase in ROS formation (p<0.0001, Figure 4.10) while pretreatment with rutin resulted in a significant reduction in ROS production at concentrations of 25 µM (p=0.0004); 50 µM (p=0.0011) and 100 µM (p<0.0001) respectively.



(A) Histogram showing SH-SY5Y cells treated with MPP^+ only vs (A) untreated SH-SY5Y cells. (B) SH-SY5Y cells pretreated with 25 µM.rutin (C) SH-SY5Y cells pretreated with 50 µM.rutin (D) SH-SY5Y cells pretreated with 100 µM.rutin (E) Box and whisker plot showing the activity of ROS in untreated and treated SH-SY5Y cells. Bars represent the mean ± SEM from three independent experiments. $^{\#}$ P<0.0001 vs untreated SH-SY5Y cells; **P<0.005, ***P< 0.0005 and ****P<0.0001 vs. SH-SY5Y cells treated with MPP^+ only.

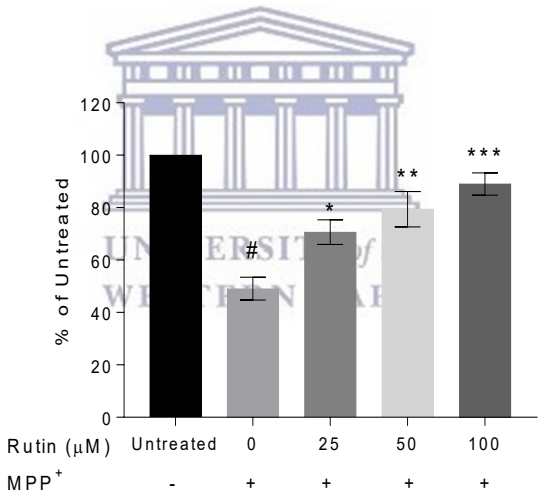
Figure 4.10: Effect of rutin on MPP^+-induced ROS production evaluated by flow cytometry.

4.7. EFFECT OF RUTIN ON MPP$^+$-TRIGGERED DISRUPTION OF ANTIOXIDANT ENZYMES

To gain better understanding into inhibition of ROS by rutin, changes in endogenous antioxidant enzymes involved in the regulation of ROS and free radicals was evaluated in treated and untreated SH-SY5Y cells.

4.7.1. Determination of SOD activity

The function of SOD is to catalyze the dismutation of O^{-}_{2} to O_2 and H_2O_2, a less toxic molecule (Hollman and Katan, 1999). Findings show that MPP$^+$ treatment resulted in a significant decrease (p=0.0001, Figure 4.11) in SOD activity; however, pretreatment of SH-SY5Y cells with rutin significantly increased SOD activity at concentrations of 25 µM (p=0.0470); 50 µM (p=0.0060) and 100 µM (p=0.0008) respectively.



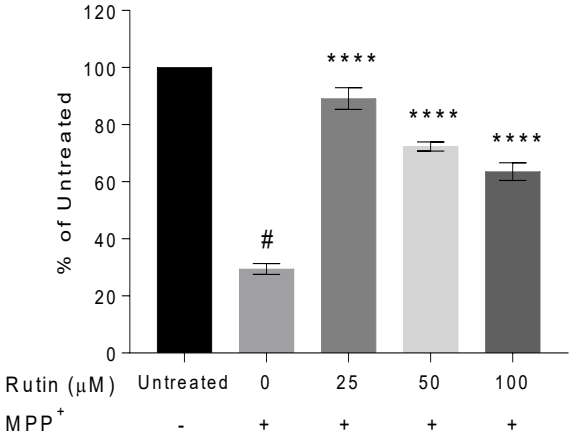
Bars represent the mean ± SEM from three independent experiments. # P<0.0005 vs untreated SH-SY5Y cells; * P<0.05, ** P< 0.01 and *** P < 0.001 vs. SH-SY5Y cells treated with MPP$^+$ only.

Figure 4.11: Effect of rutin on MPP$^+$ induced impairment of SOD activity

4.7.2. Determination of CAT activity

CAT is involved in the detoxification of H_2O_2 generated by SOD through the conversion of two molecules of H_2O_2 to form two molecules of H_2O and one molecule of O_2 (Nagata et al., 1999, Agati et al., 2012). Following treatment of SH-SY5Y cells with MPP$^+$, we observed a significant reduction in catalase activity (p<0.0001, Figure 4.12). Following pretreatment of

SH-SY5Y cells with rutin, all concentrations of rutin resulted in a significant increase in catalase activity ($p<0.0001$).

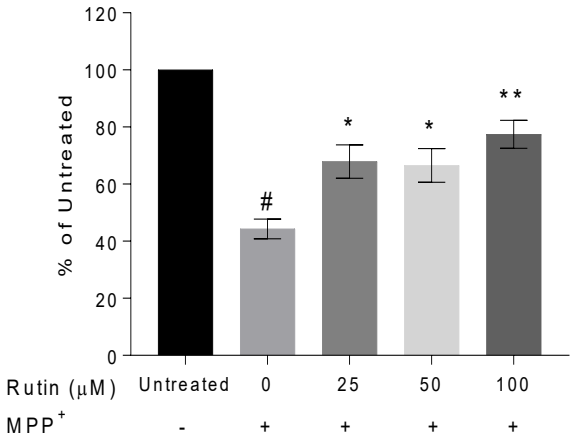


Bars represent the mean ± SEM from three independent experiments. # P<0.0001 vs untreated SH-SY5Y cells; ****P<0.0001 vs. SH-SY5Y cells treated with MPP^+ only.

Figure 4.12: Effect of rutin on MPP^+ induced impairment of CAT activity

4.7.3. Determination of GSH activity

Low levels of GSH activity are associated with PD and other neurodegenerative disorders (Pizzorno and Katzinger, 2012). When SH-SY5Y cells were treated with MPP^+, a significant reduction in the activity of GSH ($p<0.0001$, Figure 4.13) was noticed and pretreatment with rutin resulted in a significant increase in the activity of GSH at concentrations of 25 µM ($p=0.0288$); 50 µM ($p=0.0396$) and 100 µM ($p=0.0032$) respectively.

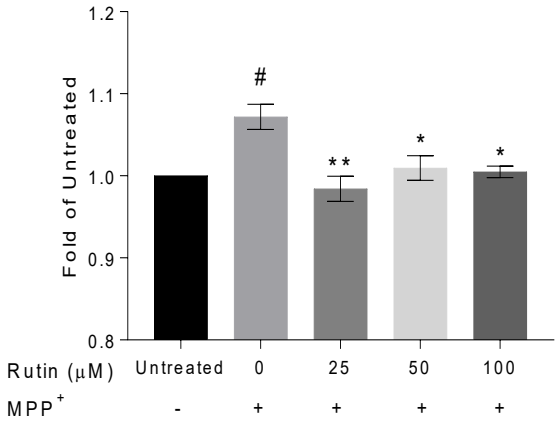


Bars represent the mean ± SEM from three independent experiments. # P<0.0001 vs untreated SH-SY5Y cells; * P<0.05 and ** P<0.005 vs. SH-SY5Y cells treated with MPP^+ only.

Figure 4.13: Effect of rutin on MPP^+ induced impairment of GSH activity

4.8. EFFECT OF RUTIN ON MPP^+-INDUCED NITRIC OXIDE PRODUCTION

NO reacts with O_2 radicals to easily oxidise proteins, lipids and DNA, thus resulting in cellular damage (Deniz et al., 2006). In this study, treatment of SH-SY5Y cells with MPP^+ treatment resulted in a significant increase in NO production (p=0.0133, Figure 4.14). Conversely, SH-SY5Y cells pretreated with rutin showed significantly reduced levels of NO at concentrations of 25 µM (p=0.0035); 50 µM (p=0.0311) and 100 µM (p=0.0205) respectively.

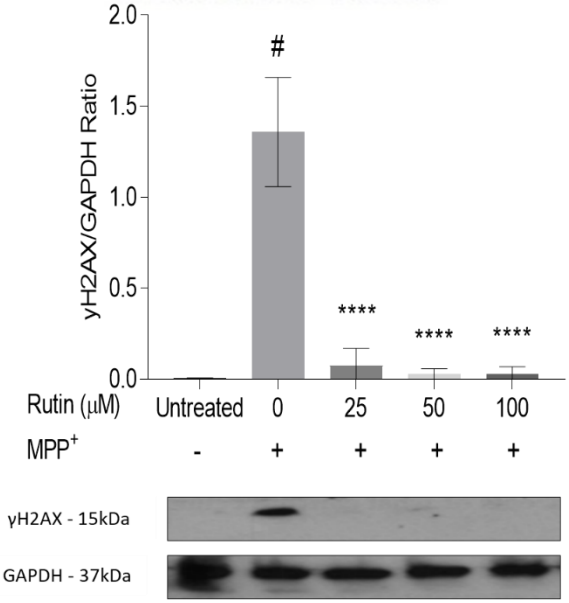


Bars represent the mean ± SEM from three independent experiments. # P<0.05 vs untreated SH-SY5Y cells; * P<0.05 and ** P<0.005 vs. SH-SY5Y cells treated with MPP^+ only.

Figure 4.14: Effect of rutin on NO production

4.9. EFFECT OF RUTIN ON MPP$^+$-INDUCED DNA DAMAGE IN SH-SY5Y CELLS

One popular target of oxidative stress-induced damage is the DNA (Avery, 2011). Within minutes of DNA damage, γH2AX is phosphorylated on Ser139 at sites of DNA damage. This very early event is required in the course of enlisting DNA damage response proteins following DNA double-strand breaks and repair (Burgess and Misteli, 2015). To gain further insights into MPP$^+$-induced oxidative damage in SH-SY5Y cells, one of the most common targets of oxidative damage (DNA) was investigated. The western blot technique was used to examine γH2AX, a common indicator of DNA damage. Findings revealed that treatment of SH-SY5Y cells with MPP$^+$ resulted in a significant increase in expression levels of γH2AX ($p<0.0001$, Figure 4.15), thus indicating that MPP$^+$ induced a double-stranded break in the cells. Conversely, when SH-SY5Y cells were pretreated with rutin, we observed that all concentrations of rutin significantly reduced γH2AX expression in SH-SY5Y cells ($p<0.0001$). The reduced expression of γH2AX appears to indicate that rutin could have protective effect against MPP$^+$ induced DNA damage in SH-SY5Y cells.

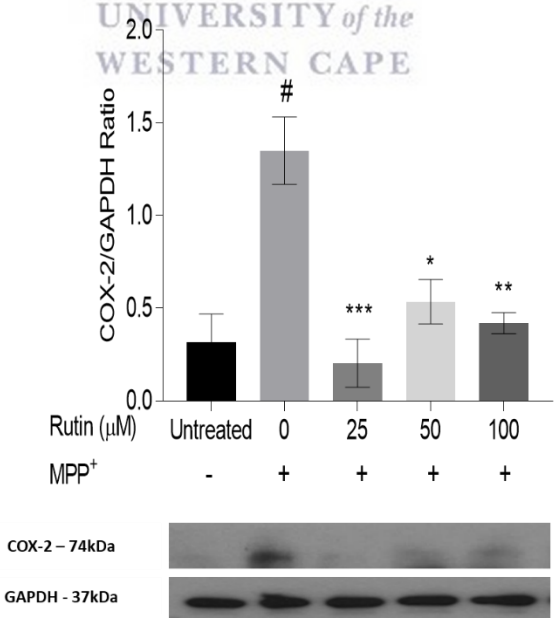


Western blot image showing γH2AX and the loading control GAPDH. Bars represent the mean ± SEM from three independent experiments. #P<0.0001 vs untreated SH-SY5Y cells; ****P<0.0001 vs SH-SY5Y cells treated with MPP$^+$ only.

Figure 4.15: Representative blot showing detection of DNA damage marker γH2AX

4.10. EFFECT OF RUTIN ON MPP$^+$-INDUCED INFLAMMATION IN SH-SY5Y CELLS

Inflammation which is a normal reaction to injury/infection, has been implicated in PD (Knott et al., 2000). Reports show that sustained oxidative stress can induce inflammation, which in turn could potentially trigger such diseases as cancer and neurodegenerative disorders (Reuter et al., 2010). The protein expression levels of the enzyme cyclooxygenase-2 (COX-2), possibly indicating inflammation (Clària, 2003) was measured in untreated and treated SH-SY5Y cells. Previously studies have shown increased expression levels of COX-2 indicates inflammation (Knott et al., 2000). Findings revealed that MPP$^+$ treatment resulted in a significant increase ($p=0.0021$, Figure 4.16) in the expression of COX-2 in SH-SY5Y cells, indicating increased inflammation in these cells. Conversely, when SH-SY5Y cells were pretreated with rutin, a significantly reduction in COX-2 expression was observed at concentrations of 25 µM ($p=0.0010$); 50 µM ($p=0.0110$) and 100 µM ($p=0.0045$) respectively, possibly indicating protective effect of rutin against MPP$^+$-induced inflammation in SH-SY5Y cells.



Western blot image showing COX-2 and the loading control GAPDH. Bars represent the mean ± SEM from three independent experiments. #P<0.005 vs untreated SH-SY5Y cells; *P<0.05, **P<0.005 and ***P<0.0005 vs SH-SY5Y cells treated with MPP$^+$ only.

Figure 4.16: Representative blot showing detection of COX-2

CHAPTER 5

RUTIN MITIGATES MPP$^+$-INDUCED NEUROTOXICITY THROUGH THE REGULATION OF AKT, AMPK AND NF-ΚB SIGNALING PATHWAYS.

5.1. INTRODUCTION

PD is the second most common neurodegenerative disorder following Alzheimer's disease (Reeve et al., 2014). Although the pathogenesis of PD is not entirely understood, aging, genetic susceptibility, inflammation and apoptosis have been implicated (Abou-Sleiman et al., 2006, McNaught and Olanow, 2006, Zhou et al., 2008, Chiang et al., 2017). There is also some evidence to suggest that autophagy plays a critical role in the progression of PD (Anglade et al., 1997, Pan et al., 2008, Nixon, 2013). Therefore, a better understanding on the involvement of apoptosis and autophagy might help in the search for new and efficient treatment options for PD.

Apoptosis is a form of PCD (Kroemer et al., 2005) previously associated with the PD pathophysiology (Olanow and Tatton, 1999, Vila and Przedborski, 2003, Bredesen et al., 2006). The pathways mediating apoptosis may be instigated by extrinsic or intrinsic factors (Benn and Woolf, 2004) resulting in the initiation of a set of caspases (Shiozaki and Shi, 2004). Alterations linked to apoptosis have been previously described in post-mortem PD tissues, such as increased caspase-3 activity in the SNpc (Hartmann et al., 2000, Tatton, 2000).

On the other hand, autophagy involves the degradation of cellular constituents in autophagolysosomes (Yorimitsu and Klionsky, 2005) initiated by sequestration of intracellular components in autophagosomes, which is then fused with lysosomes to form autophagolysosomes. Although autophagy removes unused long-lived proteins and impaired organelles, it can also act as a survival mechanism in stress conditions (White, 2008). When

autophagy is initiated incorrectly and is extensive, it acts as a cell-death pathway (Ghavami et al., 2008, Ghavami et al., 2010, Maycotte and Thorburn, 2011). Accordingly, it is suggested that autophagy can either maintain neuronal homeostasis or result in neuronal loss when extensively activated (Batlevi and La Spada, 2011, Lee, 2012).

Different intracellular signaling cascades exist in all eukaryotic cells playing vital roles in several cellular activities. For instance, the PI3K/Akt pathway controls cellular activities like neuronal cell proliferation, migration and plasticity (Jha et al., 2015). Activation of the PI3K/Akt pathway encourages cell survival, although several mechanisms involving PI3K/Akt and their association with progressive neurodegeneration remains an area of active curiosity in PD research (Jha et al., 2015). Similarly, the nuclear factor κB (NF-κB) is expressed in numerous cells and tissues such as microglia, astrocytes, and neurons (Flood et al., 2011) while the NF-κB pathway is involved in the stimulation and control of inflammatory mediators during inflammation. Earlier reports show that increased expression of NF-κB subdues the initiation of apoptosis while decreased expression mitigates neuronal survival (Camandola and Mattson, 2007). Furthermore, AMPK is a sensor of AMP:ATP ratio facilitating adaptive measures in response to low energy conditions (Rosso et al., 2016). AMPK regulates whole organism and is linked to neuronal proliferation and differentiation, synaptic connectivity, and neuroprotection (Rosso et al., 2016). Reports from previous studies suggest that AMPK has a crucial role in the physiopathology of PD owing to its activation during oxidative stress and autophagy conditions (Hardie, 2007, Arsikin et al., 2012).

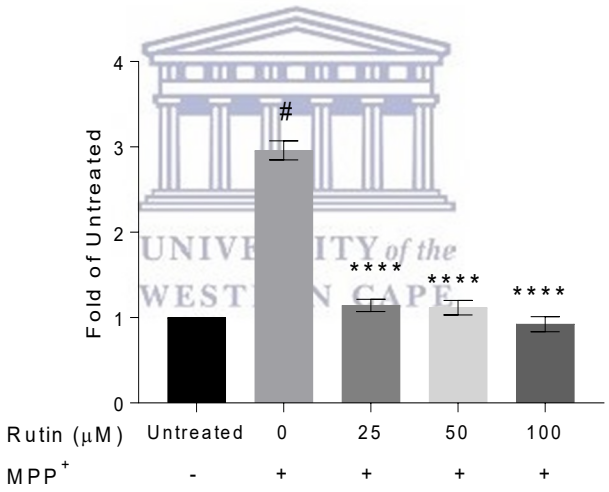
Bioactive compounds targeting the impaired Akt/AMPK/NF-κB balance could meaningfully contribute to neuroprotection in PD-challenged brains. Accordingly, to gain more insight into the mechanism of rutin action in MPP^+-treated SH-SY5Y cells, intracellular apoptotic activity was investigated in SH-SY5Y cells using assay-based kits and flow cytometry. Changes in nuclear morphology using Hoechst fluorescence staining, ultrastructural changes in morphology and autophagy using the transmission electron microscope as well as changes

in protein expression levels of apoptotic, autophagic and cell signaling pathway markers were studied in untreated and treated SH-SY5Y cells.

5.2. EFFECT OF RUTIN ON MPP$^+$-INDUCED APOPTOSIS

5.2.1. Effect of rutin on caspase 3/7 activation

To establish whether the effector caspases -3/7 are initiated in this model of PD, caspase-3/7 activation was investigated in treated and untreated SH-SY5Y cells. Findings show that treatment of SH-SY5Y cells with MPP$^+$ resulted in a significant increase ($p<0.0001$, Figure 5.1) in caspase 3/7 activation whereas pretreatment of these cells with 25 µM, 50 µM and 100 µM concentrations of rutin significantly reduced the activation of caspase 3/7 in these cells ($p<0.0001$).



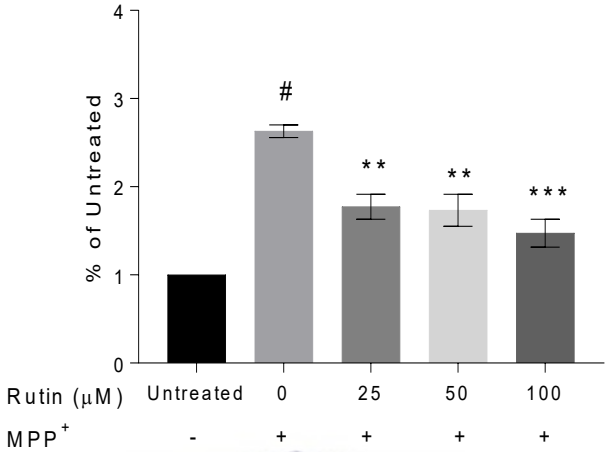
Bars represent the mean ± SEM from three independent experiments. # P<0.0001 vs untreated SH-SY5Y cells; ****P<0.0001 vs. SH-SY5Y cells treated with MPP$^+$ only.

