# NEUROPROTECTIVE EFFECTS OF ZINC AND LINOLEIC ACID IN RAT MODEL OF PARKINSONISM INDUCED WITH ROTENONE

**NGALA ELVIS MBIYDZENYUY**

**BSc (Human Physiology) Ahmadu Bello University, Zaria Nigeria (2008)**

MSc.PHY/0001/143/DF

A DISSERTATION SUBMITTED TO THE DIRECTORATE OF POSTGRADUATE STUDIES AND RESEARCH, IN PARTIAL FULFILMENT FOR THE AWARD OF MASTER OF SCIENCE DEGREE IN PHYSIOLOGY OF KAMPALA INTERNATIONAL UNIVERSITY, WESTERN CAMPUS UGANDA



**August 2017**

## DECLARATION

I, Ngala Elvis Mbiydzenyuy declare that the dissertation titled "Neuroprotective effects of zinc and linoleic acid in rat model of Parkinsonism induced with rotenone" was carried out by me as an original piece of scientific work and has never been presented for any award. All sources of information have been cited and all contributions acknowledged.

Signature and Date 17/08/17

## CERTIFICATION

We the undersigned would like to confirm that the dissertation hereby titled "Neuroprotective effects of zinc and linoleic acid in rat model of Parkinsonism induced with rotenone" submitted by Ngala Elvis Mbiydzenyuy was carried out with our guidance and supervision

SUPERVISORS

Signature/Date

Herbert Izo Ninsiima
DCM, BSc, MSc Physiology KIU
Department of Physiology, Faculty of Biomedical Sciences
Kampala International University, Uganda

Miriela Betancourt Valladares
DDS, MSc Dental Emergency, 2nd Degree Specialist in Physiology and Pathophysiology
Associate Professor of Physiology, Department of Human Physiology
University of Medical Sciences, Camaguey Cuba

Pieme Constant Anatole
BSc, MSc, PhD Biochemistry (UYI)
Associate Professor of Biochemistry,
Department of Biochemistry and Physiological Sciences
Faculty of Medicine, University of Yaounde I, Cameroon

Pr. PIEME Constant Anatole
Maître de Conférences de Biochimie
FMSB / UYI

## ACKNOWLEDGEMENTS

All praises, honour and thanksgiving are given to God almighty for provision, preservation and favour all through my studies. I express warm gratitude to my supervisors Professors Miriela Betancourt Valladares and Pieme Constant Anatole and Mr. Herbert Izo Ninsiima for their support with my graduate research, for their patience, great wealth of knowledge and above all encouragement. Their mentorship has added great value to this piece of scientific work. Besides my supervisors, I would like to thank the Head of Department, Physiology, Mr Okpanachi Alfred for his encouragement and commitment to see that I come this far .

I thank my fellow laboratory mates at the University of Yaounde 1 Cameroon for the stimulating discussions and my MSc course mates at Kampala International University, Western Campus Uganda, for great sessions of academic debate and intercourse we have had for the past years. I am also grateful to Mr Nono of the Department of Pharmacology and Toxicology, University of Yaounde 1, Cameroon for enlightening me the first glance of animal research and for working tooth and nail with me to see that the laboratory part of the study was done.

Last but not the least; I would like to thank my wife Mrs Ngala Fienmaih Jane for her unwavering support in all forms to see that this MSc dissertation came to being. I am also indebted to family: my parents Mr. Ngala Augustine and Mrs. Ngala Joan, for the first lessons they gave me life, for supporting me all through my academic journey.

**TABLE OF CONTENTS**

DECLARATION ..... ii
CERTIFICATION ..... iii
List of Tables ..... viii
List of Figures ..... viii
List of Abbreviations ..... ix
ABSTRACT ..... x
INTRODUCTION ..... 2
CHAPTER ONE ..... 2
1.1 Background ..... 2
1.2 Problem Statement ..... 4
1.3 Main Objective ..... 4
1.4 Specific Objectives ..... 4
1.5 Research Questions ..... 5
1.6 Justification ..... 5
1.7 Conceptual Framework ..... 6
1.7.1 Description of the conceptual framework ..... 6
1.8 Scope of study ..... 7
CHAPTER TWO ..... 8
LITERATURE REVIEW ..... 8
2.1 Parkinson's Disease and Parkinsonism ..... 8
2.2 Behavioural Deficits in Parkinson's disease ..... 8
2.2.1 Motor Deficits in Parkinson's disease ..... 9
2.2.2Anxiety ..... 9
2.2.3 Olfactory Disturbances ..... 10
2.3 Oxidant and Antioxidant Imbalance in Parkinson's disease ..... 10
2.4 Rotenone Model of Parkinsonism ..... 11
2.5 Zinc and Neurodegenerative disorders ..... 12
2.6 Neurological Effects of Omega-6 PUFA ..... 12
CHAPTER THREE ..... 14
MATERIALS AND METHODS ..... 14
3.0 Study design ..... 14
3.1 Materials and Methods ..... 14