Figure 5.1: Effect of rutin on caspase 3/7 activation

5.2.2. Effect of rutin on caspase 9 activity.

To determine whether the intrinsic/mitochondrial apoptotic pathway is activated in this model of PD, the activity of caspase 9 was investigated in treated and untreated SH-SY5Y cells. MPP$^+$ treatment resulted in a significant increase in the activity of caspase 9 ($p<0.0001$, Figure 5.2)

whereas pretreatment with rutin resulted in a significant decrease in the activity of caspase 9 at concentrations of 25 µM (p=0.0060); 50 µM (p=0.0044) and 100 µM (p=0.0006) respectively. These findings indicate that in the presence of MPP^+, the intrinsic or mitochondrial cell death pathway was activated but pretreatment with rutin inhibited this activation (Figure 5.2).

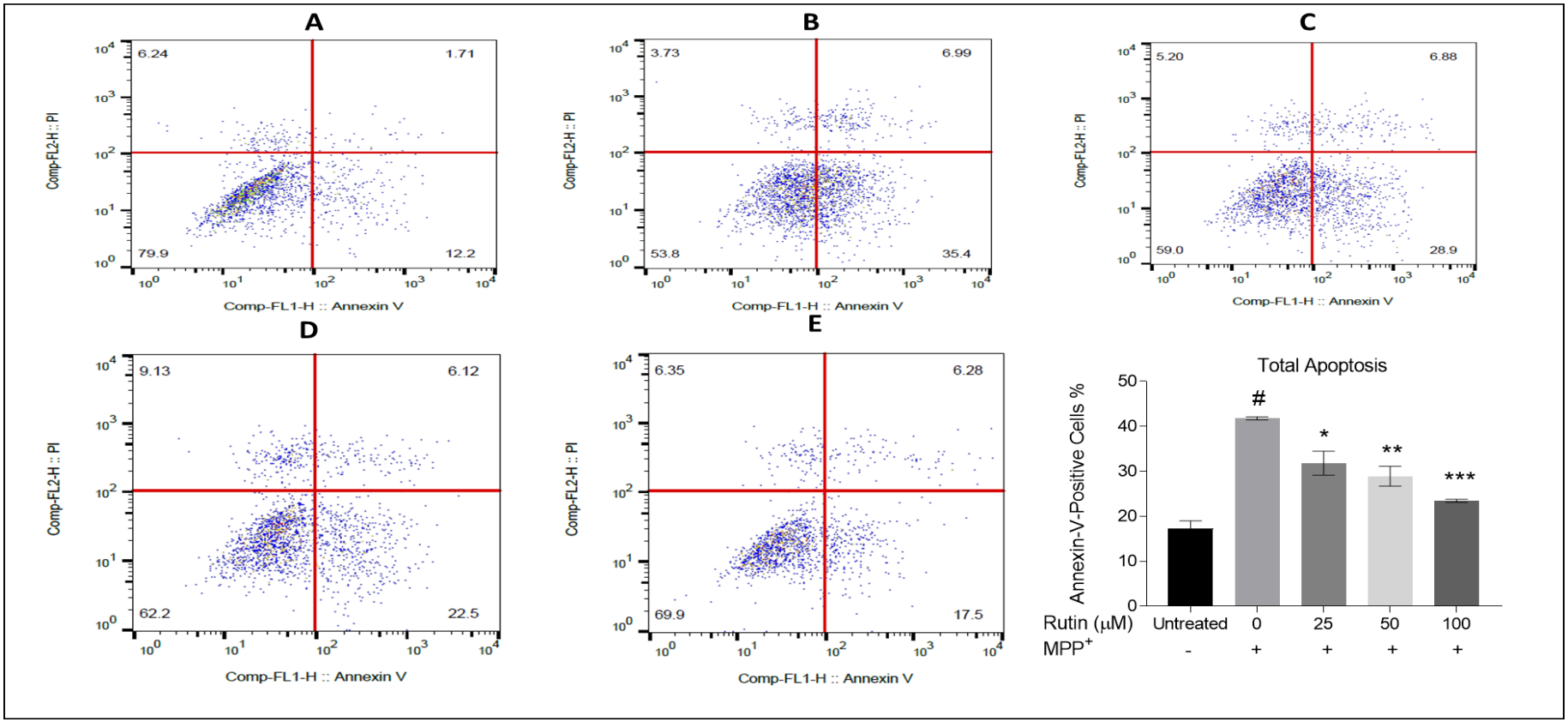


Bars represent the mean ± SEM from three independent experiments. # P<0.0001 vs untreated SH-SY5Y cells; **P<0.01 and ***P<0.001 vs. SH-SY5Y cells treated with MPP^+ only.

Figure 5.2: Effect of rutin on caspase 9 activity

5.2.3. Effect of rutin on apoptosis using flow cytometry

A flow cytometric analysis was performed to evaluate apoptotic activity in untreated and treated SH-SY5Y cells. Figure 5.3 shows a demonstration of PI versus V-FITC fluorescence. The lower left quadrant signifies the live cells and the lower right quadrant signifies the early apoptotic cells. The upper right quadrant signifies the late apoptotic cells while the upper left quadrant signifies the necrotic cells. We observed that in the untreated SH-SY5Y cells, 13.53% of cells were bound to annexin V-FITC indicating low apoptotic activity. In cells treated with MPP^+ only, 44.81% of the cells bound to annexin V-FITC indicating a significant increase (p<0.0001) in apoptotic activity. Conversely, following rutin pretreatment in SH-SY5Y cells, we observed that significantly fewer cells bound to annexin V-FITC at concentrations of 25 µM (32.95%, p=0.0066); 50 µM (27.63%, p=0.0004) and 100 µM (22.38%, p<0.0001) respectively..



(A) Untreated SH-SY5Y cells (B) SH-SY5Y cells treated with MPP^+ only (C) SH-SY5Y cells pretreated with 25 µM rutin and then treated with MPP^+ (D) SH-SY5Y cells pretreated with 50 µM rutin and then treated with MPP^+ (E) SH-SY5Y cells pretreated with 100 µM rutin and then treated with MPP^+ (F) Graph showing the effect of rutin on MPP^+ induced apoptosis in SH-SY5Y cells. Bars represent the mean ± SEM from three independent experiments. $^{\#}P<0.0001$ vs untreated SH-SY5Y cells; *P<0.05, **P<0.005 and ***P<0.005 vs. SH-SY5Y cells treated with MPP^+ only

Figure 5.3: Representative flow cytometric dot plots showing the effect of rutin on MPP^+-induced apoptosis.

5.2.4. Effect of rutin on MPP^+-induced damage in nuclear morphology

The alterations in nuclear morphological was investigated using Hoechst 33342 staining and images obtained showed that nuclei of untreated SH-SY5Y cells displayed a normal and ovum shapes, while nuclei of cells treated with MPP^+ displayed features of apoptosis typically characterized by nuclear fragmentation and condensation. On the other hand, the SH-SY5Y cells pretreated with rutin were protected from this MPP^+-induced nuclear damage (Figure 5.4).

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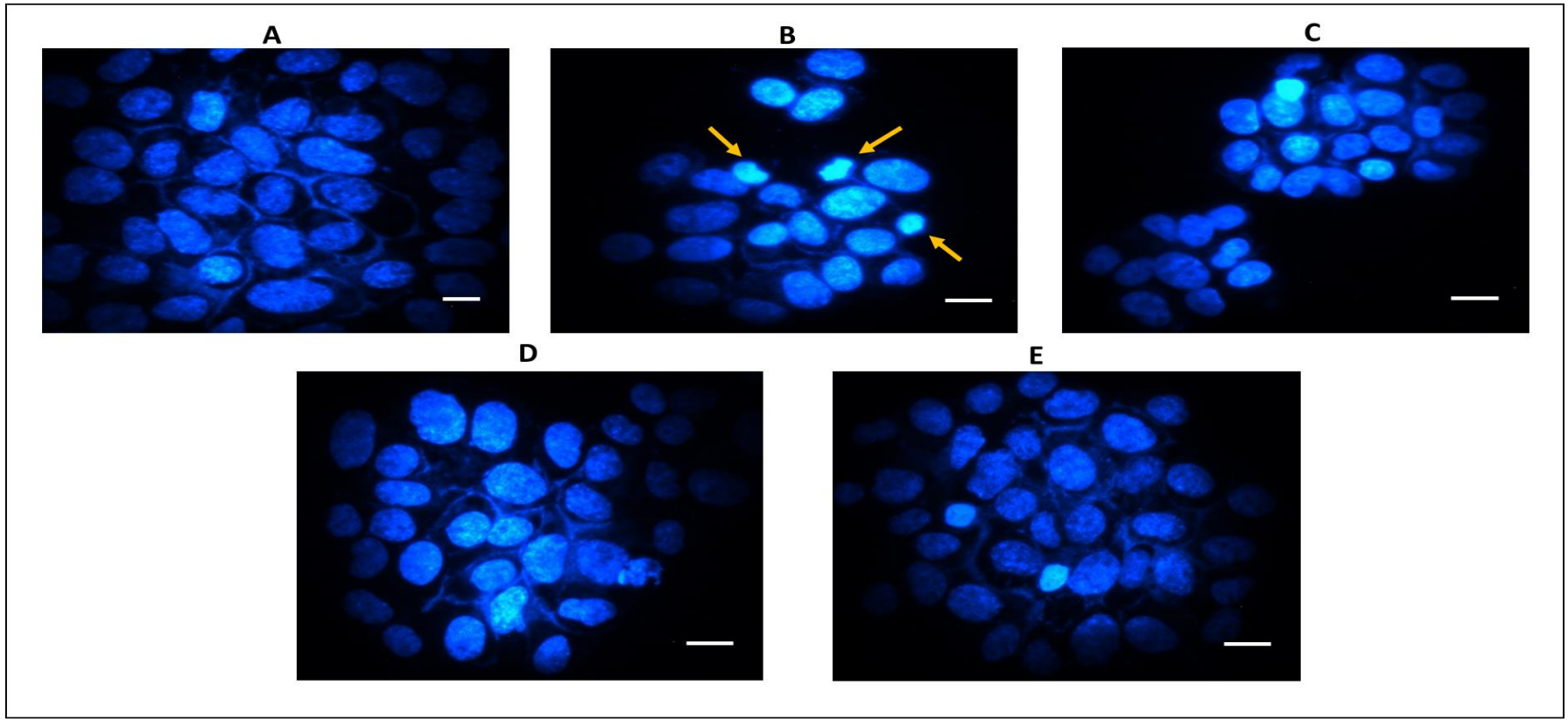


Fluorescence micrographs of Hoechst 33342 stained nuclear morphology. (A) Untreated SH-SY5Y cells (B) SH-SY5Y cells treated with MPP^+ only (C) SH-SY5Y cells pretreated with 25 µM rutin and then treated with MPP^+ (D) SH-SY5Y cells pretreated with 50 µM rutin and then treated with MPP^+ (E) SH-SY5Y cells pretreated with 100 µM and then treated with MPP^+. Scale bar indicates 100µM

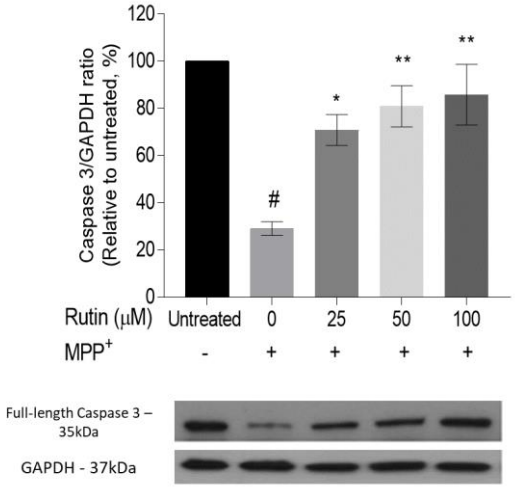
Figure 5.4: Nuclear morphological evaluation of SH-SY5Y cells by fluorescence microscopy

5.3. DETECTION OF APOPTOSIS USING WESTERN BLOT

To confirm the earlier findings on apoptosis, expression levels of three apoptotic markers - full length caspase 3, cleaved PARP and cytochrome c were evaluated. Increased apoptosis has been reported to manifest as a reduction in the expression levels of full length caspase 3 and an increase in the expression levels of both cleaved PARP and cytochrome c (Wang and Xu, 2005, Van der Merwe et al., 2017).

5.3.1. Full-length caspase 3

Western blots were used to detect expression levels of full-length caspase 3 and results obtained showed that treatment of SH-SY5Y cells with MPP^+ alone resulted in a significant decrease in the expression of full length caspase 3 ($p=0.0005$, Figure 5.5). Following pretreatment of SH-SY5Y cells with rutin, the expression levels of full length caspase 3 were significantly increased at concentrations of 25 µM ($p=0.0205$); 50 µM ($p=0.0051$) and 100 µM ($p=0.0026$) respectively. The decrease in the full length caspase 3 appears to confirm the previous results on the activation of caspase 3/7 in SH-SY5Y cells treated with MPP^+ only. Conversely, the increase in the expression levels of full length caspase 3 following rutin pretreatment could also be confirmation of the previous results on the ability of rutin to inhibit caspase 3 activity in MPP^+ treated SH-SY5Y cells.

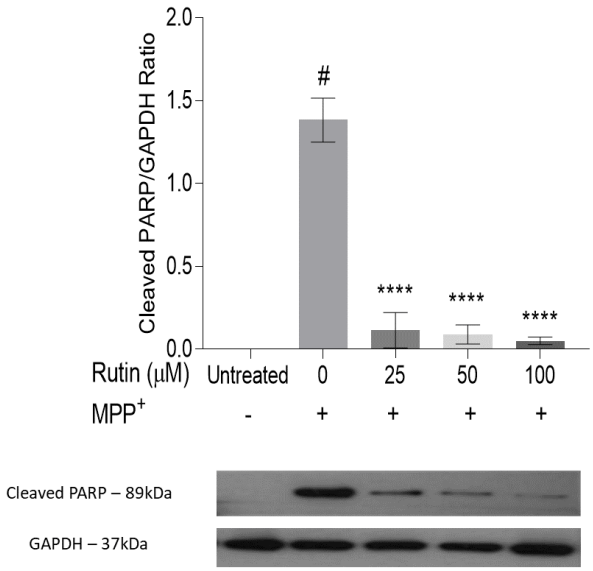


Western blot image showing full length caspase 3 and the loading control GAPDH. Bars represent the mean ± SEM from three independent experiments. #P<0.0001 vs untreated SH-SY5Y cells; *P<0.05 and **P<0.005 vs SH-SY5Y cells treated with MPP^+ only.

Figure 5.5: Representative blot showing detection of full length caspase 3

5.3.2. Cleaved PARP

To determine whether PARP is activated by the effector caspases, western blots were utilized to detect expression levels of cleaved PARP. Following quantification of western blot images, treatment of SH-SY5Y cells with MPP^+ only resulted in a significant increase in the expression of cleaved PARP ($p<0.0001$, Figure 5.6), thus indicating increased apoptosis in these cells. Conversely, when the cells were pretreated with all concentrations of rutin, significant reduction in the expression levels of cleaved PARP ($p<0.0001$) was observed, suggesting decreased apoptosis and also confirming the previous finding that rutin inhibits MPP^+-induced apoptosis in SH-SY5Y cells.

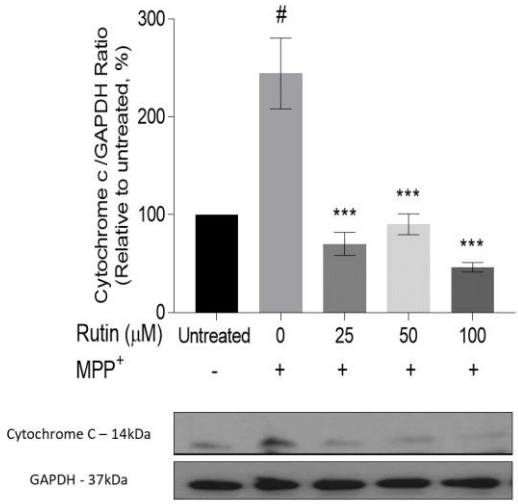


Western blot image showing cleaved PARP and the loading control GAPDH. Bars represent the mean ± SEM from three independent experiments. #P<0.0001 vs untreated SH-SY5Y cells; ****P<0.0001 vs SH-SY5Y cells treated with MPP^+ only.

Figure 5.6. Representative blot showing detection of cleaved PARP.

5.3.3. Cytochrome C

To confirm the activation of the intrinsic/mitochondrial apoptotic pathway in this PD model, the release of cytochrome c was investigated in treated and untreated SH-SY5Y cells. Treatment of SH-SY5Y cells with MPP^+ was found to result in a significant increase in cytochrome c expression (p=0.0014, Figure 5.7), possibly suggesting the initiation of the intrinsic apoptotic pathway of cell death. Conversely, expression levels of cytochrome c were significantly decreased following pretreatment with rutin at concentrations of 25 µM (p=0.0003); 50 µM (p=0.0008) and 100 µM (p=0.0001) respectively. This finding further confirms the ability of rutin to inhibit apoptosis in SH-SY5Y cells treated with MPP^+ and therefore verify previous findings from the full length caspase 3 and cleaved PARP blots.



Western blot image showing cleaved PARP and the loading control GAPDH. Bars represent the mean ± SEM from three independent experiments. #P<0.005 vs untreated SH-SY5Y cells; ***P<0.001 vs SH-SY5Y cells treated with MPP^+ only.

Figure 5.7: Representative blot showing detection of cytochrome c

In summary, all results above showed that MPP^+ treatment could cause increased apoptosis in SH-SY5Y cells. This is demonstrated by the reduction in expression levels of full length caspase 3 and increased expression levels of cleaved PARP and cytochrome c. Rutin could therefore be said to attenuate the effects of MPP^+ in SH-SY5Y cells, highlighting its protective role against MPP^+-induced apoptosis and cell death.

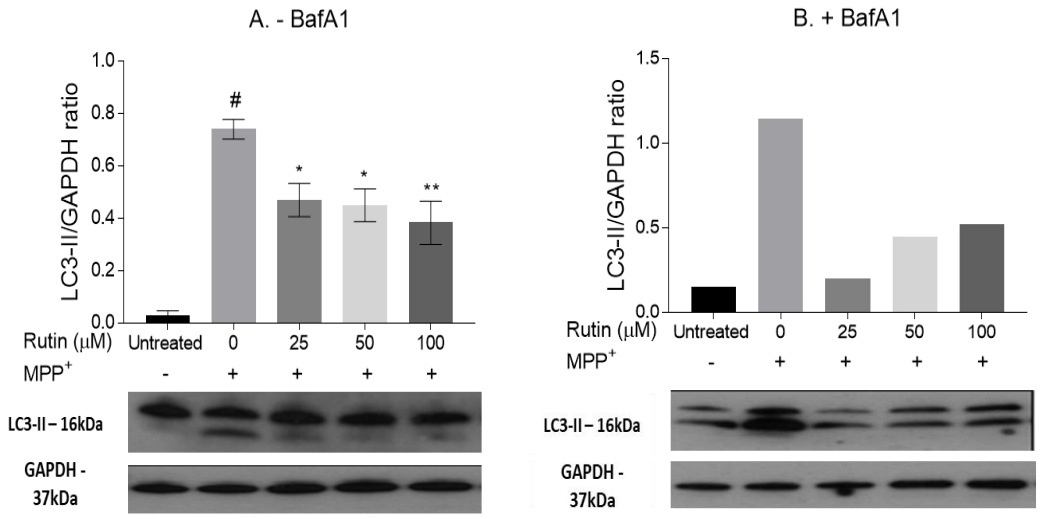
5.4. DETECTION OF AUTOPHAGY

The expression levels of two autophagy markers - LC3-II and p62 – was evaluated to determine autophagic activity in treated and untreated SH-SY5Y cells.