3.2.1 Behavioural Assessment ..... 15
3.2.2 Locomotor Assessment ..... 15
3.2.3 Anxiety Assessment ..... 16
3.3 Methodology for Objective Two ..... 18
3.3.1 Brain tissue Processing ..... 18
3.4 Brain Oxidative Stress Marker Assessment ..... 18
3.4.1 Brain Lipid Peroxidation ..... 18
3.4.2 Brain Total Antioxidant Capacity ..... 18
3.4.3 Brain Reduced Glutathione ..... 18
3.4.4 Brain Superoxide Dismutase Activity ..... 19
3.4.5 Brain Catalase Activity ..... 19
3.6 Statistical Analysis ..... 20
3.7 Ethical considerations ..... 20
3.8 Limitations ..... 21
CHAPTER FOUR ..... 22
RESULTS ..... 22
4.1 Observations of effects of rotenone treatment ..... 22
4.1.1 Motor responses ..... 22
4.2 Percentage Survival ..... 22
4.3 Behavioral Assessment ..... 22
4.3.1. Locomotor Assessment ..... 22
4.3.1.1. Latency of movement initiation ..... 24
4.3.1.2. Ambulation frequency ..... 25
4.3.1.3. Freezing Duration ..... 26
4.3.1.4 Grooming Duration ..... 27
4.3.1.5. Rearing frequency ..... 28
4.3.2 Anxiety Assessment ..... 29
4.3.3. Assessment of Olfactory Acuity ..... 29
4.3.4 Assessment of olfactory discrimination ..... 30
4.4 Biochemical Measurements ..... 31
4.4.1 Lipid Peroxidation by MDA measurement ..... 31
4.4.2. Total Antioxidant Capacity ..... 33
4.4.3. Reduced Glutathione Concentration. ..... 34

4.4.4 Superoxide dismutase. ..... 35
4.4.5 Glutathione Peroxidase ..... 36
4.4.6. Catalase ..... 37
4.5 Histopathology ..... 38
CHAPTER FIVE ..... 40
DISCUSSION, CONCLUSION AND RECOMMENDATIONS ..... 40
5.1 Discussions ..... 40
5.2 Conclusions ..... 45
5.3 Recommendations ..... 45
REFERENCES ..... 46

## List of Tables

Table 1: Showing effect of Zinc and Linoleic acid and their combination on parameters of open field test ..... 23

Table 2: Effect of Zinc and Linoleic acid and their combination on parameters of elevated plus maze ... 29

Table 3: Effect of Zinc and Linoleic acid and their combination on parameters of novel scent test ..... 30

Table 4: Effect of Zinc and Linoleic acid and their combination on parameters of block test ..... 30

Table 5: Effect of Zinc and Linoleic acid and their combination on LPO and the antioxidant system enzymes in mid brain of rotenone treated rats. ..... 31

## List of Figures

Figure 1: Showing study conceptual framework ..... 6

Figure 2: Showing picture of open field apparatus ..... 16

Figure 3: Effect of Zinc, Linoleic acid and their combination on the latency for initiation of movement by rats ..... 24

Figure 4: Effect of Zinc, Linoleic acid and their combination on the ambulation frequency of rats ..... 25

Figure 5: Effect of Zinc, Linoleic acid and their combination on inactive sitting duration of rats. ..... 26

Figure 6: Effect of Zinc, Linoleic acid and their combination on the grooming duration of rats. ..... 27

Figure 7: Effect of Zinc, Linoleic acid and their combination on rearing frequency of rats. ..... 28

Figure 8: Lipid Peroxidation (MDA) levels in the midbrain of experimental groups ..... 32

Figure 9: Total Antioxidant Capacity (TAC) levels in the midbrain of experimental groups. ..... 33

Figure 10: Reduced Glutathione levels in the midbrain of experimental groups. ..... 34

Figure 11: Superoxide Dismutase level in the midbrain of the experimental groups. ..... 35

Figure 12: Glutathione Peroxidase activity in the midbrain of experimental groups. ..... 36

Figure 13: Catalase activity in the midbrain of experimental groups ..... 37

Figure 14: Histopathological changes in the mid brain of rats stained with haematoxylin and eosin ..... 39

**List of Abbreviations**

| | |
|---|---|
| ÅAP | 4-aminophenazone |
| AchE | Acetylcholinesterase |
| ANOVA | Analysis of Variance |
| ATP | Adenosine Triphosphate |
| CAT | Catalase |
| DA | Dopamine |
| DHBS | 3,5-dichloro-2-hydroxy-benzene sulfonic acid |
| DNA | Deoxyribonucleic acid |
| DTNB | Dithiobis-2-nitrobenzoic acid |
| EPM | Elevated Plus Maze |
| GI | Gastrointestinal |
| GPi | Globus Pallidus internal |
| GSH | Reduced Glutathione |
| I.P | Intra-peritoneal |
| LPO | Lipid peroxidation |
| MDA | Malondialdehyde |
| MPP+ | 1-methyl-4-phenylpyridinium ion |
| PTP | Methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine |
| MT | Metallothionein |
| NO | Nitric Oxide |
| Non-DA | Non-Dopamine |
| NSAID | Non-steroidal anti-inflammatory drug |
| PD | Parkinson's disease |
| PDD | Parkinson's disease and Dementia |
| PUFA | Polyunsaturated Fatty Acids |
| REM | Rapid Eye Movement |
| ROS | Reactive Oxygen Species |
| SE | Standard Error |
| SN | Substantia Nigra |
| SNpc | Substantia nigra pars compacta |
| SOD | Superoxide Dismutase |
| TAC | Total Antioxidant Capacity |
| TBA | Thiobarbituric acid |

## ABSTRACT

**Introduction:** Studies have investigated the neuroprotective effects of either zinc or linoleic acid but none has investigated the combined effects. Little is known about the behavioural effect of either zinc or linoleic acid or their combination in the management of Parkinson's disease.

**Aim**: This study was designed to investigate the neuroprotective effects of zinc and linoleic acid in rotenone-induced Parkinsonism in rats.

**Materials and Methods**: Thirty six young adult female rats weighing 100-150 grams divided into six groups were used. Rats were induced with Parkinsonism by subcutaneous administration of rotenone (2.5mg/kg) once a day for seven consecutive days. The rats received DMSO/Olive oil or rotenone dissolved in (Dimethyl sulfoxide) DMSO/Olive oil. Groups III and IV received zinc (30mg/kg) or linoleic acid (150µl/kg) while group V received a combination of both, two weeks prior to rotenone injection. Groups II and VI served as negative (rotenone group) and positive (Levodopa groups) controls respectively. Measurement and analysis of behavioural function in rats employed a battery of tests including elevated plus maze (EPM), open field test (OFT), novel scent and block tests. The oxidative stress levels were assessed by estimating Lipid peroxidation, Total Antioxidant Capacity, Superoxide Dismutase, reduced Glutathione, Glutathione Peroxidase and Catalase in the midbrain. Histological examination was done to assess structural changes in the midbrain

**Results**: Rats receiving rotenone displayed bradykinesia and motor impairment in the OFT, anxiety, decrease in olfactory acuity and discrimination in EPM, FST and Novel Scent Test respectively. In addition, histological examination revealed that parkinsonian rat brains exhibited neuronal damage. There was a significant reduction in lipid peroxidation and improvement in the antioxidant status in intervention-treated groups. The significant increase in postural instability, impaired motor activity/coordination, increase anxiety and the decrease in rearing behaviour caused by rotenone induction was attenuated significantly by treatment with zinc and linoleic acid, but not their combination.

**Conclusion**: These results suggest that zinc and linoleic acid and their combination showed significant neuroprotective activity most likely due to the antioxidant effect.


Keywords: Anxiety, antioxidant, olfactory deficit, sensorimotor assessment, ageing

# CHAPTER ONE

# INTRODUCTION

## 1.1 Background

Parkinson's disease (PD) is a degenerative condition of the neurons that affects diverse brain regions, namely the red nuclei in the midbrain and brainstem, the cerebral cortex, the olfactory tubercle, and some parts of the peripheral nervous system (Kalia and Lang, 2015; Muangpaisan, Hori, and Brayne, 2009). These changes result into motor impairments together with several non-motor symptoms (Braak *et al.*, 2004). Parkinsonism is a general term that refers to neurological disorder that causes movement disorders. These disorders include supranuclear palsy, vascular Parkinsonism, dementia with Lewy bodies, corticobasal degeneration and drug induced-Parkinsonism (Shin and Chung, 2012). The pathophysiology of the latter is related to drug-induced changes in the basal ganglia motor circuit secondary to dopaminergic neuron destruction (Hirose, 2006). The consequence of nigro-striatal fibre degeneration is a loss of nerve endings in the striatum which contain tyrosine hydroxylase, and thus a reduced production of dopamine in the striatum. This produces tremor bradykinesia, rigidity and postural instability (Hallett, 2014).

The aetiology of PD is considered to be associated with environmental exposures to various factors and genetic mutations (Elbaz and Tranchant, 2007). Suggested environmental factors related to the aetiology of PD include exposure to herbicides and pesticides, intake of contaminated well-water and neurotoxins for example rotenone. Rotenone inhibits the Fe-S centres in the complex-I of the mitochondrial electron transport chain. The oxidative stress brings about death of dopaminergic neurons in the substantia nigra pars compacta. This brings about loss of nigral cells, decreased dopamine production in the striatum and thus deficits in behaviour (Meredith and Rademacher, 2011). None of these has however been identified as the one and only causative agent of PD (Elbaz and Tranchant, 2007; Nuti *et al.*, 2004). Genetically, parkin, LRRK2 and α-synuclein gene mutations have been described in familial PD in certain multiethnic populations (Lesage and Brice, 2009). Such mutations however are unusual in idiopathic early PD, which make up for a greater part of the people suffering from PD (Clark *et al.*, 2007; Madegowda, Kishore, and Anand, 2005).