5.4.1. LC3-II

Western blots were used to detect expression levels of autophagosome-linked LC3-II, a widely accepted marker of autophagy (Kabeya et al., 2000). Findings revealed that in the absence of BafA1 (inhibitor of vacuolar ATPase and autophagosome-lysosome fusion),

treatment of SH-SY5Y cells with MPP^+ resulted in a significant increase in the expression levels of LC3-II ($p<0.0001$, Figure 5.8a). Autophagic activity was significantly reduced in rutin pretreated SH-SY5Y cells at concentrations of 25 µM ($p=0.0473$); 50 µM ($p=0.0320$) and 100 µM ($p=0.0092$) respectively. The increase in LC3-II expression levels in the presence of BafA1 indicates that there was either an increase in autophagic flux or a defect in the autophagy process, thereby resulting in the accumulation of LC3-II in SH-SY5Y cells treated with MPP^+ only (Figure 5.8b). To confirm whether the increase in autophagy is as a result of higher autophagic flux or an accumulation of autophagosomes, we investigated the expression levels of p62.



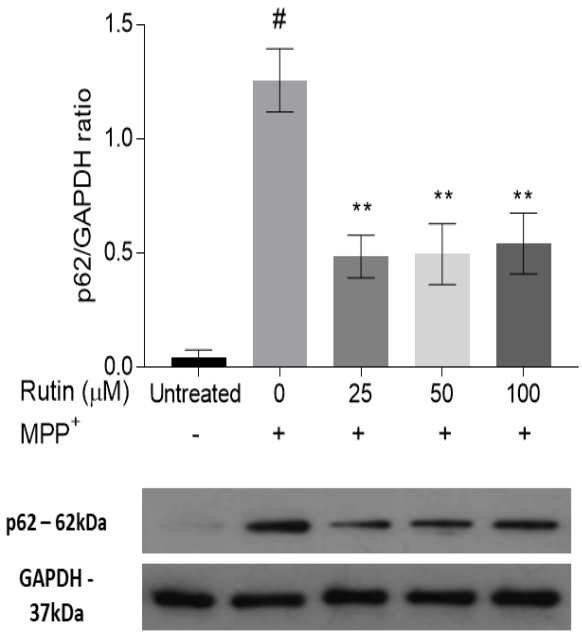
Western blot image showing LC3-II and the loading control GAPDH. Bars represent the mean ± SEM from three independent experiments. #P<0.0001 vs untreated SH-SY5Y cells; *P<0.05 and **P<0.01 vs SH-SY5Y cells treated with MPP^+ only.

Figure 5.8: Representative blot showing detection of LC3-II

5.4.2. p62

p62 is an autophagy marker known to link ubiquitinated proteins to LC3 for degradation via autophagy (Pankiv et al., 2007). p62 is an important indicator of autophagy because its accumulation is unusually elevated when there is a block or defect in the activation of autophagy (Lynch-Day et al., 2012). Treatment of SH-SY5Y cells with MPP^+ only resulted in

a significant increase in the expression of p62 in SH-SY5Y cells (p=0.0001, Figure 5.9), thus confirming our earlier hypothesis of a defective autophagy process in the SH-SY5Y cells treated with MPP^+ only. However, p62 activity significantly reduced in rutin pretreated SH-SY5Y cells at concentrations of 25 µM (p=0.0050); 50 µM (p=0.0055) and 100 µM (p=0.0083) respectively, indicating inhibition of the aberrant autophagic process triggered by MPP^+ and thus confirming the previous findings from LC3-II.

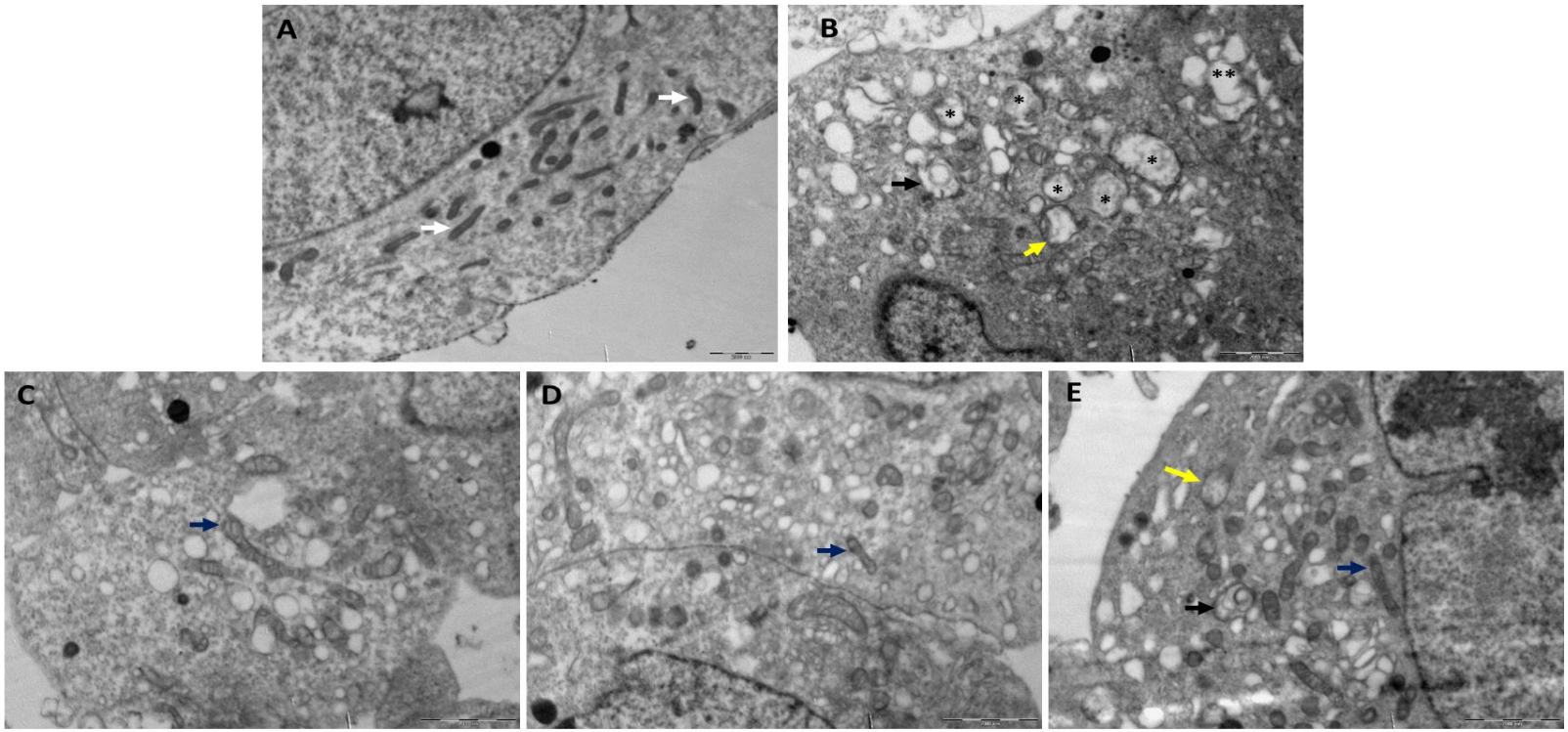


Western blot image showing p62 and the loading control GAPDH. Bars represent the mean ± SEM from three independent experiments. #P<0.0005 vs untreated SH-SY5Y cells; **P<0.01 vs SH-SY5Y cells treated with MPP^+ only.

Figure 5.9: Representative blot showing detection of p62

5.4.3. Rutin inhibits MPP^+-induced aberrant autophagy and mitochondrial changes in SH-SY5Y cells.

To further verify and validate the findings from western blots in Figure 5.8-5.9, transmission electron microscopy (TEM) was utilized to examine autophagy and ultrastructural changes in the morphology of untreated and treated SH-SY5Y cells. TEM images revealed the presence of autophagy in treated SH-SY5Y cells (Figure 5.10). Cells treated with MPP^+ only contained numerous autophagic vacuoles (with more visible autophagosomes), showed distorted mitochondria structure, mitochondrial swelling and cristae rupturing in the electron micrographs. Expectedly and in confirmation of our hypothesis, the general ultrastructure in rutin pretreated SH-SY5Y cells showed relatively normal, lesser swellings and better mitochondrial architecture as well as marked improvement and less autophagosomes when compared to the SH-SY5Y cells treated with MPP^+ only. All these suggest that rutin possibly inhibited MPP^+-induced accumulation of autophagic vacuoles in SH-SY5Y cells.



(A) Untreated SH-SY5Y cells (B) SH-SY5Y cells treated with MPP^+ only (C) SH-SY5Y cells pretreated with 25 µM and then treated with MPP^+ (D) SH-SY5Y cells pretreated with 50 µM and then treated with MPP^+ (E) SH-SY5Y cells pretreated with 100 µM and then treated with MPP^+. White arrows indicate healthy mitochondria; black arrows show different autophagic vacuoles: * represents autophagosomes (with cytoplasm); ** represents autophagolysosomes at the late stage of degradation. Yellow arrows indicate swollen or degrading mitochondria with distorted or disorganized cristae; blue arrows indicate improved mitochondria structure. Scale bar represents 2 µM

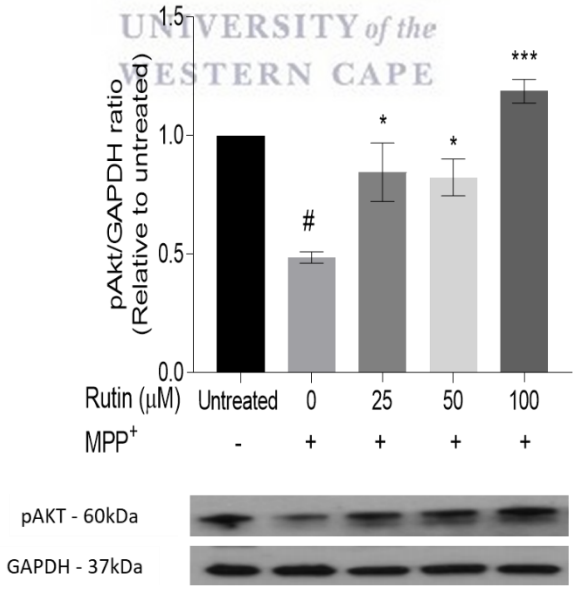
Figure 5.10: Ultrastructural images of SH-SY5Y cells and detection of autophagy using transmission electron microscope

5.5. INVOLVEMENT OF SIGNALING PATHWAYS

Four markers from the cell signaling cascade were measured – namely phosphorylated-Akt, phosphorylated-AMPKα, NF-κB and phosphorylated-NF-κB

5.5.1 AKT

Earlier studies have established that Akt mediates pro-survival effects when cells are exposed to various apoptotic stimuli (Zhao et al., 2016b, Kim and Park, 2018). Findings revealed that treatment of SH-SY5Y cells with MPP^+ only resulted in a significant decrease (p=0.0028, Figure 5.11) in the expression of p-Akt, thus indicating that there was downregulation of pro-survival signaling. Conversely, following pretreatment of SH-SY5Y cells with rutin, a significant increase in the expression of p-Akt was observed at concentrations of 25 µM (p=0.0283); 50 µM (p=0.0400) and 100 µM (p=0.0002) respectively, possibly indicating an upregulation of pro-survival signaling in MPP^+-treated SH-SY5Y cells which could be linked to the potential neuroprotective effects of rutin against MPP^+ induced apoptosis and cell death.

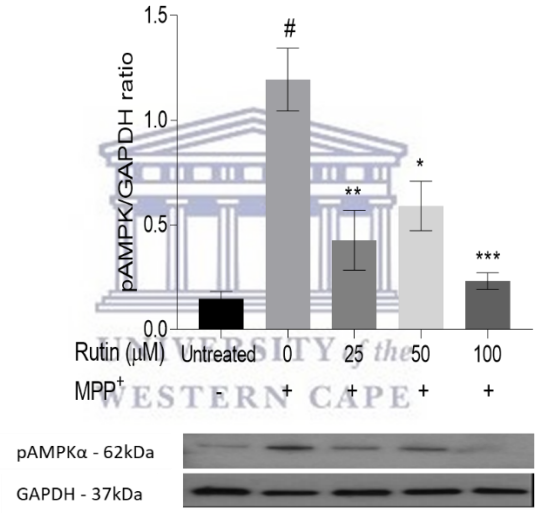


Western blot image showing p-Akt and the loading control GAPDH. Bars represent the mean ± SEM from three independent experiments. #P<0.0005 vs untreated SH-SY5Y cells; *P<0.05 and ***P<0.0005 vs SH-SY5Y cells treated with MPP^+ only.

Figure 5.11: Representative blot showing detection of phosphorylated Akt

5.5.2. AMPKα

AMPK is a molecular measure of energy status and becomes activated during oxidative stress, autophagy and other conditions that lead to the depletion of cellular energy (Ju et al., 2014, Wen et al., 2018). Findings revealed that treatment of SH-SY5Y cells with MPP^+ only resulted in a significant increase in the expression of p-AMPKα ($p=0.0004$, Figure 5.12). Following pretreatment of SH-SY5Y cells with rutin, a significant reduction in the expression of p-AMPKα was observed at concentrations of 25 µM ($p=0.0040$); 50 µM ($p=0.0193$) and 100 µM ($p=0.0007$) respectively. These findings indicate that rutin was able to inhibit MPP^+-induced activation of AMPK in SH-SY5Y cells.



Western blot image showing p-AMPKα and the loading control GAPDH. Bars represent the mean ± SEM from three independent experiments. #P<0.0005 vs untreated SH-SY5Y cells; *P<0.05, **P<0.005 and ***P<0.001 vs SH-SY5Y cells treated with MPP^+ only.

Figure 5.12: Representative blot showing detection of phosphorylated AMPKα

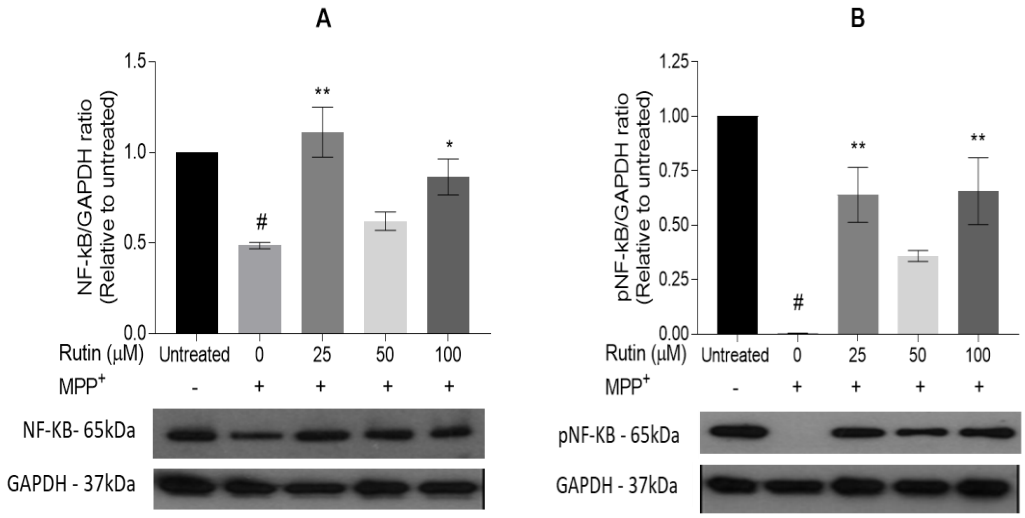
5.5.3 NF-κB

NF-κB is a transcription factor that plays an essential role in cell survival, inflammation and cell cycle (Li and Verma, 2002, Mattson, 2005, Ledoux and Perkins, 2014). NF-κB acts as an anti-apoptotic or pro-apoptotic mediator depending on the apoptosis-triggering agent and cell-type (Baichwal and Baeuerle, 1997). Although previous studies have shown that the inhibition of NF-κB triggers apoptosis (Liu et al., 1996b, Wang et al., 1996a), a post-mortem study

demonstrated that NF-κB translocation was elevated in neurons of PD patients (Hunot et al., 1997). Due to the lack of clarity and contrasting reports on the role of NF-κB in PD pathogenesis, it was necessary to evaluate the activity of NF-κB in SH-SY5Y cells pretreated with rutin and then MPP^+.

Quantification of western blot images showed that treatment of SH-SY5Y cells with MPP^+ resulted in a significant decrease in the expression of NF-κB (p=0.0071, Figure 5.13A). Conversely, when SH-SY5Y cells were pretreated with rutin, a significant increase in the expression of NF-κB was observed at concentrations of 25 µM (p=0.0017) and 100 µM (p=0.0441) respectively, but no significant increase was observed in cells pretreated with 50 µM (p=0.7549).

Similarly, we observed that treatment of SH-SY5Y cells with MPP^+ resulted in a highly significant decrease in the expression levels of p-NF-*κ*B (p=0.0001, Figure 5.13B) but expression levels of p-NF-*κ*B significantly increased following pretreatment with rutin at concentrations of 25 µM (p=0.0038) and 100 µM (p=0.0031) respectively. As for cells pretreated with rutin at 50 µM, the increase in expression of p-NF-*κ*B was not significant (p=0.1071).



Western blot image showing NF-κB, p-NF-κB and the loading control GAPDH. Bars represent the mean ± SEM from three independent experiments. #P<0.05 vs untreated SH-SY5Y cells; *P<0.05 and **P<0.005 vs SH-SY5Y cells treated with MPP^+ only.

Figure 5.13: Representative blots showing detection of NF-κB and phosphorylated NF-*κ*B

CHAPTER 6

RUTIN ATTENUATES ENDOPLASMIC RETICULUM STRESS, IMPAIRED CALCIUM HOMEOSTASIS AND ALTERED MITOCHONDRIAL FUNCTIONS IN CELLULAR MODELS OF PARKINSON'S DISEASE.

6.1. INTRODUCTION

PD is a progressive neurodegenerative disorder characterized by the loss of dopaminergic neurons in the SNpc of the midbrain, resulting in depletion of dopamine in the striatum. Clinically, its cardinal motor symptoms include bradykinesia, tremor at rest, postural instability and rigidity while non-motor symptoms include autonomic and sensory disturbances, cognitive, sleep and neuropsychiatric disorders (Park and Stacy, 2009).

It is thought that PD is caused by complex pathophysiologic mechanisms involved in dopaminergic neuronal death (Michel et al., 2016). These proposed mechanisms include impairment of intracellular Ca^{2+} homeostasis, endoplasmic reticulum (ER) stress and mitochondrial dysfunction. For example, it has been demonstrated that cytosolic Ca^{2+} levels have to be sustained within limited concentrations for optimal survival of dopaminergic neurons in the substantia nigra, whereas changes in Ca^{2+} homeostasis especially in storage organelles, ER and mitochondria reduce neuronal survival in PD (Bezprozvanny, 2009, Michel et al., 2013). Moreover, a growing body of evidence tends to indicate that the ER plays a vital role in cell signaling, thus mediating many neurodegenerative processes (Ghribi et al., 2003, Egawa et al., 2011, Cai et al., 2016). The ER is a large Ca^{2+} ion-storing organelle essential for regulating protein translation, membrane folding, and protein secretion (Balch et al., 2008). An impairment of the ER or Ca^{2+} homeostasis leads to the accumulation of unfolded/misfolded proteins in the ER lumen, thereby causing ER stress (Paschen and Mengesdorf, 2005). In response, cells activate the unfolded protein response (UPR), thus increasing ER chaperones

such as BiP which in turn activates an ER-associated degradation pathway that is essential to alleviate ER stress and ultimately improve cell survival (Hiller et al., 1996, Boyce and Yuan, 2006). However, sustained activation of the UPR due to severe ER dysfunction results in programmed cell death (Harding et al., 2002).

Similarly, it is well established that mitochondrial dysfunction plays a key role in the development of PD (Schapira, 2006, Hang et al., 2015). Impairment of the mitochondrial electron transport chain and defects in the regulation of mitochondrial dynamics are widely reported in post-mortem studies of PD brains (Keeney et al., 2006, Parker Jr et al., 2008) and in experimental PD models (Schapira et al., 1990a, McCoy and Cookson, 2012, Koyano et al., 2013, Requejo-Aguilar and Bolaños, 2016). These changes are associated with ATP deficiency, however, in an effort to compensate for mitochondrial dysfunction, neurons may upregulate glycolysis as a low-efficient mode of energy production in PD models (Requejo-Aguilar and Bolaños, 2016). Thus, any process damaging mitochondria may lead to metabolic switching intended to compensate for their diminished ability to produce ATP.

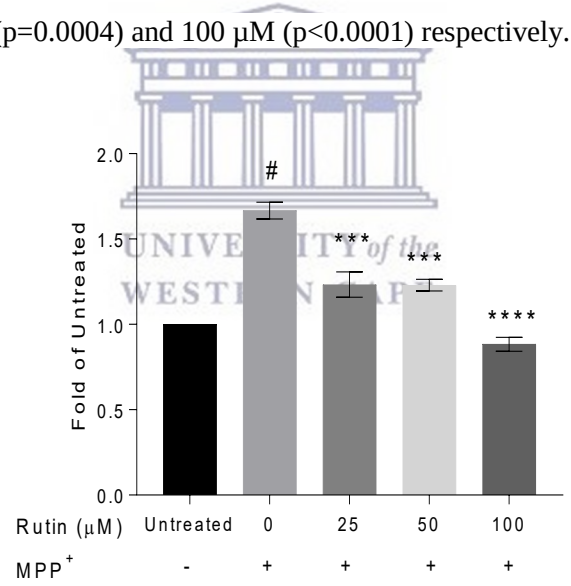
Several potentially therapeutic compounds targeting the proper regulation of Ca^{2+} homeostasis, ER stress and mitochondrial functions have been investigated in numerous PD models (Song et al., 2010, Van der Merwe et al., 2017, Wang et al., 2018). One such compound is rutin, an antioxidant and bioflavonoid, widely contained in fruits and vegetables (Yang et al., 2008). Reports show that it has numerous pharmacological activities such as cytoprotective (La Casa et al., 2000, Magalingam et al., 2016), anticarcinogenic (Webster et al., 1996, Perk et al., 2014a), anti-inflammatory (Guardia et al., 2001, Yoo et al., 2014) and anti-diabetic properties (Ghorbani, 2017). Earlier studies have also indicated that rutin strengthens the capillaries of blood vessels due to its high radical scavenging activity and antioxidant capacity (Couch, 1943).

Based on previous reports on the pharmacological effects of rutin and its potent antioxidant properties, this study provides the first research evidence of the role of rutin on MPP^+-induced

changes in mitochondrial membrane potential, ER stress as well as impaired Ca^{2+} homeostasis in SH-SY5Y cells. This chapter highlights the effects of rutin on MPP^+-induced alterations in bioenergetic status using SH-SY5Y cells and primary dermal fibroblasts as models of PD.

6.2. EFFECT OF RUTIN ON MPP^+-INDUCED DYSREGULATION OF CALCIUM HOMEOSTASIS

Previous reports indicate that Ca^{2+} dysregulation is associated with PD pathogenesis (Surmeier et al., 2012b, Zaichick et al., 2017). To gain better understanding on the role of rutin in the regulation of Ca^{2+} homeostasis, the level of intracellular Ca^{2+} in treated and untreated SH-SY5Y cells was investigated. Results showed that treatment of SH-SY5Y cells with MPP^+ resulted in a significant increase in intracellular Ca^{2+} levels ($p<0.0001$, Figure 6.1). However, this increase in Ca^{2+} levels was significantly attenuated following pretreatment with rutin at 25 µM ($p=0.0004$), 50 µM ($p=0.0004$) and 100 µM ($p<0.0001$) respectively.



Bars represent the mean ± SEM from three independent experiments. # P<0.0001 vs untreated SH-SY5Y cells; ***P<0.0005 and ****P<0.0001 vs. SH-SY5Y cells treated with MPP^+ only.

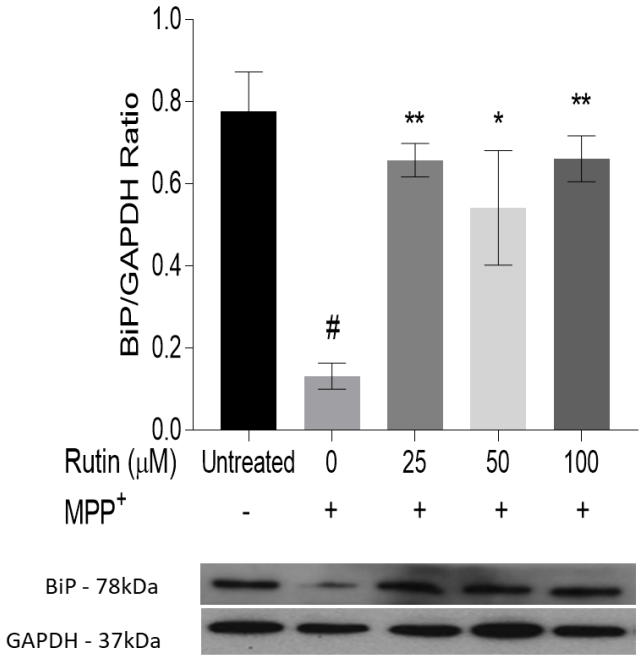
Figure 6.1: Effect of rutin on MPP^+ induced dysregulation of calcium homeostasis

6.3. EFFECT OF RUTIN ON MPP$^+$-INDUCED ENDOPLASMIC RETICULUM STRESS.

In order to detect levels of ER stress in SH-SY5Y cells, two protein markers of ER stress, namely BiP and C/EBP homologous protein (CHOP) were evaluated.

6.3.1. BiP

Activation of BiP is essential for regulating protein folding and the initiation of UPR signaling in the ER (Gorbatyuk and Gorbatyuk, 2013). Findings revealed that treatment of SH-SY5Y cells with MPP$^+$ resulted in a significant decrease in the expression of BiP (p=0.0019, Figure 6.2). Conversely, the expression levels were significantly increased following pretreatment with rutin at 25 µM (p=0.0082), 50 µM (p=0.0370) and 100 µM (p=0.0078) respectively.

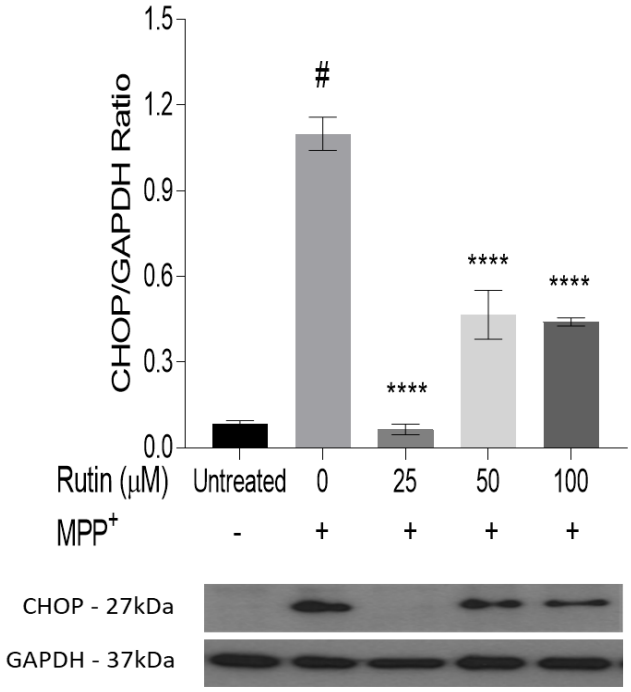


Western blot image showing BiP and the loading control GAPDH. Bars represent the mean ± SEM from three independent experiments. #P<0.005 vs untreated SH-SY5Y cells; *P<0.05 and **P<0.01 vs SH-SY5Y cells treated with MPP$^+$ only.

Figure 6.2: Representative blot showing detection of BiP

6.3.2. CHOP

CHOP is a transcription factor generally activated during ER stress (Choi et al., 2011). Findings showed that treatment of SH-SY5Y cells with MPP^+ resulted in a significant increase ($p<0.0001$, Figure 6.3) in the expression of CHOP, indicating a marked elevation of MPP^+-induced ER stress. Conversely, the expression level of CHOP was significantly decreased following pretreatment with rutin at 25 µM ($p<0.0001$); 50 µM ($p<0.0001$) and 100 µM ($p<0.0001$) respectively, signifying that rutin pretreatment prevented against ER stress in the cells.



Western blot image showing CHOP and the loading control GAPDH. Bars represent the mean ± SEM from three independent experiments. #P<0.005 vs untreated SH-SY5Y cells; ****P<0.0001 vs SH-SY5Y cells treated with MPP^+ only.

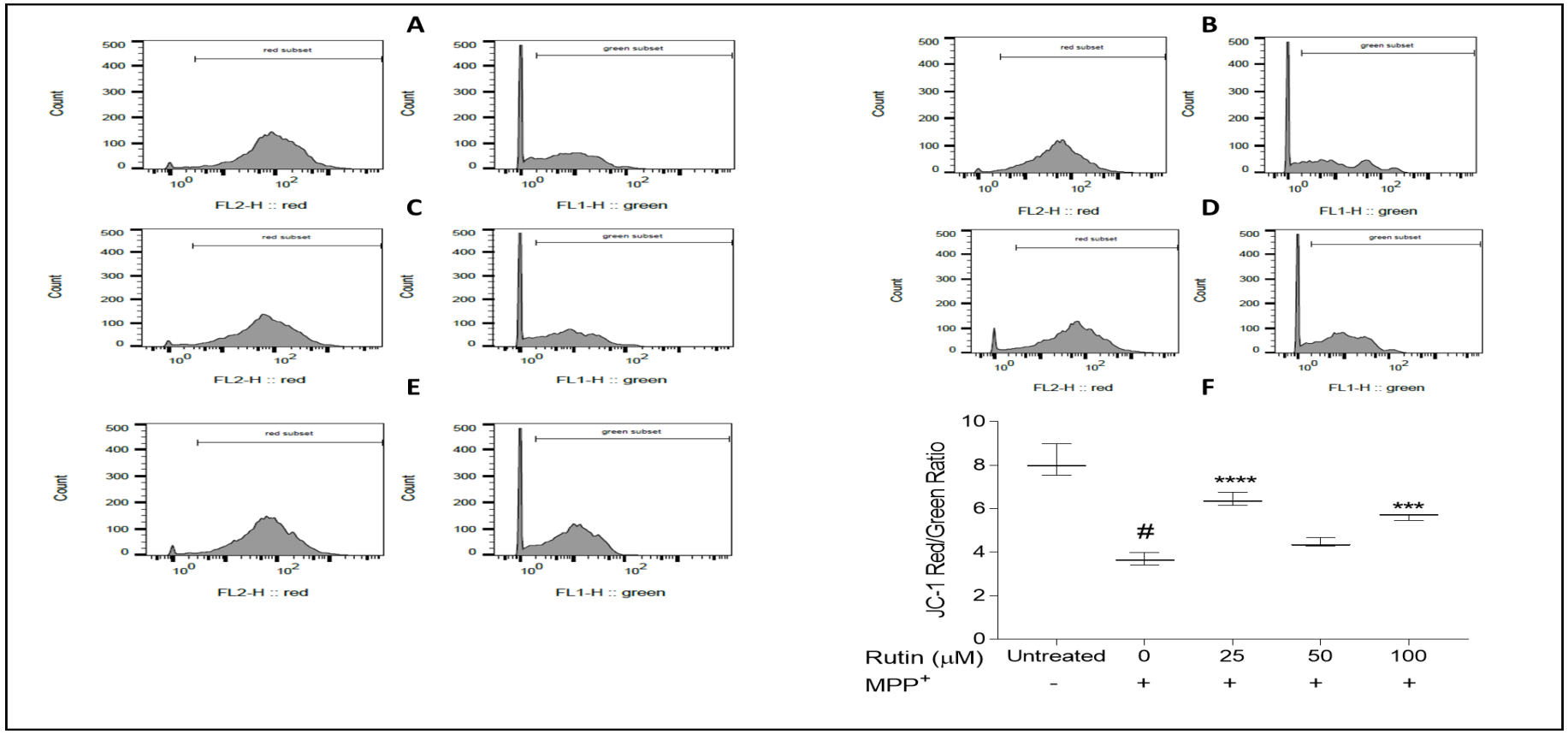
Figure 6.3: Representative blot showing detection of CHOP

6.4. ANALYSIS OF MITOCHONDRIAL MEMBRANE POTENTIAL

Having established that rutin attenuates Ca^{2+} dysregulation and ER stress, the effect of rutin and MPP^+ treatment on two parameters of mitochondrial function, namely mitochondrial membrane potential (MMP) and mitochondrial respiration was investigated, given that rutin might protect cells from MPP^+ toxicity at the mitochondrial level owing to its established antioxidant effects (Magalingam et al., 2013). MMP was assayed in SH-SY5Y cells by means two complementary potentiometric mitochondrial probes, JC-1 and Rhodamine123.

6.4.1. JC-1

When added to live cells, the JC-1 dye enters mitochondria in a MMP-dependent manner where it forms red-fluorescing aggregates. Disaggregated JC-1 and cytosolic JC-1 fluoresces green following loss of MMP, hence, the ratio of red to green fluorescence can be used as a sensitive gauge of MMP (Smiley et al., 1991). Findings revealed a significant decrease in the red/green ratio in MPP^+ treated SH-SY5Y cells ($p<0.0001$, Figure 6.4), indicating loss of MMP and mitochondrial dysfunction. Conversely, pretreatment with rutin significantly prevented the decrease in MMP at 25 µM ($p<0.0001$) and 100 µM ($p=0.0010$) respectively. The increase in MMP following pretreatment with rutin at 50 µM was however not significant ($p=0.2189$).

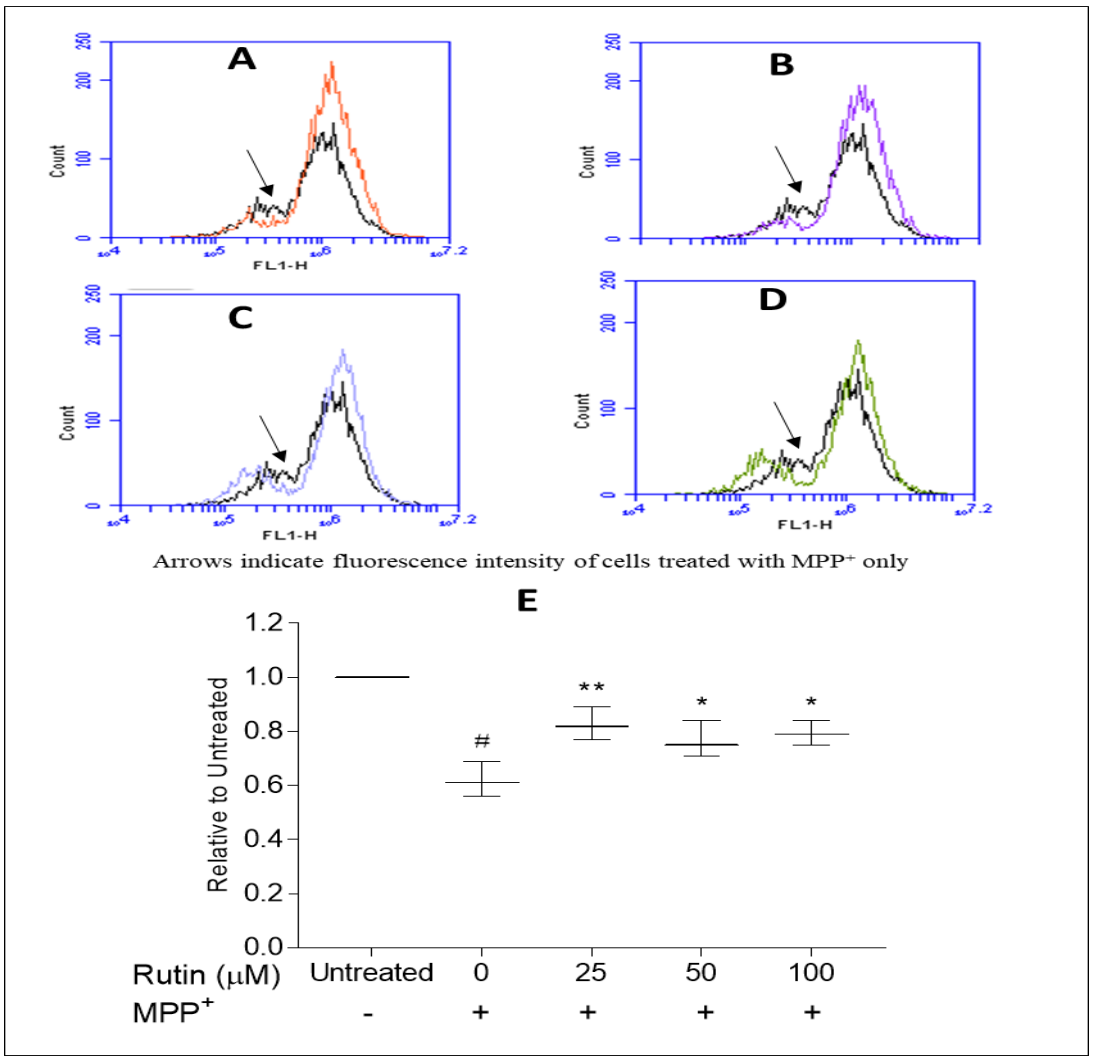


(A). Control SH-SY5Y cells (B) SH-SY5Y cells treated with MPP^+ only (C) SH-SY5Y cells treated with 25 µM rutin and then treated with MPP^+ (D) SH-SY5Y cells treated with 50 µM rutin and then treated with MPP^+ (E) SH-SY5Y cells treated with 100 µM rutin and then treated with MPP^+ (F) Box and whisker plot showing the mitochondrial membrane potential in untreated and treated SH-SY5Y cells. Bars represent the mean ± SEM from three independent experiments. $^{\#}$ P<0.0001 vs untreated SH-SY5Y cell; ***P<0.0005 and ****P< 0.0001 vs. SH-SY5Y cells treated with MPP^+ only.

Figure 6.4: Mitochondrial membrane potential shown by a ratio of red/green fluorescence intensity of JC-1

6.4.2. Rhodamine123

For Rhodamine123, a decrease in fluorescence intensity indicates a loss of MMP (Wang and Xu, 2005). Findings further confirmed the observation of lower MMP in MPP^+ treated cells, as the fluorescence intensity in MPP^+ treated SH-SY5Y cells was significantly decreased ($p<0.0001$, Figure 6.5), thus confirming loss of MMP and possible mitochondrial dysfunction. However, the MMP was significantly increased following pretreatment with rutin at 25 µM ($p=0.0057$); 50 µM ($p=0.0458$) and 100 µM ($p=0.0179$) respectively.

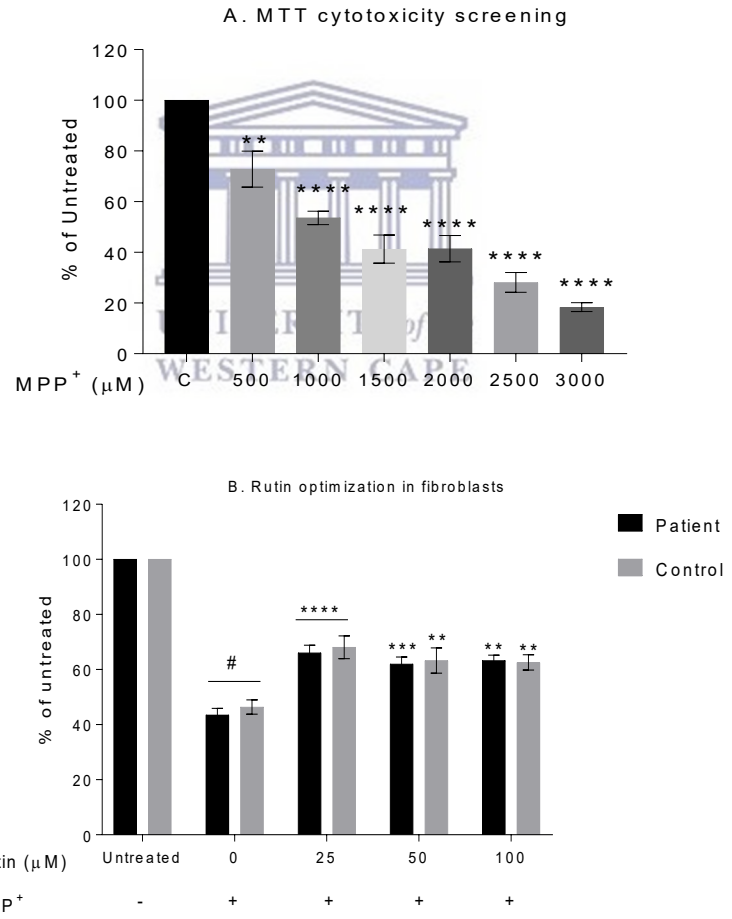


Histogram showing SH-SY5Y cells treated with MPP^+ only vs (A) untreated SH-SY5Y cells. (B) SH-SY5Y cells pretreated with 25 µM.rutin (C) SH-SY5Y cells pretreated with 50 µM.rutin (D) SH-SY5Y cells pretreated with 100 µM.rutin (E) Box and whisker plot showing the MMP in untreated and treated SH-SY5Y cells. Bars represent the mean ± SEM from three independent experiments. $^{\#}P<0.0001$ vs untreated SH-SY5Y cells; $^*P<0.05$ and $^{**}P< 0.01$ vs. SH-SY5Y cells treated with MPP^+ only.