Oxidative stress has been suggested as the common underlying mechanism in both sporadic and genetic cases of PD. This leads to cellular dysfunction (Henchcliffe and Beal, 2008). This maybe

reason why there is increased levels of oxidized DNA, proteins and lipids (Bosco *et al.*, 2006) in the substantia nigra of PD patients and low levels of glutathione (GSH) (Zeevalk, Razmpour, and Bernard, 2008). The initiators of these cascade of processes associated with generation of oxidative stress in the nigral dopaminergic neurons are thought to be the reactive oxygen species (ROS) produced during inflammation of the neurons, dysfunction of the mitochondria and metabolism of dopamine (Hwang, 2013).

Micronutrients and trace elements have been described as key components in the combat against these ROS and hence the onset and progression of neurodegenerative disorders (Blesa, Trigo-Damas, Quiroga-Varela, and Jackson-Lewis, 2015). Zinc homeostasis has been implicated in several processes related to brain aging and the onset and development of age-related neurodegenerative disorders. Serum Zinc did not significantly correlate with age of onset and duration of the disease in a study of Zinc status in PD patients (Zawada *et al.*, 2011) suggesting that Zinc does not play any role. It should however be noted that serum Zinc only represents less than 1% of total body amount and therefore cannot be used as a reliable method for determining Zinc status (Prasad, 2012b). Other studies have found that Zinc deficiency accompanies many cases of PD as shown by significant low levels of Zinc in the cerebrospinal fluid (CSF) of PD patients. Zinc has been shown to play an important role in protecting dopaminergic neurons against free radicals and toxins from the environment by stimulating metallothionein production (Samardzic *et al.*, 2013).

Another micronutrient essential for influencing signal transduction, neurochemistry, enzymes, membrane proteins and gene expression is the omega-6 polyunsaturated fatty acids of which Linoleic acid is the major long chain PUFA (Calon and Cole, 2007). Omega-6 PUFAs also modulates transmission in cholinergic, serotoninergic and dopaminergic systems. Dopamine storage vesicle formation have been shown to significantly reduce in prolonged omega-6 PUFA deficiency (Zimmer *et al.*, 2000). Omega-6 PUFA is involved in inflammation, neurotrophic support, and oxidative stress through modulation of expression of genes responsible for that. Despite all these, the neuroprotective effects of omega-6 PUFA in PD are yet to be investigated elaborately (Luchtman, Meng, and Song, 2012).

There is therefore the need to investigate the potentials of micronutrients and trace elements offering protective effects in the brain or slowing down the degeneration processes that occur in toxin-induced neurodegeneration.

## 1.2 Problem Statement

One point eight percent of persons above the age of 60 years have been estimated to be suffering from PD. Approximately 16-19 cases per 100,000 appear every year (Checkoway and Nelson, 1999). The reported low PD prevalence in Africa compared with the West, reflects a difference in susceptibility within the African populace (Blanckenberg, Bardien, Glanzmann, Okubadejo and Carr, 2013). Lack of information, adequate health personnel and facility for proper diagnosis and underreporting have been attributed to the reported low values (Chaudhuri and Schapira, 2009). With increase use of antipsychotic and psychostimulant drugs among youths, there is increase risk of incidence of Parkinsonism, as toxin-induced PD has also been observed in individuals younger than 40 years (Erro, Bhatia, and Tinazzi, 2015; Morley *et al.*, 2014).

Current treatment of Parkinson's disease (PD) is based on dopamine replacement therapy, but chronic administration may cause motor fluctuations and dyskinesias, increased free radical formation and accelerating neuronal degeneration in some PD patients. Studies in rodents showed that the Levodopa/carbidopa combined therapy does not offer neuroprotection as well (Fox and Brotchie, 2014; Vijayakumar and Jankovic, 2016). Several agents have been investigated for neuroprotection against development of Parkinson disease or for slowing down the degeneration of dopaminergic neurons (Ojha, Javed, Azimullah, Khair, and Haque, 2015; Patil, Jain, Ghumatkar, Tambe, and Sathaye, 2014a); none has yet successfully offered neuroprotection. A combination of a micronutrient (linoleic acid) and a trace element (zinc) may improve mitochondrial function, prevent oxidative injury, protect dopaminergic cells and improve behavioural deficits in Parkinsonism.

## 1.3 Main Objective

The aim of the study is to determine the neuroprotective effects of zinc and linoleic acid against the development of rotenone-induced Parkinsonism in rats.

## 1.4 Specific Objectives

**1.4.1** To determine the effect of pre-treatment with zinc and linoleic acid and their combination on the behaviour of rats in rotenone-induced Parkinsonism in rats.

**1.4.2** To determine the effect of pre-treatment with zinc and linoleic acid and their combination on brain antioxidant markers in rotenone-induced Parkinsonism in rats.

**1.4.3** To determine the effect of pre-treatment with zinc and linoleic acid and their combination on the histopathological changes in the midbrain of rats with rotenone-induced Parkinsonism

## 1.5 Research Questions

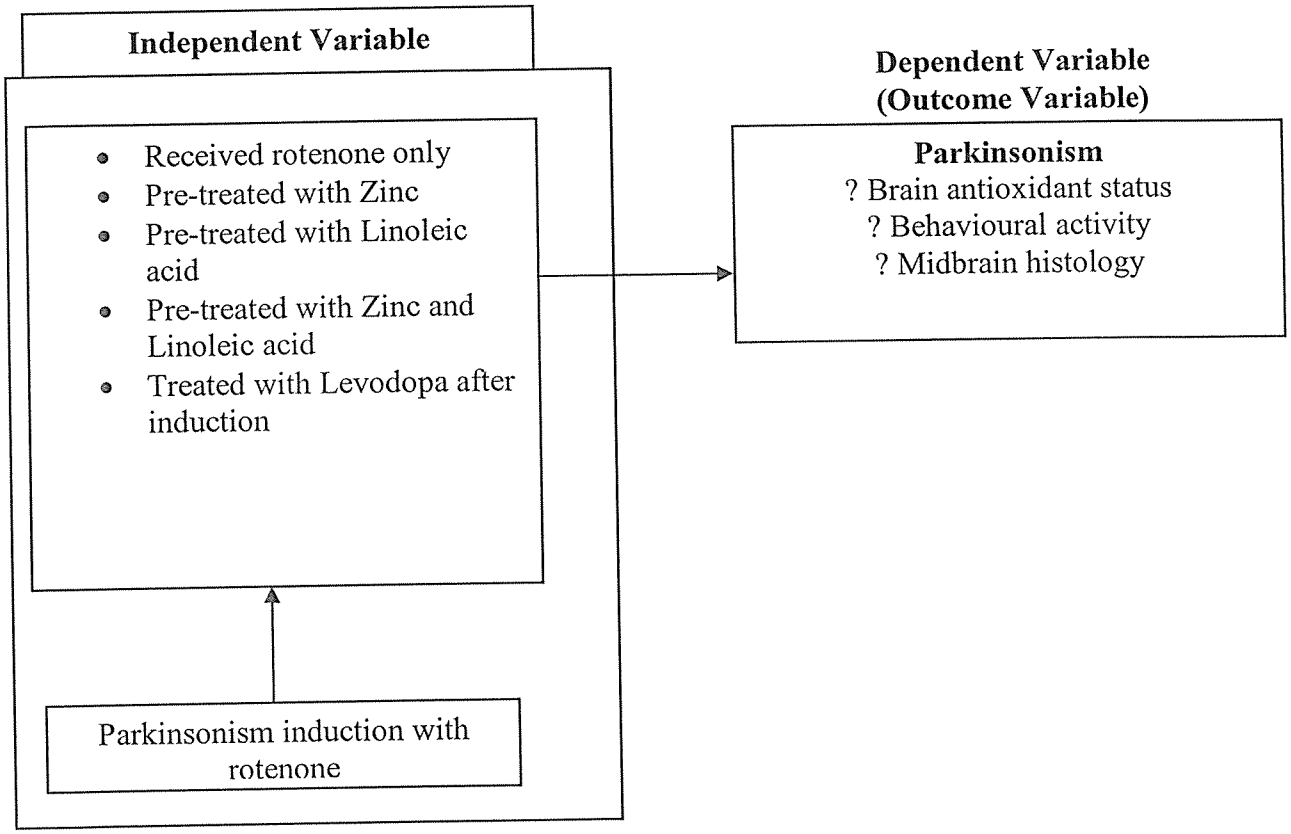
1. What are the effects of pre-treatment with zinc, linoleic acid or their combination on behaviour in rat model of Parkinsonism induced with rotenone?
2. What are the effects of pre-treatment with zinc, linoleic acid or their combination on brain antioxidant status in rat model of Parkinsonism induced with rotenone?
3. What are the effects of pre-treatment with zinc, linoleic acid or their combination on midbrain histological changes in rat model of Parkinsonism induced with rotenone?

## 1.6 Justification

Zinc has been used as a protective agent in the management of diarrhoea (Abdullah *et al.*, 2006), acute lower respiratory infection (Sazawal *et al.*, 1998) and male infertility (El-Tawil, 2003). Linoleic acid, a nutraceutical has been shown to protect the brain from stroke (Iso *et al.*, 2002) and dementia (Sydenham, Dangour, and Lim, 2012), yet their protective effect against the development of PD has not been investigated. The choice of zinc for its use in PD is motivated by significant low levels of zinc in the cerebrospinal fluid (CSF) of PD patients in several studies. Chronic Linoleic acid deficiency significantly decreases formation of vesicles that store dopamine (Zimmer *et al.*, 2000).

It is known that dysfunction of the mitochondria and oxidative injury is the basis for degeneration of neurons in PD; use of modifiers of metabolism may provide alternatives for early intervention. In order to investigate this, a toxin-induced model of Parkinson's disease was used to assess the protective effect of zinc and linoleic acid. Zinc and linoleic acid or their combination may provide a potential neuroprotective treatment aimed for use as xenobiotic interventions to slow the progressive nature of PD.