Figure 6.5: Mitochondrial membrane potential shown by fluorescence intensity of Rhodamine123

6.5. RUTIN PROTECTS FIBROBLASTS FROM MPP$^+$ TOXICITY

As previously described (section 3.14), a range of MPP$^+$ and rutin concentrations were screened, after which 1000µM MPP$^+$ was selected because it significantly reduced ($p<0.0001$) cell viability to 53.6% when compared to untreated fibroblasts (Figure 6.6A). To investigate the most protective concentration of rutin, fibroblasts were pretreated with 25 µM, 50 µM and 100 µM concentrations of rutin before tretment with 1000 µM MPP$^+$. Results showed that pretreatment of fibroblasts with 25 µM rutin was most protective and significantly enhanced ($p<0.0001$) cell viability (Figure 6.6B). We did not observe differences in the effect of rutin pretreatment between PD patient-derived and control fibroblasts (Figure 6.6B).



Bars represent the mean ± SEM (n=3) from three independent experiments. #P<0.0001 vs untreated patient derived fibroblasts; **P<0.005, ***P<0.0005 and ****P<0.0001 vs fibroblasts treated with MPP$^+$ only.

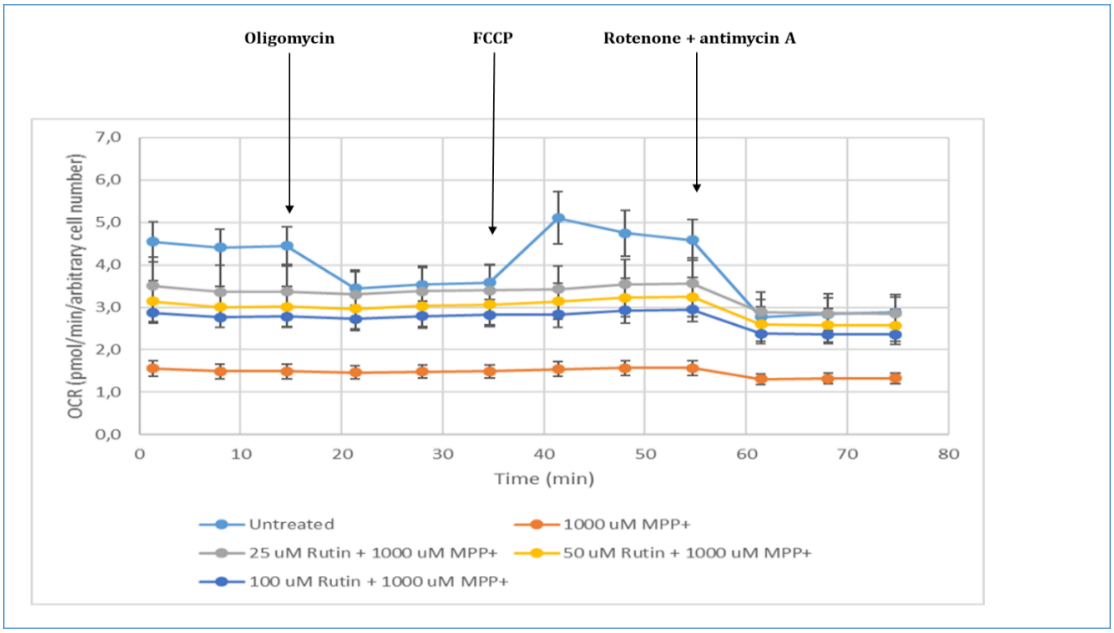
Figure 6.6: Effect of rutin and/or MPP$^+$ on cell viability in fibroblasts

6.6. THE MITOCHONDRIAL STRESS TEST

The mitochondrial stress test was measured under four conditions:

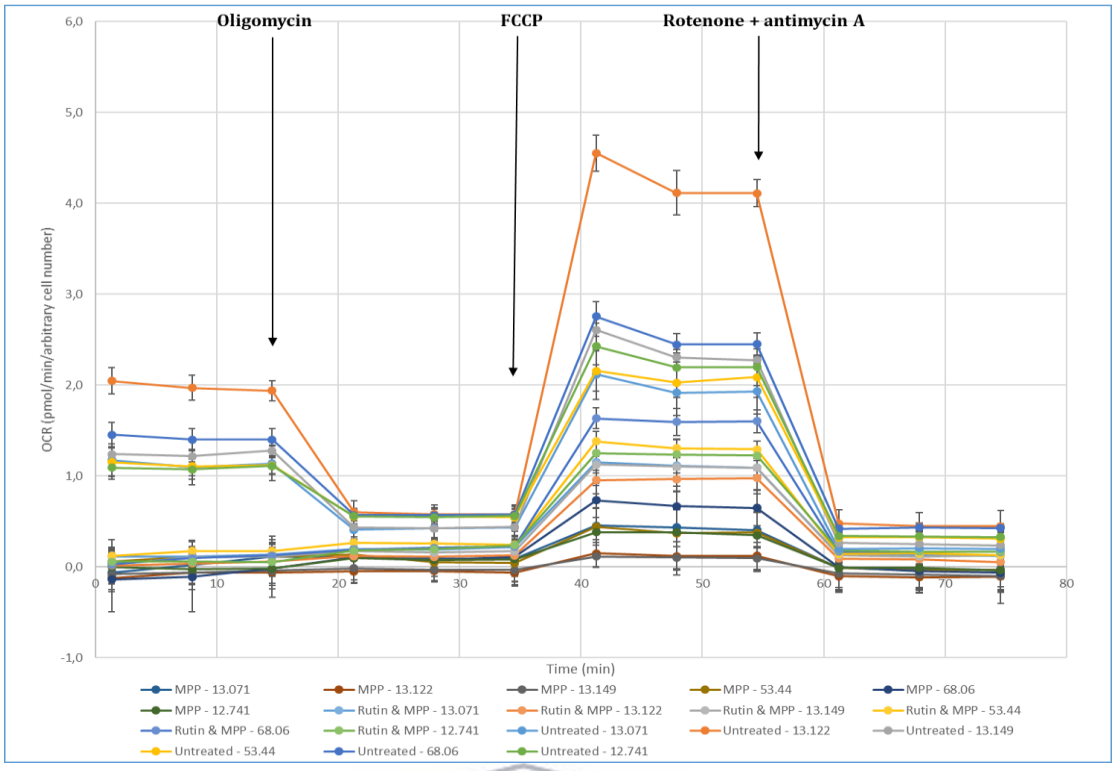
1. Basal respiration
2. Addition of a complex V inhibitor, oligomycin, which enabled the calculation of overall ATP-coupled respiration
3. Addition of FCCP to obtain maximal respiration and spare respiratory capacity
4. Addition of rotenone and antimycin A, a complex I and III inhibitor respectively.

The overall mitochondrial respiration results for SH-SY5Y cells and fibroblasts can be seen in Figure 6.7 and 6.8.



Each measurement represents 9min 12sec interval. (n=15) replicates.

Figure 6.7: Line graphs showing oxygen consumption rate (OCR) in untreated and treated SH-SY5Y cells after the introduction of drug compounds

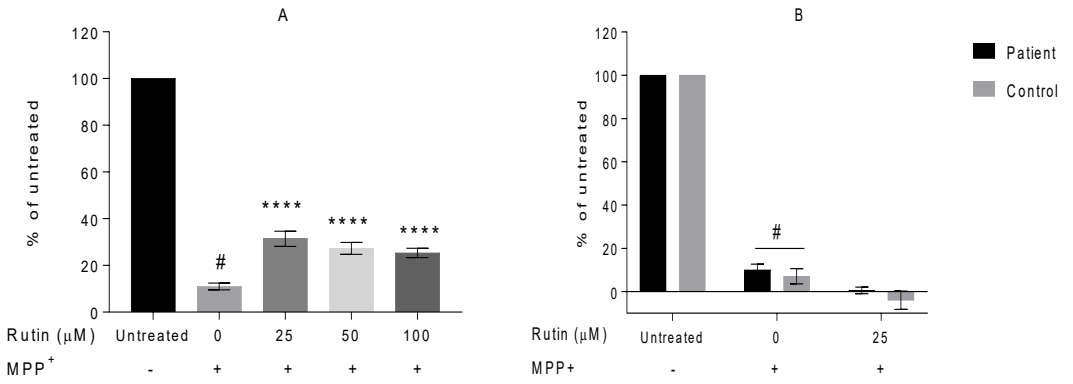


Each measurement represents 9min 12sec interval. (n=5) replicates.

Figure 6.8: Line graphs showing oxygen consumption rate (OCR) in treated and untreated fibroblasts after the introduction of drug compounds

6.6.1. Basal respiration

Basal respiration indicates the energetic need of the cell under baseline conditions (Agilent Technologies user guide). Findings showed that MPP^+ treatment resulted in a significant decrease ($p<0.0001$, Figure 6.9) in basal respiration in SH-SY5Y cells and fibroblasts. All concentrations of rutin significantly increased basal respiration ($p<0.0001$) in SH-SY5Y cells, but no significant changes ($p>0.05$) were observed in fibroblasts pretreated with rutin.

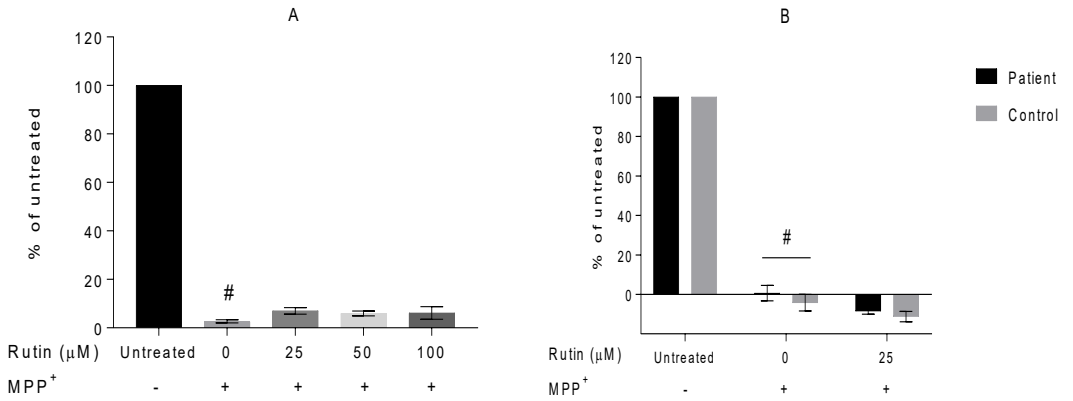


Each bar represents mean ± SEM from 15 wells of a 96-well plate except for SH-SY5Y cells and fibroblasts treated with MPP^+ only (14). #P<0.0001 vs untreated SH-SY5Y cells and fibroblasts; ****P<0.0001 vs. SH-SY5Y cells and fibroblasts treated with MPP^+ only.

Figure 6.9: Basal respiration in SH-SY5Y cells and fibroblasts

6.6.2. ATP-coupled respiration

ATP-coupled respiration indicates the ATP generated by the mitochondria that contributes to the energetic demand of the cell (Agilent Technologies user guide). Findings revealed that treatment of SH-SY5Y cells and fibroblasts with MPP^+ resulted in a significant decrease ($p<0.0001$, Figure 6.10) in ATP-coupled respiration, however, there were no significant changes ($p>0.05$) following pretreatment with rutin in SH-SY5Y cells and fibroblasts.

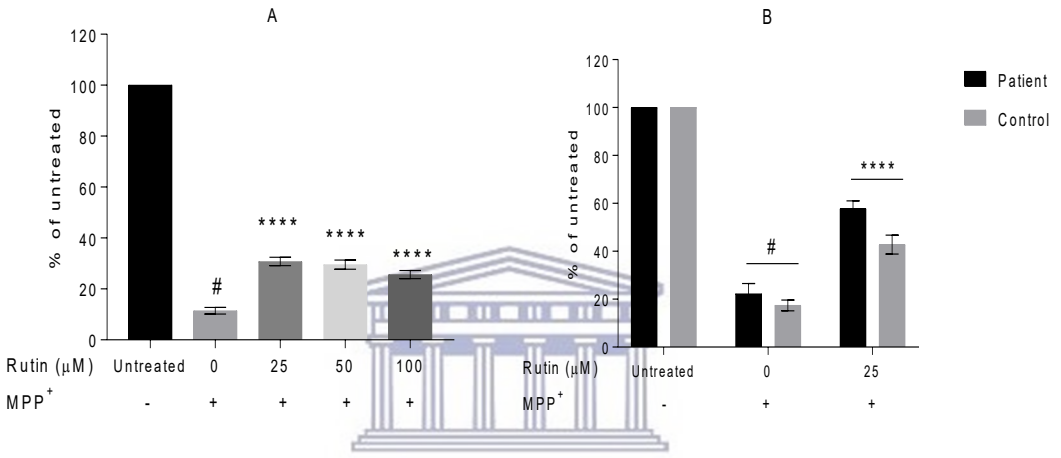


Each bar represents mean ± SEM from 15 wells of a 96-well plate except for SH-SY5Y cells and fibroblasts treated with MPP^+ only (14). #P<0.0001 vs untreated SH-SY5Y cells and fibroblasts.

Figure 6.10: ATP-coupled respiration in SH-SY5Y cells and fibroblasts

6.6.3. Maximal respiration

Maximal respiration indicates the maximum rate of respiration that the cell can attain (Agilent Technologies user guide). Following treatment and after the addition of FCCP (stimulates the respiratory chain to function at maximum capacity), it was observed that MPP^+ caused a significant decrease ($p<0.0001$, Figure 6.11) in maximal respiration in SH-SY5Y cells and fibroblasts. Conversely, rutin pretreatment resulted in a significant increase ($p<0.0001$) in maximal respiration.



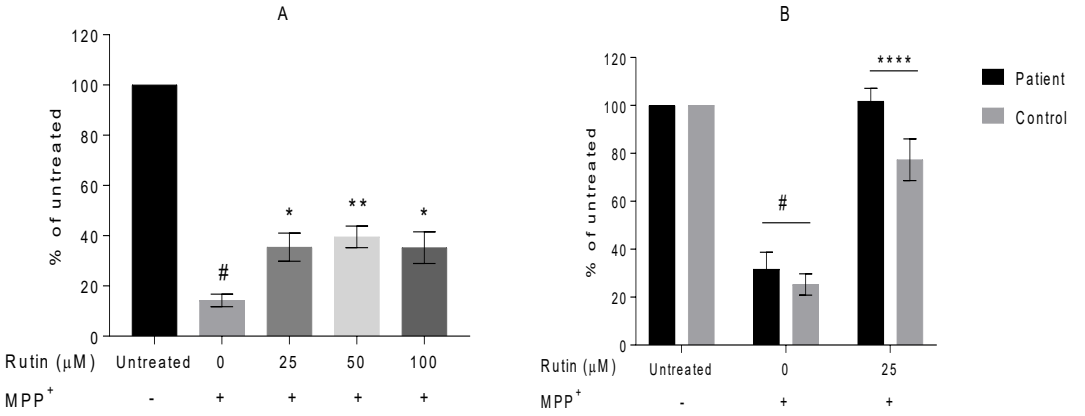
Each bar represents mean ± SEM from 15 wells of a 96-well plate except for SH-SY5Y cells and fibroblasts treated with MPP^+ only (14). $^{\#}P<0.0001$ vs untreated SH-SY5Y cells and fibroblasts; ****$P<0.0001$ vs. SH-SY5Y cells and fibroblasts treated with MPP^+ only.

Figure 6.11: Maximal respiration in SH-SY5Y cells and fibroblasts

6.6.4. Spare respiratory capacity

Spare respiratory capacity indicates the ability of the cell to respond to an energetic need (Agilent Technologies user guide). After addition of FCCP to treated SH-SY5Y cells and fibroblasts, MPP^+ treatment caused a significant decrease ($p<0.0001$, Figure 6.12) in spare respiratory capacity. However, pretreatment with rutin significantly increased ($p<0.05$) spare respiratory capacity in SH-SY5Y cells and fibroblasts.

These findings indicate a potential protective effect of rutin on impaired mitochondria.



Each bar represents mean ± SEM from 15 wells of a 96-well plate except for SH-SY5Y cells and fibroblasts treated with MPP^+ only (14). #P<0.0001 vs untreated SH-SY5Y cells and fibroblasts; *P<0.05 and **P<0.005 vs. SH-SY5Y cells treated with MPP^+ only; ****P<0.0001 vs fibroblasts treated with MPP^+ only.

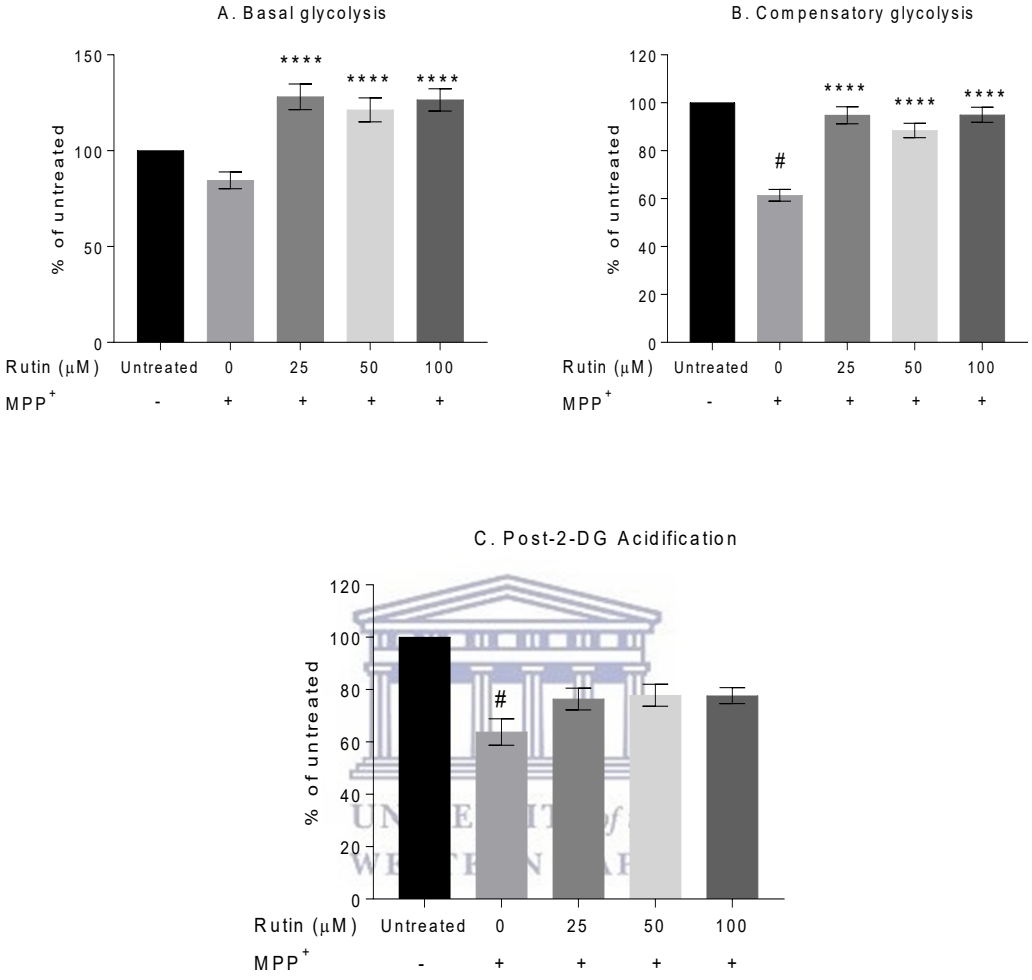
Figure 6.12: Spare respiratory capacity in SH-SY5Y cells and fibroblasts

6.7. THE GLYCOLYSIS STRESS TEST

OXPHOS is primarily responsible for the production of ATP through a process of mitochondrial respiration. However, when OXPHOS is impaired, glycolysis becomes primarily responsible for the production of ATP. It is thus essential to examine glycolytic function for an in-depth understanding of cellular energy demands. The glycolysis stress test accessed the following parameters – basal glycolysis, compensatory glycolysis and post-2-DG acidification.