## 1.7 Conceptual Framework



### 1.7.1 Description of the conceptual framework

Rats pre-treated with zinc, linoleic acid or their combinations for a period of time were then challenged with a toxin that induces Parkinsonism in rats. The brain antioxidants status in normal rats, as indicated by the levels and activity of antioxidant markers served as control and were considered to be in normal ranges. Behaviour was assessed by assessing locomotor activity, anxiety and olfactory function. Induction of Parkinsonism by administration of rotenone decreases the brain antioxidant status in untreated rats. Pre-treatment of rats with zinc and or linoleic acid may improve the brain antioxidant status and behavioural deficits. Treatment with Levodopa improves locomotor activity, brain antioxidant status, but not all of the behavioural parameters.

## 1.8 Scope of study

This study which involved the investigation of the neuroprotective effects of zinc and linoleic acid in laboratory rats induced with Parkinsonism using rotenone was carried out in a time span of 3 weeks from February 10$^{th}$ to March 5$^{th}$, 2017. The study set-out to investigate the behavioural effects on living rats and biochemical and histological effects on isolated rat brain. The brain region used for biochemical and histological assessment was the midbrain. Behavioural assessment was limited to only locomotor, anxiety and olfaction (detection and discrimination).

# CHAPTER TWO

# LITERATURE REVIEW

## 2.1 Parkinson's Disease and Parkinsonism

Parkinson's disease is a common neurodegenerative disease first described in 1817 by James Parkinson (Parkinson, 2002). Bradykinesia, rigidity, tremors at rest and postural instability have been observed as hallmark clinical features (Halliday *et al.*, 2006). Despite these prior descriptions, the causation of PD is still unclear. Several genes implicated in familial forms of PD (which are rare) have been identified in the last many years (Veeriah *et al.*, 2010). This has revealed new pathways and proteins that may produce degeneration of the dopaminergic neurons and also a clinical Parkinsonian syndrome. Parkinsonism is a general term that refers to neurological disorder that causes movement disorders. These disorders include supranuclear palsy, vascular Parkinsonism, dementia with Lewy bodies, corticobasal degeneration and drug induced-Parkinsonism (Shin and Chung, 2012). The pathophysiology of the latter is related to drug-induced changes in the basal ganglia motor circuit secondary to dopaminergic neuron destruction (Hirose, 2006). It should be noted that the preclinical phase of PD appears quite long even before the degeneration of dopamine producing neurons in the substantia nigra (Braak *et al.*, 2004), suggesting that assessment of symptoms within this phase can increase knowledge of the disease process.

## 2.2 Behavioural Deficits in Parkinson's disease

The diagnosis of PD is usually never until over 80% of the dopaminergic neurons in the striatum are lost (Kalia and Lang, 2015). Decrease dopamine levels in the striatum follows death of neurons in the substantia nigra pas compacta (SNPc). This decrease in dopamine levels results in a loss of voluntary movements. The appearance of tremors, rigidity, bradykinesia and postural instability have been a large part of the basis of behavioural testing in rodent models of PD (Chaudhuri and Schapira, 2009). The appearance of non-motor symptoms (nausea, depression, olfactory disturbance, anxiety) in PD patients well before the motor symptoms has greatly influenced the modelling of PD in animals.

### 2.2.1 Motor Deficits in Parkinson's disease

PD is an age-related progressive neurodegenerative disease that manifest clinically with tremor at rest, bradykinesia, rigidity and postural instability as cardinal motor manifestations (Kalia and Lang, 2015). Following degeneration of dopaminergic neurons in the SNPc that project into the striatum, several biochemical and electrophysiological changes can be predicted. Many of these changes have been confirmed by findings obtained from in vivo and in vitro animal models and humans (Goldman and Postuma, 2014a). Progressive neurodegeneration also impact non-dopamine neurotransmitter systems such as cholinergic, noradrenergic, and serotonergic systems (Wright and Harding, 2012) and their overall effect is to decrease motor activity. These classic signs are not presented by every patient all at the same time. Muscle stiffness or motor weakness is often the first signs, followed by postural instability and tremors. There are often risks of misdiagnoses which are done on the basis medical history and neurologic examination (Suttrup and Warnecke, 2016). Tremor at rest which occur in approximately 70% of PD patients is one of the most common characteristic features (Hallett, 2014). A prolonged absence of tremor in patients may be suggestive of signs of parkinsonism such as degeneration, multiple system atrophy, supranuclear palsy and others (Martinu and Monchi, 2013). Rigidity is stiffness in the skeletal muscles often appreciated on examination and is detected as a resistance to passive movement of the limbs. Bradykinesia which can be observed in the absence of limb rigidity refers to a slowness and paucity of movement. Postural instability is the most potentially dangerous, because it can lead to falls with resulting fractures (Politis and Niccolini, 2015).