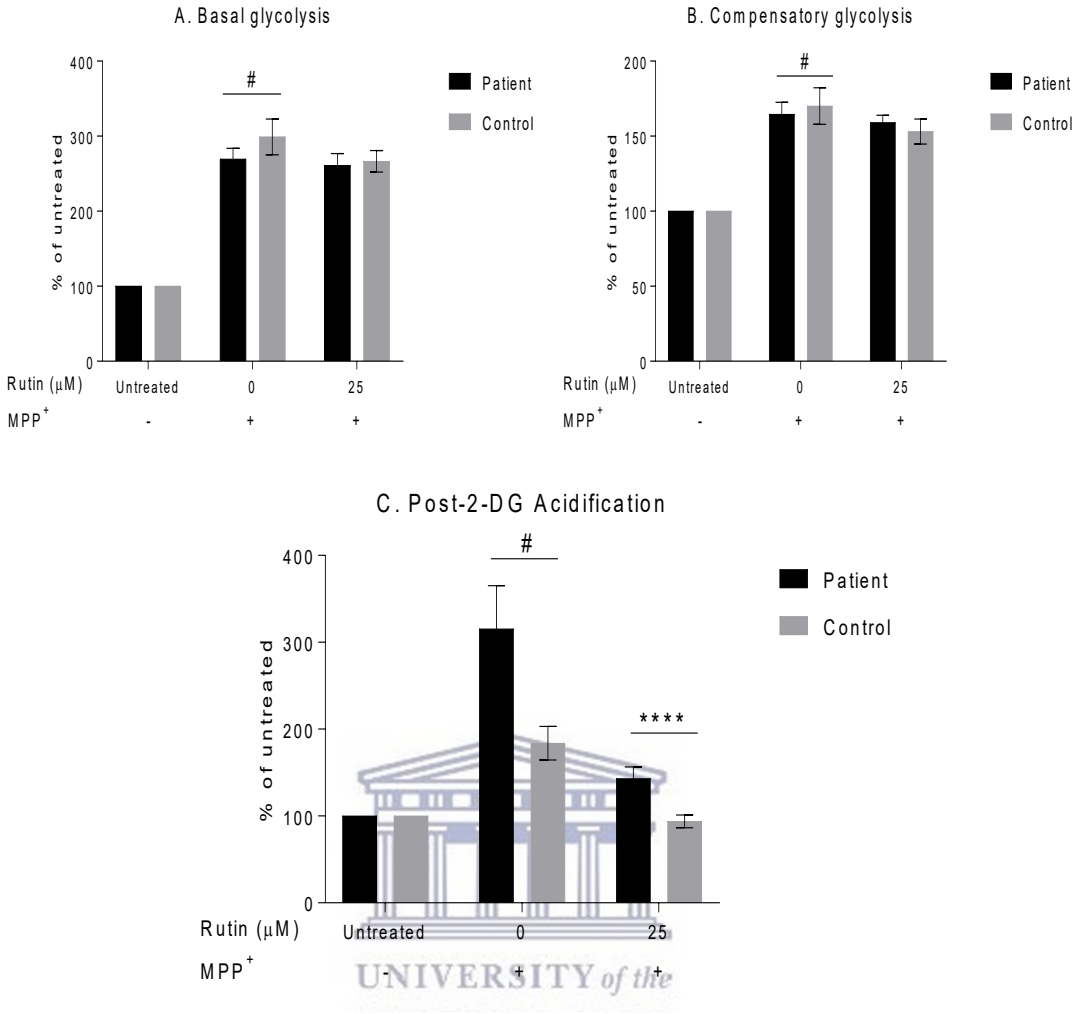
Our findings show that treatment of SH-SY5Y cells with MPP^+ significantly reduced basal and compensatory glycolysis ($p<0.0001$, Figure 6.13) while rutin pretreatment significantly improved basal glycolysis and compensatory glycolysis in SH-SY5Y cells treated with MPP^+ ($p<0.0001$). An opposite effect of increased basal and compensatory glycolysis was observed in fibroblasts treated with MPP^+ ($p<0.05$, Figure 6.14). Inhibition of glycolysis by 2-DG was not effective in fibroblasts treated with MPP^+ ($p<0.0001$, Figure 6.14), but was significant in fibroblasts pretreated with rutin ($p<0.0001$). The high levels of post-2-DG acidification in MPP^+-treated fibroblasts after inhibition of glycolysis by 2-DG indicates that the increase in basal and compensatory glycolysis observed in fibroblasts could be due to other sources of

extracellular acidification and not necessarily attributed to mitochondrial TCA activity or glycolysis.



Each bar represents mean ± SEM from 14 wells of a 96-well plate. #P<0.0001 vs untreated SH-SY5Y cells; ****P<0.0001 vs SH-SY5Y cells treated with MPP^+ only.

Figure 6.13: Effect of rutin on glycolysis stress test in SH-SY5Y cells



Each bar represents mean ± SEM from 15 wells of 96-well plate except for patient fibroblasts pretreated with 25µM rutin (13). #P<0.0001 vs untreated fibroblasts; ****P<0.0001 vs fibroblasts treated with MPP^+ only.

Figure 6.14: Effect of rutin on glycolysis stress test in fibroblasts

CHAPTER 7

DISCUSSION, CONCLUSION AND FUTURE DIRECTIONS

SECTION A

7.1. RUTIN PROTECTS SH-SY5Y CELLS FROM TOXIN-INDUCED OXIDATIVE STRESS, DNA DAMAGE AND INFLAMMATION

7.1.1. Rutin inhibits ROS generation and regulates antioxidant enzymes in SH-SY5Y cells treated with MPP^+.

Many important drugs and bioactive compounds are designed based on structural characteristics of naturally occurring molecules (Rates, 2001, Harvey, 2008). In the last two decades, there have been dedicated search for potential neuroprotective compounds from plant materials. For instance, bioactive compounds such as curcumin (Van der Merwe et al., 2017), resveratrol (Jin et al., 2008), ginsenoside (Xu et al., 2005) and quercetin (Karuppagounder et al., 2013) have been utilized in *in-vivo* and *in-vitro* models of PD. In this regard, *in-vitro* modelling of PD has been achieved through the specific neurotoxic effect of several toxins such as paraquat, MPP^+ and 6-OHDA on dopaminergic neurons (Hou et al., 2008, Magalingam et al., 2016, Kim and Park, 2018). To our knowledge, there is no available report in literature on the neuroprotective activity of rutin in an MPP^+-induced PD model.

Our findings from cell viability and cytotoxicity assays indicate that rutin significantly attenuated MPP^+ toxicity in the SH-SY5Y cells. Further investigations provided useful information on possible mechanisms involved in the neuroprotective activities of rutin. In this study, evaluation of SH-SY5Y neuroblastoma cells revealed that MPP^+ resulted in morphological changes such as cell deformation, shrinkage and detachment. These changes,

often linked to cellular death, were however attenuated in SH-SY5Y cells pretreated with rutin. This is in line with earlier reports on toxin-induced changes in cellular morphology (Wang et al., 2013, Wang et al., 2015a, Kao et al., 2017, Yang et al., 2017)

ROS mediated oxidative damage is a vital component of neuronal degeneration in PD (Cadet and Brannock, 1998). Also, high metabolism of dopamine in PD brains may explain the build-up of harmful radicals in the brain (Gerlach et al., 2003). As a primary mechanism of toxicity in cells, MPP^+ inhibits complex I thereby increasing ROS generation (Johannessen et al., 1986, Adams Jr et al., 2001). Since prolonged generation of ROS initiates key events leading to cell death, inhibition of ROS may partially prevent dopaminergic cell death. Results from this study showed that rutin suppressed MPP^+-triggered increase in ROS generation and therefore regulated multiple targets at the downstream of ROS.

As a way of defending against oxidative stress, SOD emerges as the most vital enzyme for the detoxification of ROS through a process that transforms $O^{-}{}_2{}^{\cdot}$ to the less toxic H_2O_2 (Greenlund et al., 1995). SOD exists in three forms in mammals; i.e. cytosolic (Cu/Zn-SOD) or SOD1, mitochondrial (Mn-SOD) or SOD2 and extracellular (Ec-SOD) or SOD3 (Majima et al., 1998). In the current study, SOD activity was significantly reduced in SH-SY5Y cells treated with MPP^+ only, possibly because MPP^+ damages the anti-oxidative defence system in the cells and increases the susceptibility of neurons to oxidative injury. Rutin on the other hand was found to attenuate the effects by upregulating the activity of SOD in the SH-SY5Y cells, in line with previous studies which reported the ability of rutin to increase SOD levels in other models of PD (Khan et al., 2012, Magalingam et al., 2013, 2016, Sharma et al., 2016).

CAT is a tetrameric abundant antioxidant enzyme found in every living organism exposed to O_2 (Nazıroğlu, 2012). CAT detoxifies H_2O_2 generated by SOD (Nagata et al., 1999, Agati et al., 2012) and catalyses the reduction of two molecules of H_2O_2 to two molecules of H_2O and one molecule of O_2^{-}. CAT is localised in peroxisomes and is likewise detected in the mitochondria and cytoplasm. CAT can remove organic hydro-peroxides (Nazıroğlu, 2012) and

has a negligible role at low levels of H_2O_2 production but becomes more important at higher levels of H_2O_2 production (Gandhi and Abramov, 2012). Post-mortem investigation reveals a reduction in the activity of CAT in PD brains (Ambani et al., 1975). In our findings, we observed a significant reduction in CAT activity following treatment of SH-SY5Y cells with MPP^+ while rutin increased CAT activity in the cells. This is in line with earlier reports which demonstrated the ability of rutin to increase CAT levels in other models of PD (Khan et al., 2012, Magalingam et al., 2013, 2016, Sharma et al., 2016). These findings suggest that rutin possibly has a coordinated effect with CAT by triggering its activation to reduce the levels of ROS in SH-SY5Y cells.

GSH, known as the most ubiquitous non-protein thiol in cells (Dickinson and Forman, 2002, Zeevalk et al., 2008), is critical for clearing metabolic waste, maintaining redox homeostasis and acting as an amino acid pool in the central nervous system (Mischley et al., 2016). GSH acts alone or works with other enzymes to decrease O^{-}_2, $^{\bullet}OH$ and $ONOO^-$ accumulation in the body (Dringen, 2000). GSH deficit has long been implicated in PD and was first postulated in 1982 to play a causative role in PD (Perry et al., 1982, Zeevalk et al., 2008, Martin and Teismann, 2009). Also, post-mortem investigation of nigral tissue of PD patients showed deficiency of GSH when compared to healthy controls (Sofic et al., 1992, Sian et al., 1994). Understandably, GSH deficit undermines the capacity of the cell to metabolize cellular waste, thus impairing its defence against RNS, ROS and H_2O_2 (Mischley et al., 2016).

In the present study, MPP^+ treatment induced oxidative stress by decreasing GSH activity, whereas rutin treatment demonstrated potent antioxidant capacity by upregulating GSH activity. This is in line with reports signifying the ability of rutin to increase GSH activity in other models of neurodegenerative diseases (Magalingam et al., 2013, Xu et al., 2014a, Yu et al., 2015). Taken together, our findings suggest that rutin could regulate cellular antioxidant enzymes as part of its neuroprotective effects in PD.

7.1.2. Rutin inhibits DNA damage and inflammation through proper regulation of the ROS-NO pathway in SH-SY5Y cells treated with MPP^+.

NO is linked to a range of biological processes in humans (Zhang et al., 2006). For instance, in the nervous system, NO is a crucial neural modulator that partakes in neuronal excitability, neurotransmitter discharge, learning and memory (Kiss and Vizi, 2001, Prast and Philippu, 2001, Boehning and Snyder, 2003). NO also participates in pathogenic pathways underlying PD and other neurodegenerative disorders (Torreilles et al., 1999, Boje, 2004), and has been linked with common pathogenic mechanisms such as protein modifications, excitotoxicity and DNA damage (Aarts et al., 2002, Hong et al., 2004). In conditions of prolonged oxidative stress and in cells incapable of eliminating ROS, NO reacts with ROS to form $ONOO^-$ which in turn reacts with other compounds to generate additional harmful peroxide products, thus damaging the DNA and activating cell death pathways (Beckman and Koppenol, 1996, Hong et al., 2004).

Studies on postmortem PD brains and MPTP-treated mice reveal that NO is linked to PD pathogenesis (Langston et al., 1983, Heikkila et al., 1984a) and additional reports demonstrate its involvement in MPP^+ -triggered DNA damage and cell death (Liberatore et al., 1999, Cleeter et al., 2001, Dennis and Bennett, 2003). For example, treatment of mice with 7-nitroindazole (a NO inhibitor) showed resilience to MPP^+-triggered toxicity (Przedborski et al., 1996, Liberatore et al., 1999) while Mandir and co-workers explained that the protective effects of NO inhibition were not due to a defect in the uptake of MPP^+ but was due to a reduction in NO production, which ultimately reduced DNA damage (Mandir et al., 1999). Findings from this study showed that MPP^+ significantly increased intracellular NO production and γH2AX expression in SH-SY5Y cells, whereas rutin significantly decreased NO production and γH2AX expression in the cells, thus suggesting that rutin protected the cells from MPP^+-induced toxicity through adequate regulation of the ROS-NO pathway, leading to attenuation of DNA damage.

Neuroinflammation has been previously associated with PD pathogenesis (Wyss-Coray and Mucke, 2002), as several markers of inflammation have been shown to be upregulated following microglia activation and toxin exposure in PD (McGeer et al., 1988, Hunot et al., 1999). Neuronal injury/stress often results in microglial activation, resulting in the generation of several neurotoxic factors through additional production of cytotoxic factors including NO, ROS, interleukin-1β and TNF-α (Banati et al., 1999, Calabrese et al., 2007, Dai et al., 2011). For example, previous studies have demonstrated increases in striatal TNFα following injection of 6-OHDA in rats (Mladenović et al., 2004). It is believed that the increase in pro-inflammatory cytokines triggers signaling pathways that result in the activation and upregulation of COX-2. Generally, COX translates arachidonic acid into prostaglandin H2 (the key originator of different prostaglandins) (Teismann, 2012), and exists in three different isoforms, COX-1, COX-2 and COX-3 (Yamagata et al., 1993, Beiche et al., 1998, Chandrasekharan et al., 2002). COX-2 is involved in the formation of pro-inflammatory prostaglandins, and an increase in the expression of COX-2 has been reported in dopaminergic neurons of postmortem PD patients (Knott et al., 2000). Similarly, using an MPTP model of COX-2 deficient mice, the importance of COX-2 was further confirmed when the mice exhibited significant protection against MPTP-induced neurodegeneration (Feng et al., 2002, Teismann et al., 2003). Accordingly, bioactive compounds such as acetylsalicylic acid, salicylate, resveratrol, ginsenosides, omega-3 fatty acid and docosahexaenoic acid have been used to inhibit COX-2 expression as a mechanism of neuroprotection in PD models (Aubin et al., 1998, Teismann and Ferger, 2001, Bousquet et al., 2008, Jin et al., 2008, Lee et al., 2013). Findings from this study showed that rutin decreased COX-2 expression in SH-SY5Y cells, thus suggesting that this could be a mechanism to validate its neuroprotective activity. We suggest that COX-2 is an important factor in PD pathogenesis and bioactive compounds which can attenuate COX-2 activity could be effective as possible neuroprotective agents.

In conclusion, this study provides the first evidence that rutin protects dopaminergic SH-SY5Y neuroblastoma cells from MPP^+-induced toxicity, possibly mediated, at least in part, by the proper regulation of the ROS-NO pathway as a neuroprotective mechanism.

SECTION B

7.2. RUTIN MITIGATES MPP^+-INDUCED NEUROTOXICITY THROUGH THE REGULATION OF AKT, AMPK AND NF-ΚB SIGNALING PATHWAYS.

7.2.1. Rutin downregulates apoptotic signals and prevents PARP proteolysis in SH-SY5Y cells treated with MPP^+.

The present study provides the first evidence of the neuroprotective activity of rutin through its inhibition of apoptosis in MPP^+-treated SH-SY5Y cells. It is believed that apoptosis is a consequence of activated caspase proteolysis of numerous cellular mechanisms (Serviddio et al., 2011). Caspase-dependent and caspase-independent mechanisms have been proposed as essential mechanisms leading to dopaminergic neuronal death in the SNpc of PD patients (Schulz, 2006). The mechanisms of apoptosis are multifaceted and involve a cascade of reactions. One of such crucial phases is the mitochondrial release of cytochrome c and initiation of caspase-3 (Wang et al., 2014b). Cytochrome c, a member of the mitochondrial ETC necessary for the production of ATP, initiates the caspase cascade that is essential for cellular energetics (Friedlander, 2003). Cytochrome c–facilitated initiation of cell-death pathways ensues when cytochrome c is released into the cytoplasm from the mitochondria (Friedlander, 2003). It binds to Apaf-1 in the cytoplasm to form an apoptosome (a molecular complex consisting of cytochrome c, Apaf-1, ATP, and procaspase 9). Thereafter, apoptosome activates caspase 9 (an upstream initiator of apoptosis) which in turn activates caspase 3 (Liu et al., 1996a, Li et al., 1997). This mechanism makes cytochrome c release a vital phase in the activation of apoptosis (Hengartner, 2000, Wang, 2001). In this study, the activation of apoptosis by MPP^+ was found to be initiated by the release of cytochrome c from the

mitochondria, however, the ability of rutin to inhibit cytochrome c release demonstrates its potent neuroprotective activity in PD.

Similarly, PARP enzymes are involved in neurodegenerative disorders, including PD (Martire et al., 2015). PARP, a downstream target of caspase-3, is a ubiquitous nuclear enzyme involved in DNA repair, however, when PARP levels are incredibly high, cell death is initiated (Tewari et al., 1995, Bürkle, 2001, Le et al., 2002). During cell death processes, PARP is cleaved into fragments which are specific to various apoptotic signals. The action of caspase 3/7 activation most likely produced the 89-kDa fragment detected in this study, which is in line with previous reports showing detection of PARP (Lazebnik et al., 1994, Nicholson et al., 1995).

Different neuroprotective compounds studied in various PD models have demonstrated their ability to affect the expression of caspases, cytochrome c and PARP. For example, in PC12 cells, curcumin was reported to inhibit cytochrome c release and PARP activation (Raza et al., 2008) and also protect against MPP^+-induced apoptosis (Chen et al., 2006). The polyphenol hesperidin was also found to reduce caspase-3/9 activities and inhibit cytochrome c release in an *in-vitro* human SK-N-SH cellular model of rotenone-induced PD (Tamilselvam et al., 2013). Baicalein, a flavonoid was also reported to inhibit apoptotic cell death in both 6-OHDA and rotenone-treated SH-SY5Y and PC12 cells by decreasing caspase-3/7 and 9 activities (Lee et al., 2005, Li et al., 2012, Wang et al., 2013). In addition, curcumin acted as an anti-apoptotic agent by reducing caspase-3/9 activities as well as inhibiting cytochrome c release in PC 12 cells (Liu et al., 2011). Findings from this study show that rutin attenuates MPP^+ induced neuronal apoptotic death, at least in part, by down-regulating apoptotic signals, such as caspase 3/7, caspase 9, cytochrome c and cleaved PARP. Also, rutin attenuated MPP^+-induced reduction of full-length caspase 3 in SH-SY5Y cells. These findings indicate that the protective effect of rutin is associated with the inhibition of upstream and downstream apoptotic cascades which prevented PARP proteolysis.