### 2.2.2Anxiety

Approximately 40% of patients with PD are anxious or depressed. The reason for this is however poorly understood (Cummings, 1992). Depressed PD patients have been observed with corresponding loss of noradrenergic and serotonergic projections (Gjerstad, Alves, Wentzel-Larsen, Aarsland, and Larsen, 2006). Mood fluctuations observed in PD patients have also been observed to occur independent of motor deficits and are often improved by medications used for managing PD (Czernecki *et al.*, 2002; Poewe, 2008). Anxiety-like behaviours have been measured effectively in rats using light-dark exploration, open field test and the elevated plus maze (EPM) (File, Baldwin, Johnston, and Wilks, 1988; Paine, Jackman, and Olmstead, 2002).

### 2.2.3 Olfactory Disturbances

Disturbances in sense of olfaction are one of the earliest non-motor symptoms observed in PD. Deficits in odour detection, differentiation, and identification have been observed in PD patients (Doty, 2012; Tissingh *et al.*, 1998). The buried pellet test has been used to measure general olfactory function in rodents. This test relies on the rodent locating an object that is hidden, most often a food pellet; by odour and measuring the time an animal that has been restricted from food takes to find the food pellet (Fleming *et al.*, 2008; Nathan *et al.*, 2004). The rodent's ability to discriminate between odours is crucial for feeding, social communication and mating organization. It is also crucial for the processes of learning and memory related with these behaviours (Mackay-Sim and Dreosti, 1989). The block and novel scent tests have been used to measure olfactory discrimination and acuity which are simple ways to quantify time spent sniffing/exploring a new odour (Doty, Shaman, Kimmelman, and Dann, 1984a; Goldman and Postuma, 2014). Disturbances in the sense of olfaction occur in a similar frequency to resting tremor and are not responsive to dopaminergic therapies. Behavioural testing of olfactory disturbances can ease earlier detection of PD, since deficits in olfaction has been positively linked with an high risk of developing PD (Doty, 2012).

## 2.3 Oxidant and Antioxidant Imbalance in Parkinson's disease

Animals as well as humans metabolize biofuels using oxygen which has the potential of being part of damaging molecules called free radicals. These molecules attack healthy body cells and have been involved in the pathogenesis of several diseases (Ames, Shigenaga, and Hagen, 1993). The body however has a natural defence system called the antioxidant system that wards off the oxidant system triggered by these free radicals. When the oxidant assault, to the body overwhelms this inherent antioxidant system, damage sets in. This damage is cumulative.

There is evidence of the interaction between environmental toxins and internal toxins arising from normal metabolism and genetic components (Parron, Requena, Hernandez, and Alarcon, 2011). Oxidative stress is a major intermediary factor that could initiate neurone degeneration or its progression.

Studies conducted in PD patients showed increased levels of superoxide dismutase and lipid peroxidation, decreased glutathione levels with no change in glutathione reductase and glutathione peroxidase (Sharma, Kaur, Kumar, Prabhakar, and Gill, 2008; Vinish, Anand, and Prabhakar, 2011). Sharma and collaborators (2008) observed significant levels of plasma

thiobarbituric acid and nitric oxide but significantly low levels of superoxide dismutase (SOD), glutathione reductase (GPX), Catalase (CAT), selenium, copper and zinc in PD patients in comparison to the control subjects. The conclusions of their study suggested that increased oxidative stress plays a vital role in loss of dopaminergic neurons in the SNPc and thus in the pathogenesis of PD.

Evidence suggests that damage to the nigral neurons by free radicals which are very cytotoxic are generated under normal and pathological conditions (Jenner and Olanow, 2006; Simonian and Coyle, 1996). The most common cellular free radicals are hydroxyl radical (OH•), superoxide radical ($O2^{-}$ •), and nitric oxide (NO•). Exposure of cell membranes to these free radicals stimulates the process of lipid peroxidation. The various classes of macromolecules are vulnerable to attack by these cytotoxic free radicals but lipids are the most susceptible. Cell membranes are rich sources of fatty acids, which are prone to attack by free radicals, whose destruction is most damaging as once begin it proceeds in a self-perpetuating chain reaction ( Sharma *et al.*, 2008).

Oxidative stress in PD patients may be as a result of diminished efficiency of endogenous antioxidant systems. We should note that under normal conditions, the continuously produced free radicals are always combated by antioxidant enzymes of which SOD, CAT and GPx are the major ones present in the body. SOD catalyses the breakdown of superoxide anion to $O_2$ and $H_2O_2$. Peroxides are also destroyed by GPx or CAT reactions (Fridovich, 1995; Sun *et al.*, 1995; Teixeira, Schumacher, and Meneghini, 1998).

CAT reacts with $H_2O_2$ to form $H_2O$ and $O_2$ and with $H_2$ donors. CAT protects cells from $H_2O_2$ generated within them by detoxifying them. On the other hand, GPx uses reduced GSH to reduce $H_2O_2$. The cells are thus protected against. The metabolism of glutathione has been described as the most vital defence mechanism in combat against oxidants (Grazioli *et al.*, 1998; Rikans and Hornbrook, 1997; Sigalov and Stern, 1998).