7.2.2. Rutin elicits efficient autophagic clearance in SH-SY5Y cells treated with MPP^+

In recent years, compelling evidence obtained from PD patients and PD models have helped to unravel the involvement of autophagy in PD pathogenesis (Cheung and Ip, 2011). Early reports reveal the accumulation of autophagic vacuoles in postmortem brain tissues of PD patients (Anglade et al., 1997), and significant activation of the autophagy response has been subsequently detected in blood samples from PD patients (Prigione et al., 2010). Thus, there is growing interest in autophagic deregulation observed in PD since the autophagic pathway is responsible for the clearance of aberrant proteins. It is believed that many of the neurotoxins used to induce PD also influence the autophagy pathway. For example, MPP^+ causes increased autophagy in SH-SY5Y cells (Zhu et al., 2007), and reports show that the substantia nigra of mice treated with MPTP exhibits autophagic cell death (Meredith et al., 2009, Liu et al., 2013a). Variations in the control of autophagic responses in conditions of organelle damage as opposed to nutrient deficiency and ineffective conclusion of degradative recycling may underlie diverse autophagic effects (Zhu et al., 2007).

In an attempt to fully understand autophagy, different markers of autophagy have been used to study underlying mechanisms in various models of PD. For instance, LC3 exists in two forms, I and II (Klionsky et al., 2016). LC3, originally produced in an unrefined form, proLC3, is converted into LC3-I and further modified into the PE-conjugated form, LC3-II which is continuously associated with completed autophagosomes and autophagy-related structures (Klionsky et al., 2016). In normal conditions, during activation of autophagy, the expression of LC3-II increases. However, there is no clear relationship between LC3-I and LC3-II owing to the cell type-specific conversion of the former to the latter and dependence on the treatment utilized in autophagy induction. LC3-II is known to sometimes localize ectopically on non-autophagosome structures that are not turned over in the lysosome (Tanida et al., 2005, Mizushima and Yoshimori, 2007, Mizushima et al., 2010), and an accumulation of LC3-II can be detected by impairing the fusion of autophagosome and lysosome (Yamamoto et al., 1998,

Jahreiss et al., 2008, Klionsky et al., 2008). Similarly, p62 is another marker frequently used to monitor autophagic activity due to its ability to bind to LC3 directly (Bjørkøy et al., 2005, Pankiv et al., 2007). p62 and p62-bound proteins are integrated into the formed autophagosome and are subsequently degraded in autolysosomes, therefore serving as an index of autophagic degradation. In mammals, inhibition of autophagy and a defect in the autophagy process corresponds to increased p62 expression while reduced p62 levels are linked to efficient activation of autophagy (Lee et al., 2010a, Bartlett et al., 2011, Cui et al., 2012).

While it is agreed that deficient autophagy activation would impede clearance of protein aggregates, it is suggested that the magnitude of autophagy produced may determine cell survival, with low levels linked to homeostatic functions and high levels stimulating cell death, either directly or indirectly (Wang et al., 2009a). In line with previous reports (Liu et al., 2013b), our findings show that MPP^+ causes aberrant/extreme activation of autophagy in SH-SY5Y cells. This may be due to excessive autophagic demand in the cells which could not be balanced by cellular reserves, thus resulting in cell death. Also, MPP^+ may have impaired the membrane integrity of lysosomes leading to protease leakage that caused damage to other cellular components (Button et al., 2015). A deficit in lysosomal function is known to stimulate autophagy, thereby leading to a pathological accumulation of autophagosomes that cannot be successfully cleared (Gomez-Suaga et al., 2011). Furthermore, in line with previous reports (Anglade et al., 1997, Zhu et al., 2003), our TEM images also revealed abnormal presence/accumulation of autophagosomes in SH-SY5Y cells treated with MPP^+ only. This is in sharp contrast to the rare detection of autophagosomes in control and rutin pretreated SH-SY5Y cells, probably due to the efficient and speedy clearance of autophagic vacuoles. The presence of several autophagosomes in our PD model besides signifying a flaw in the activation of autophagy, might be as a result of a defective clearance which led to its accumulation. This is demonstrated by the increased expression of both LC3-II and p62 proteins in SH-SY5Y cells treated with MPP^+ only. However, attenuation of abnormal autophagy activation by rutin provides the first evidence of its beneficial role in autophagy-related mechanism in PD models.

7.2.3. Rutin initiates a crosstalk between the Akt/AMPK/NF-kB signaling pathways in SH-SY5Y cells treated with MPP^+.

Findings from this study show that rutin pretreatment prevents MPP^+-induced toxicity partly through the regulation of the Akt signaling pathway. Akt is a substrate of PI3K and is deeply involved in the anti-apoptotic pathway (Pap and Cooper, 1998, Manning and Cantley, 2007). Total activation of Akt involves sequential phosphorylation at two sites (Thr308 and Ser473). Even though phosphorylation at Thr308 is adequate for Akt activity, phosphorylation at both sites produces full catalytic activation of the enzyme (Zhang et al., 2016). Once activated, Akt pathway controls some downstream signaling cascades that affect a variety of cellular activities including proliferation, survival, migration, differentiation, metabolism and polarity in different cell types including neurons (Manning and Cantley, 2007, Greene et al., 2011, Zhang et al., 2016). Some reports support compromised Akt signaling in PD. For example, immunostaining of post-mortem brains reveals that Akt phosphorylation at Thr308 and Ser473 is significantly reduced in the dopaminergic SNpc of PD patients when compared with non-PD patients (Timmons et al., 2009). Akt phosphorylation at both Thr308 and Ser473 was significantly diminished by treatment of neuronal cells with PD neurotoxins (Malagelada et al., 2008, Rodriguez-Blanco et al., 2008, Tasaki et al., 2010). On the other hand, treatments that inhibit loss of Akt phosphorylation are protective in such PD models (Malagelada et al., 2010, Tasaki et al., 2010). Also, numerous animal studies provide evidence for the protective effects of Akt signaling in models of PD. For instance, estrogen and IGF-1 treatment significantly reduced impairment of motor behavior, protected SN dopaminergic neurons from death and preserved TH^+ axons in the striatum of PD rats. This evidence was based on western blot images which showed that both treatments improved Akt phosphorylation in the SN of rats (Quesada et al., 2008).

Similarly, several bioactive compounds are reported to exhibit their neuroprotective properties through their ability to activate the Akt pathway in various *in-vitro* models of PD (Nakaso et al., 2008, Liu et al., 2009, Fukui et al., 2010, Malagelada et al., 2010, Tasaki et al., 2010, Qin

et al., 2011). These reports suggest that most neuroprotective PD agents share a common characteristic in their ability to improve Akt activation. Accordingly, our findings show that MPP^+ treatment caused a reduction in phosphorylated Akt levels in SH-SY5Y cells while rutin pretreatment upregulated phosphorylated Akt expression and thus inhibited some downstream cellular activities associated with MPP^+ toxicity.

AMPK is a heterotrimeric enzyme that consists of an α catalytic subunit, a β and γ regulatory subunits (Oakhill et al., 2009). It is considered a key regulator of cellular energy metabolism and is activated by phosphorylation of the α subunit at Thr172 (Hawley et al., 1996, Birnbaum, 2005). Activation of AMPK is regulated by ROS, Ca^{2+} and cellular AMP/ATP ratio (Weekes et al., 1994, Hawley et al., 2005, Park et al., 2006, Jung et al., 2008), and has a range of effects that may be relevant to PD such as mitochondrial quality control, changes in cellular metabolism, promotion of autophagy, reduced inflammation and improved antioxidant capacity (Choi et al., 2010b). Conversely, reports show that AMPK also plays a role in facilitating PCD, particularly in situations of bioenergetic failure or severe cellular stress. Therefore, it is quite important to balance the potentially positive effects of AMPK activation with its potentially negative effects. Previous studies showed that activation of AMPK by PD-inducing neurotoxins expedited neuronal death by facilitating excessive autophagy and impaired protein synthesis possibly through a mechanism mediated by AMPK's inhibition of mTOR (Xilouri and Stefanis, 2011, Xu et al., 2014b).

Our findings and previous reports tend to suggest that extreme autophagy may damage basic cellular machinery, or cellular stress may hinder the autophagic process such that autophagy cannot be completed (Thorburn et al., 2014). Findings from this study agree with previous reports (Gomez-Suaga et al., 2011, Xu et al., 2014b, Button et al., 2015) that activation of AMPK stimulates autophagy and leads to accumulation of autophagosomes that cannot be successfully cleared. Results from this study also suggests that a crosstalk exists between the activation of AMPK and inactivation of Akt which leads to neuronal cell death. Xu and

coworkers have proposed that this phenomenon occurs via the inhibition of the mTOR pathway (mTOR is a widely regarded as a central controller for cell growth, proliferation as well as survival) (Cornu et al., 2013, Laplante and Sabatini, 2013, Xu et al., 2014b). A certain level of mTOR activity is essential for neuronal cell survival and healthy function, however, too low or high levels of mTOR activity may be harmful to neurons (Xu et al., 2014b). These authors found that MPP^+ potently inhibited phosphorylation of mTOR, inducing a crosstalk between these signaling pathways, leading to neuronal cell death in PC12 cells and primary neurons.

Similarly, NK-κB has been reported to play a critical role in such cellular processes as growth, immune reaction and oncogenesis (Perkins, 2007). NF-κB comprises two subunits of p65, namely p50 and 52 and in the cytoplasm, it is sequestered as an inactive complex with NF-κB inhibitory subunit (IκB). When stimulated, phosphorylation of IκB occurs at the IκB kinase (IKK) complex and is degraded by the UPS after dissociating from the IKK complex (Naoi et al., 2009). Several stimuli such as toxins and cytokines activate the release of NF-κB from IκB, resulting in translocation of NF-κB into the nucleus, binding to DNA at specific sites and consequently inducing a variety of genes (Baeuerle and Henkel, 1994, Baldwin Jr, 1996). Activation of NF-κB has been linked with a wide variety of human diseases, including PD (Gupta et al., 2010, Shih et al., 2015). Previous reports show that NF-κB plays either an anti-apoptotic or a proapoptotic role, depending on the cell type and/or nature of the apoptosis-inducing stimuli (Baichwal and Baeuerle, 1997). Activation of NF-κB inhibits induction of apoptosis and its inactivation impedes survival of neurons (Camandola and Mattson, 2007). Also, other reports show that inhibition of NF-κB alerts cells to apoptosis by activating several stimuli such as TNF-α (Beg and Baltimore, 1996, Van Antwerp et al., 1996, Wang et al., 1996a) as well as inducing cell death in β-cells (Wu et al., 1996), neurons (Grilli et al., 1996), and Schwann cells (Carter et al., 1996).

Accumulating evidence further suggests that the Akt pathway is actively involved in the regulation of NF-κB (Kane et al., 1999, Ozes et al., 1999, Sizemore et al., 1999, Madrid et al.,

2000), as demonstrated in reports showing that the inhibition of Akt impedes the upregulation of NF-κB (Bai et al., 2009). Conversely, other reports indicate that through its signaling network, AMPK activation can suppress the activation of NF-κB (Salminen et al., 2011, Choi et al., 2016) which are confirmed in our findings on MPP^+-induced cell death through the inhibition of NF-κB expression and attenuation thereof by rutin through the upregulation of NF-κB expression in the SH-SY5Y cellular model of PD. This study therefore provides the first evidence that rutin attenuates MPP^+-induced downregulation of Akt and NF-κB as well as upregulation of AMPK, and tends to confirm suggestions that a crosstalk between these signaling pathways leads to the debilitating effects of MPP^+ (Pan et al., 2009, Xu et al., 2014b). Thus, the neuroprotective activity of rutin may be attributed to its ability to regulate the Akt/AMPK/NF-κB signaling pathways.

In conclusion, our study demonstrated the ability of rutin to protect SH-SY5Y cells against MPP^+ induced neurotoxicity, attenuate apoptosis and ameliorate aberrant autophagy triggered by MPP^+ in SH-SY5Y cells.

SECTION C

7.3. RUTIN ATTENUATES ENDOPLASMIC RETICULUM STRESS, IMPAIRED CALCIUM HOMEOSTASIS AND ALTERED MITOCHONDRIAL FUNCTIONS IN CELLULAR MODELS OF PARKINSON'S DISEASE.

7.3.1. Rutin protects against dysregulated Ca^{2+} homeostasis and maintains MMP in SH-SY5Y cells treated with MPP^+.

This study provides first evidence on the regulation of Ca^{2+} by rutin in an MPP^+ model of PD. Ca^{2+} is widely regarded as a common second messenger under tight homeostatic control owing to its regulation of an array of cellular events (Petersen et al., 2005a). The process of removing Ca^{2+} from the cytosol is considered to be an energy-consuming process due to higher cytosolic

Ca^{2+} levels in the extracellular space (Surmeier and Schumacker, 2013), thus, Ca^{2+} not quickly removed from the neuron are hidden away in intracellular organelles such as the lysosomes, Golgi, endoplasmic reticulum (ER) and mitochondria (Lloyd-Evans and Platt, 2011, Kaufman and Malhotra, 2014). Different mechanisms have been proposed for the association between increased demand for Ca^{2+} handling and neuronal degeneration. It is believed that due to the high basal ATP consumption rates linked to Ca^{2+} control, susceptible neurons have lower respiratory or bioenergetic reserve (Nicholls, 2008). The respiratory reserve in cells is the variance between the basal ATP consumption rate and the maximum capacity for ATP generated by OXPHOS. A lesser respiratory reserve is reported to put neurons at risk when metabolic demand increases, for example, in conditions of toxin exposure (Rivero-Ríos et al., 2014). Consequently, inadequate ATP levels result in a decline of the MMP, influx of Ca^{2+} and cell death.

MMP is essential to the organelle's several functions (Nicholls and Ferguson, 2013) and is responsible for driving ATP synthesis by OXPHOS. The magnitude of MMP is determined by the equilibrium between its production and utilization by processes such as ATP synthesis, thus making MMP a useful sign of cellular energetics. Also, many biological processes are associated with changes in MMP, for instance, a decrease in MMP may be as a result of high ATP production or from an impairment of the mitochondria while an increase can be due to reduced generation of ATP or an increase in proton pumping (Logan et al., 2016). The ability to quantify these changes in MMP enables mechanistic insights and facilitates new methods for diagnosis and therapies. In our study, we observed that MPP^+ caused a significant reduction in MMP in SH-SY5Y cells, indicating mitochondrial dysfunction, however, this loss in MMP was significantly attenuated by rutin. This suggests that rutin was able to prevent the harmful activities associated with a decline in MMP. Likewise, in modulating mitochondrial function, Ca^{2+} is taken into the mitochondrial matrix in a process that involves the MMP, thus increasing ATP production (Kirichok et al., 2004, Santo-Domingo and Demaurex, 2010). However, excess Ca^{2+} in mitochondria causes dysfunction by halting ATP production due to its initiation of a

collapse in the MMP (McCormack and Denton, 1990). Therefore, for optimal survival of dopaminergic neurons, it is essential for Ca^{2+} levels to be sustained within low range of concentrations (Michel et al., 2013). Our current study showed that MPP^+ induced a significant increase in Ca^{2+} levels in the cells, and further demonstrated that rutin significantly reduced Ca^{2+} levels, thus preventing the deleterious effects associated with excessive Ca^{2+}.

7.3.2. Rutin attenuates MPP^+ induced ER stress in SH-SY5Y cells treated with MPP^+.

The present study provides the first research evidence on the ability of rutin to inhibit ER stress in SH-SY5Y cells treated with MPP^+. BiP is activated during cytotoxic stimuli such as oxidative stress, toxicity and in response to ER stress (Kaufman, 1999, Ghribi et al., 2003). Activation of BiP during the UPR protects neurons against metabolic/excitotoxic damage (Lowenstein et al., 1991, Yu et al., 1999), which is needed to attenuate ER stress, sustain proper ER function, enable protein folding and defend cells against toxic insults (Ghribi et al., 2003). As a consequence of aging in such age-related disorders as PD, a distortion in equilibrium between the protective and pro-apoptotic signaling in the UPR occurs. With increasing age, the activity of the protective/pro-survival component of the UPR is considerably decreased while the activity of the pro-apoptotic component is upregulated (Gavilan et al., 2006, Hussain and Ramaiah, 2007, Naidoo et al., 2011). These changes are complemented by alterations in the ER causing declines in important UPR chaperones and enzymes, thus affecting the ability of the ER to sustain thorough protein folding or maintain ER equilibrium (Hinds and McNelly, 1978). This failure in ER function is believed to occur partly because of a deficiency in significant ER resident chaperones such as GRP94 and BiP (Nuss et al., 2008).

Accordingly, reports show that the UPR is a therapeutic target for proper protein homeostasis during inhibition of neurodegeneration. For example, the drug, valproate increases BiP levels and other ER chaperones (Wang et al., 1999, Bown et al., 2000) while MPP^+ resulted in a down-regulation of BiP mRNA in MN9D cells (Holtz and O'Malley, 2003a). We hypothesized that BiP was responsible for the main chaperone action in our PD model and that its upregulation

would possibly improve proper protein folding, attenuate ER stress and eventually inhibit apoptosis in SH-SY5Y cells. In line with previous findings, results from this study show that rutin significantly increased the expression of BiP, thus suggesting that a ‘‘stress response’’ may trigger the protective activity of rutin in this model of PD, thus enabling proper function of the UPR and alleviating ER stress.

Upregulation of BiP is linked to the inhibition of ER stress-induced activation of CHOP (Wang et al., 1996b, Oyadomari et al., 2001). CHOP/GADD153 is a 29kDa protein that has been associated with the control of processes necessary for cellular differentiation, proliferation, expression and energy metabolism of specific genes (Birkenmeier et al., 1989, Umek et al., 1991). Normally, the expression of CHOP is extremely low, however, it is upregulated in the nucleus during apoptosis triggered by ER stress (Lu et al., 2014). CHOP is activated by agents that disturb ER function such as 6-OHDA, thapsigargin and MPP^+ (Oyadomari and Mori, 2004). Upon activation, CHOP is translocated to the nucleus from the cytoplasm and partakes in the induction of apoptosis. The CHOP pathway is reported to be a major regulator of ER stress-induced apoptosis, thus increased levels of CHOP indicate ER stress induction. This is demonstrated in reports showing CHOP’s activity in PD models. For example, reports show that MPP^+ increases the expression of CHOP in SH-SY5Y cells (Conn et al., 2002, Zhao et al., 2016a), and authors have observed a correlation between cell death induced by 6-OHDA and CHOP induction (Ryu et al., 2002). Findings from this study show that the attenuation of MPP^+-induced increase in the expression of CHOP is possibly via the upregulation of BiP. These findings suggest that neuroprotective agents aimed at targeting proper regulation of these proteins would be a worthwhile therapeutic option in PD.

7.3.3. Rutin mitigates impaired oxidative phosphorylation in SH-SY5Y cells and fibroblasts treated with MPP^+

To gain comprehensive insights into the protective mechanism of rutin, different aspects of cellular bioenergetic status (Dranka et al., 2011) was investigated in SH-SY5Y cells and fibroblasts following treatment with MPP^+. It is well believed that a range of human pathologies

associated with mitochondrial dysfunction frequently arises as a result of OXPHOS impairment (Koopman et al., 2012, Koopman et al., 2013). By definition, OXPHOS is a process by which ATP is generated from the energy of biological oxidation. A dysfunction in OXPHOS at the cellular level initiates adaptive responses that may inhibit cell death or create other harmful consequences (Hao et al., 2010, Germain et al., 2012, Benard et al., 2013). The adaptive responses may include (but are not limited to) alterations in redox state and antioxidant reactions, upregulation of glycolysis and initiation of mitochondrial biogenesis (Koopman et al., 2010). Thus, evaluation of bioenergetic status provides critical understandings into the energetic demands, response and rate of respiration achieved by the cells in normal and stressed conditions.

Findings from this study show a significant reduction in the basal respiration in SH-SY5Y cells and fibroblasts treated with MPP^+. Also, a decrease in oxygen consumption rate after the injection of oligomycin indicates the proportion of basal respiration being used to drive ATP production. Results show that rutin did not improve ATP-coupled respiration in SH-SY5Y cells and fibroblasts treated with MPP^+. Also, rutin significantly improved spare respiratory capacity and maximal respiration in pretreated SH-SY5Y cells when compared to those treated with MPP^+ only. These findings are important because maximal respiration indicates the maximum capacity operated by the respiratory chain arising from substrate oxidation to meet its metabolic challenges, thus, a reduction in maximal respiration is a strong measure of an impairment to the mitochondria (Brand and Nicholls, 2011). Additionally, the mitochondrial spare respiratory capacity is considered as a vital aspect of mitochondrial function, and its measurement signifies the cell's ability to respire maximally as well as its capability to react to an energetic demand (Yamamoto et al., 2016). When cells are exposed to stress conditions, more ATP is needed to maintain normal functions, thus cells with higher spare respiratory capacity are capable of producing more ATP to overcome more stress (Hill et al., 2009). Consequently, our observation that rutin significantly increases maximal respiration and spare respiratory capacity confirms its role in protecting mitochondrial function.

Remarkably, under basal conditions, MPP^+ exhibited a different mode of action in its interactions with the glycolytic pathway in SH-SY5Y cells and fibroblasts. Inhibition of OXPHOS is often linked with the stimulation of glycolysis as a compensatory response to damages in mitochondrial ATP synthesis (Keuper et al., 2014, Ozawa et al., 2015). As an OXPHOS inhibitor, MPP^+ increased glycolysis in fibroblasts which correlated with a decrease in mitochondrial respiration whereas MPP^+ inhibited glycolysis in SH-SY5Y cells. This may be due to an existing assumption that despite the ability of neurotoxins to induce cell death, their effects on basal bioenergetics and metabolism are sometimes diverse. For instance, authors have observed that the toxicity of different MPP^+ concentrations occurs by inhibiting glucose (as seen in the SH-SY5Y cells) or via a metabolic collapse which triggers a shift to glycolysis from OXPHOS, thus leading to cell death (as seen in the fibroblasts) (Mazzio and Soliman, 2003). Accordingly, authors proposed a biphasic mechanism of MPP^+ toxicity which includes (i) a DAT-related mechanism which is selective for dopaminergic neurons and (ii) an oxidative mechanism (Maruoka et al., 2007).

In this regard, while neurons have a restricted capacity to stimulate glycolysis, increase in glycolysis is known to attenuate mitochondria-associated energy failure and cell death (Chaudhuri et al., 2015, Hong et al., 2016). Also, reports show that sustaining cellular ATP concentrations through glycolysis inhibits toxicity induced by MPP^+ (Chalmers-Redman et al., 1999, Mazzio and Soliman, 2003, Maruoka et al., 2007). This is in line with our observations that rutin significantly increased basal and compensatory glycolysis in the SH-SY5Y cells. To our knowledge, the current study provides the first evidence on the activity of rutin on cellular bioenergetic status in SH-SY5Y cells and fibroblasts using the XF Analyzer.

LIMITATIONS OF THE STUDY

Considerable effort was made to replicate all assays done in the SH-SY5Y neuroblastoma cells in the fibroblasts. In addition, effort was made to investigate additional cell signalling, DNA damage response, inflammatory, apoptotic and autophagy pathway markers in both SH-SY5Y

cells and fibroblasts, however, funding constraints hindered such objectives.

CONCLUSION AND FUTURE DIRECTIONS

For the first time, this thesis provides evidence on the ability of rutin to protect SH-SY5Y cells and fibroblasts from MPP^+ -induced toxicity. The key findings can be summarized as follows

1. MPP^+ disrupts the activities of antioxidant enzymes and induces a significant increase in ROS and NO production in SH-SY5Y cells. We believe that this is responsible for the DNA damage and inflammation observed in SH-SY5Y cells. Conversely, rutin regulates the activities of antioxidant enzymes and significantly attenuates MPP^+-induced ROS, inflammation and DNA damage in SH-SY5Y cells.
2. The ability of rutin to protect SH-SY5Y cells against MPP^+ induced apoptosis and autophagy may occur via a proper regulation and possible crosstalk between Akt, AMPK and NK-κB signaling pathways.
3. MPP^+ disrupts calcium homeostasis, reduces MMP and induces ER stress in SH-SY5Y cells. Also, MPP^+ impairs bioenergetic status in SH-SY5Y cells and fibroblasts. On the other hand, rutin protects against MPP^+ induced reduction in MMP and protects against MPP^+ induced dysregulation of Ca^{2+} homeostasis and ER stress in SH-SY5Y cells. Additionally, rutin rescues major bioenergetic parameters in MPP^+-treated SH-SY5Y cells and fibroblasts.

As discussed in chapter 2, previous studies have demonstrated the ability of rutin to ameliorate various neurodegenerative processes that trigger AD, PD, HD and PrDs. As revealed in this study, the ability of rutin to exert its neuroprotective effects in PD could be ascribed to its antioxidant, anti-apoptotic and anti-inflammatory activities as well as its effects on the mitochondria. In addition, the regulation of Akt, AMPK and NK-κB by rutin indicates its involvement in signaling pathways known to contribute to survival of neurons in the CNS.

The benchmark for authenticating the neuroprotective properties of rutin is clinical trials in humans. A few clinical trials have been conducted to examine the effect of one compound from

the rutin family namely - O-(β-hydroxyethyl)-rutosides (HRs) in venous disease patients with diabetes treated for a prolonged period of time (Stuard et al., 2008). HRs is obtained by substituting rutin hydroxyl groups with O-β-hydroxyethyl groups. Human clinical trials with rutin (in the form of HRs) have shown that it is safe and well tolerated (Stuard et al., 2008) but no clinical trials exploring the efficacy of rutin in PD have been reported possibly due to lack of sufficient data from animal models in PD.

Further studies to investigate its protective activities in animal models of PD would provide a solid foundation for its use in clinical trials. The ability of rutin to offer neuroprotection against pathological insult offers hope in its utilization and development as a safe and effective neuro-therapeutic agent.

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APPENDIX

I. SOLUTIONS FOR WESTERN BLOT AND FUNCTIONAL ASSAYS

Lysis buffer
1M Tris- HCl pH 7.4 (5 ml); 5M NaCl (3 ml); Triton-x (1%); 1M $MgCl_2$ (150 µl); ddH2O (150 ml)
Add fresh on day of lysis (to 5 ml lysis buffer): *β-mercapto-ethanol 0.1%; 50 mM PMSF 100 µl; One quarter protease inhibitor tablet (Roche)*

Bradford protein reagent
Coomassie Brilliant Blue 100 mg; Phosphoric acid 100 ml; 96% Ethanol 50 ml; ddH2O (*adjust to a total volume of 1 liter*). *Filter until solution is light brown in color. Store away from light at room temperature.*

TBST
5 M NaCl stock solution (30ml); 1M Tris-HCl stock solution (pH 7.6, 20 ml); Tween-20 (1 ml); ddH2O (*adjust to a total volume of 1 liter*).

10X SDS-PAGE running buffer
Tris base 30 g; Glycine 144 g; 10% SDS 100 ml; ddH_2O (*adjust to a total volume of 1 liter*)

1X SDS-PAGE running buffer
10X SDS-PAGE running buffer (100 ml); ddH_2O (*adjust to a total volume of 1 liter*)

Stripping buffer
Glycine 15 g; 10% SDS (10 ml); Tween-20 (10 ml); Adjust pH to 2.2 and add ddH2O (*adjust to a total volume of 1 liter*).

5% Milk for membrane blocking
2 g milk powder; 40 ml TBST; *Vortex till its adequately dissolved.*

5 mg/ml MTT stock solution
Thiazolyl blue tetrazolium bromide 50 mg; PBS 10 ml - *Store away from light at 4˚C for up to two weeks.*

20 mM DCFH-DA stock solution
2′,7′-Dichlorofluorescin diacetate 3 mg; DMSO 307.82 ml; *Aliquot in 40 µl volumes and store away from light at -20˚C.*

5 mg/ml JC-1 stock solution
Tetraethyl benzimidazolyl carbocyanine iodide 5 mg; DMSO 1 ml; *Aliquot in 40 µl volumes and store away from light at -20˚C.*

10 µg/ml JC-1 working solution
5 mg/ml JC-1 stock solution 2 µl; Culture media 1 ml;

20 mM Rhodamine 123 stock solution
Rhodamine 5 mg; Ethanol 656.48 ml; *Store away from light at -20˚C.*

20 µg/ml Bafilomycin A1 stock solution
100 µg Bafilomycin A1; 5 ml ethanol

II. ADDITIONAL RESULTS FROM MITOCHONDRIAL BIOENERGETICS IN SH-SY5Y CELLS

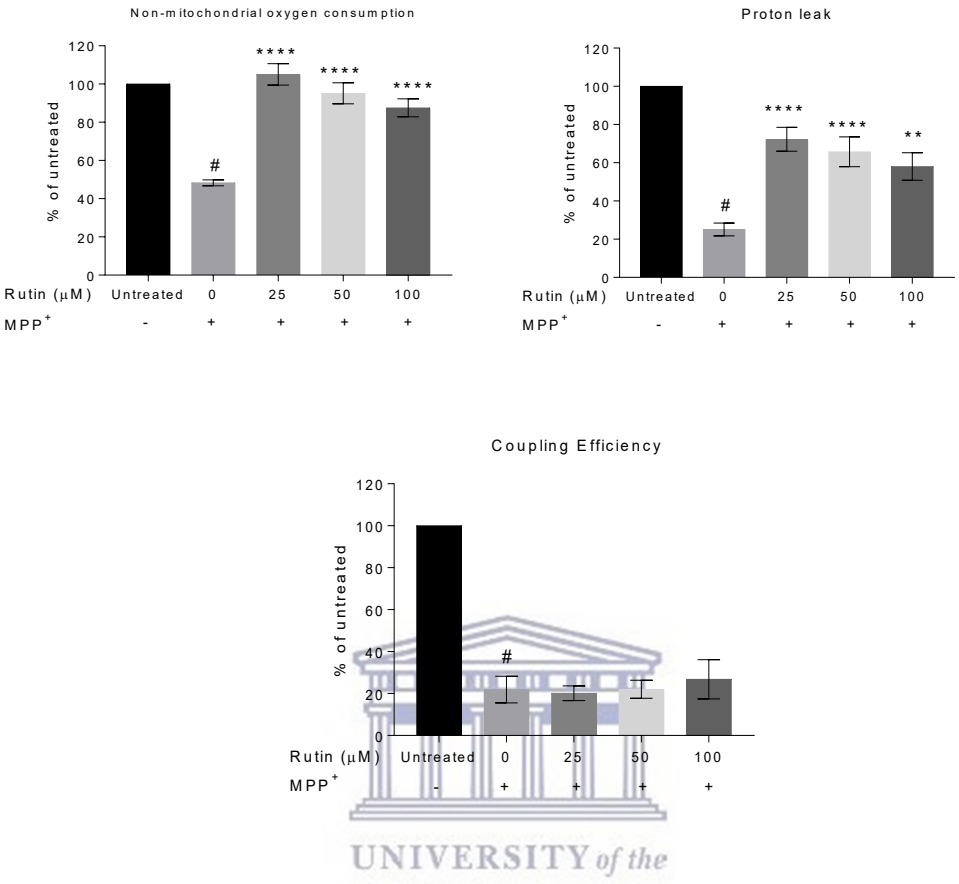

Figure 8.1: Non-mitochondrial oxygen consumption, proton leak and coupling efficiency in treated and untreated SH-SY5Y cells. Each bar represents mean ± SEM from 15 wells of a 96-well plate except SH-SY5Y cells treated with MPP^+ only (14). # P<0.0001 vs untreated SH-SY5Y cells; ****P < 0.0001 and **P<0.005 vs. SH-SY5Y cells treated with MPP^+ only.

III. ADDITIONAL RESULTS FROM GLYCOLYSIS STRESS TEST IN SH-SY5Y CELLS

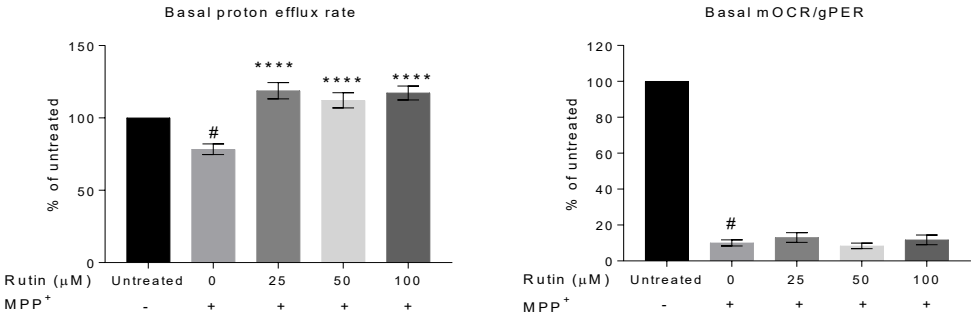

Figure 8.2: Basal proton efflux rate and Basal mOCR/gPER in treated and untreated SH-SY5Y cells. Each bar represents mean ± SEM from 14 wells of a 96-well plate. $^{\#}P<0.01$ and $^{\#\#}P<0.0001$ vs untreated SH-SY5Y cells; **** $P < 0.0001$ vs. SH-SY5Y cells treated with MPP^+ only.

IV. ADDITIONAL RESULTS FROM MITOCHONDRIAL BIOENERGETICS IN FIBROBLASTS.

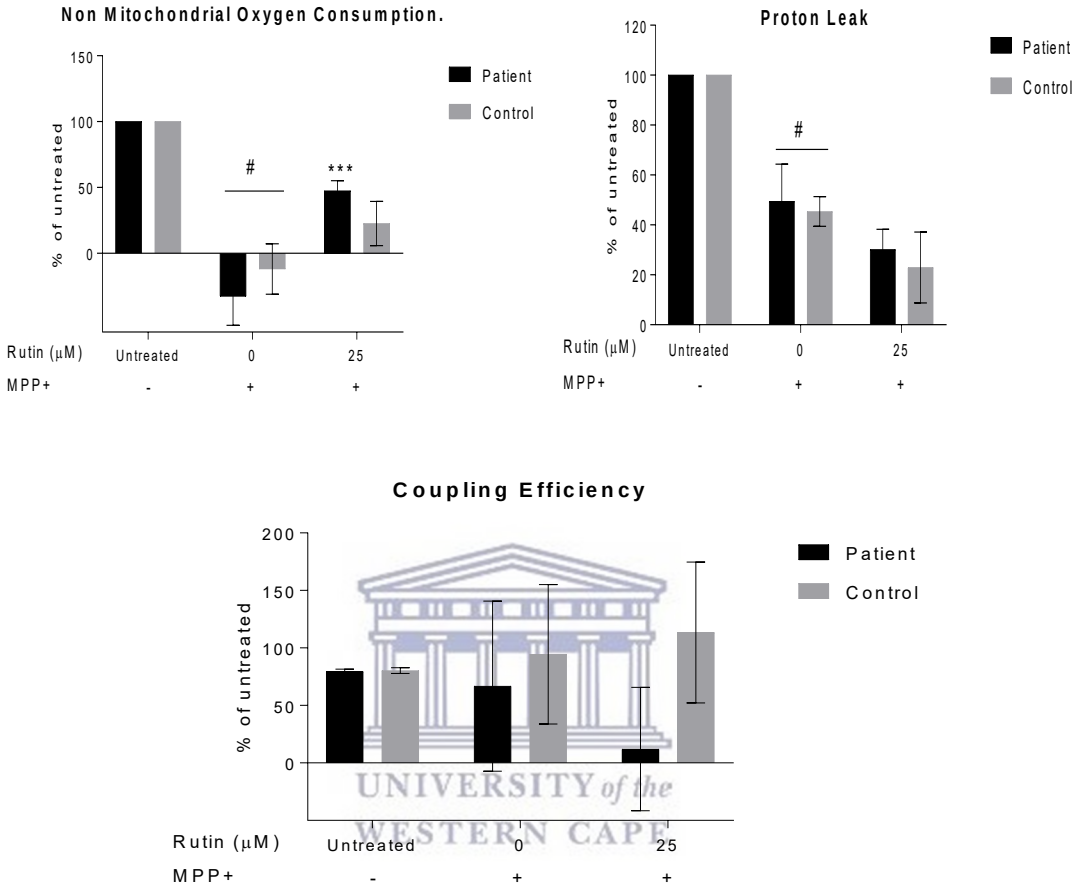

Figure 8.3: Non-mitochondrial oxygen consumption, proton leak and coupling efficiency in treated and untreated fibroblasts. Each bar represents mean ± SEM from 15 wells of a 96-well plate except for fibroblasts treated with MPP^+ only (14). $^{\#}P<0.0001$ vs untreated fibroblasts; ***$P<0.0005$ vs fibroblasts treated with MPP^+ only.

V. ADDITIONAL RESULTS FROM GLYCOLYSIS STRESS TEST IN FIBROBLASTS.

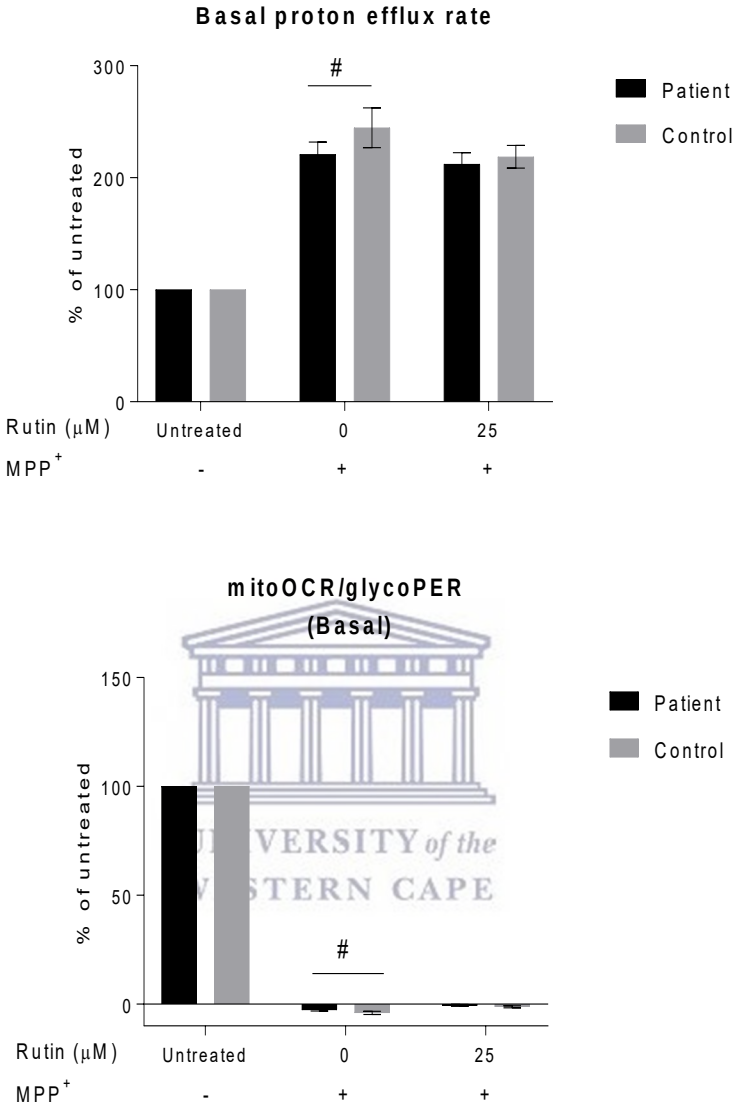

Figure 8.4: Basal proton efflux rate and Basal mOCR/gPER in treated and untreated fibroblasts. Each bar represents mean ± SEM from 15 wells of 96-well plate except for patient fibroblasts pretreated with 25 µM rutin (13). #P<0.0001 vs untreated fibroblasts.