### 2.4 Rotenone Model of Parkinsonism

An ideal PD model will be one that reproduces all relevant pathological and clinical features. It should be one that can produce the progressive pattern of PD as occurs in humans. There is hardly any of such in an animal model, notwithstanding, the rotenone model stands out in the modelling of PD pathogenesis (Liu, Li, Yang, and Smith, 2013) . In rotenone model, inhibition of complex I in the mitochondrial respiratory chain; produces selective degeneration of

nigrostriatal dopaminergic neurons. This reproduces to variable extents key pathological features of clinical PD (Fleming *et al.*, 2004).

Due to its mechanism of action, the Rotenone rat model is useful for studying dysfunctions of the mitochondrial dysfunction in toxin-induced Parkinsonism (Dauer and Przedborski, 2003; Dawson and Dawson, 2003). Administration of Rotenone affects several of the pathways including: Lewy pathology, phosphorylation and aggregation, proteasomal dysfunction, oxidative stress and alpha-synuclein accumulation (Greenamyre, Betarbet, and Sherer, 2003; Sherer *et al.*, 2003; Sherer, Betarbet, and Greenamyre, 2005). Variability in the percentage of animals that develop lesions in the dopaminergic neurons in the SNPc and striatum has been a major limitation of the Rotenone model (Cannon *et al.*, 2009).

### 2.5 Zinc and Neurodegenerative disorders

Zinc is very essential in the formation, development and functioning of the brain. Its role as a co-factor in enzymes, and stimulation of several genes have been linked to processes involved in neurotransmission, and metabolism of dopamine (Prasad, 2012a). Glutamatergic neurons have been shown as zinc abundant neurons in the brain. These are majorly found in the prefrontal lobe. The synthesis of metallothionein which has been described to acting as a scavenger of free radicals and metals was shown to be stimulated by zinc (Samardzic *et al.*, 2013).

The physiological functions of zinc may thus occur through the action of metallothionein. Besides being synthesized in response to zinc changes it also modulates the expression of zinc-dependent genes activating the antioxidant enzymes system(Bastianetto and Quirion, 2004). Low levels of zinc have been implicated in several behavioural deficits including hyperactivity, jitters anorexia and depression (Mocchegiani, Malavolta, Marcellini, and Pawelec, 2006).

### 2.6 Neurological Effects of Omega-6 PUFA

Visual function and development of the central nervous system have been tied to available sufficient amounts of polyunsaturated fatty acid (PUFA). They also play key roles in neurotransmissions in the cholinergic, serotoninergic and dopaminergic systems. It has been observed that in conditions of prolonged omega-6 PUFA deficiency, the formation of storage vesicles for dopamine (Zimmer *et al.*, 2000), and dopamine levels in the rat cortex in decreased (Delion *et al.*, 1994). Supplementation of the rats described in the study of Delion *et al.* (1994), increased dopamine levels in the rat frontal cortex area. Despite all these, sufficient evidence

describing the function of omega-6 PUFA in PD treatment has been lacking (Luchtman *et al.*, 2012).

# CHAPTER THREE

# MATERIALS AND METHODS

## 3.0 Study design

This was an experimental study which used laboratory rats with rotenone induced Parkinsonism as a model to evaluate the protective effect of zinc and linoleic acid.

## 3.1 Materials and Methods

Thirty six young adult female rats weighing 100-150 gms (Patil, Jain, Ghumatkar, Tambe, and Sathaye, 2014b), and 8-12 weeks of age (as these are 1-2 fold more sensitive to rotenone) were used (Gupta *et al.*, 1986). They were obtained from the animal house Department of Biochemistry and Physiological Sciences, University of Yaounde I, Cameroon and acclimated in a room at temperature of 25 ± 1 °C (Hawkins, 2002). The rats were housed with *ad libitum* access to water and food. All rats received the same batch food prepared by the animal house coordinator. The rat food had the following composition: cornmeal (60%), corn (10%), boiled and dried soya beans (12.8%), fish (12%), bones (1.3%), palm oil (3%), vitamin complex (0,1%) and cooking salt (0.8%).

The animals were grouped into six groups of 6 rats each as follows:

Group I served as the normal control and received Olive oil/Dimethylsulphoxide (DMSO) (which was the vehicle used) once a day through the study period of 3 weeks.

Group II served as the negative control and as described by Fujikawa *et al.* (2005) and Ojha *et al.* (2015) received 2.5mg/kg of rotenone subcutaneously once a day consecutively for the last seven days of the study.

Group III received zinc (30 mg/kg b.w.) according to Partyka, Jastrzebska-Wiesek, and Nowak (2010) and Anna Partyka *et al.* (2011) orally for 3 weeks in drinking water once per day and rotenone (2.5mg/kg) once a day for the last seven consecutive days.

Group IV received a daily dose of linoleic acid (150µg/kg) subcutaneously for 3 weeks as described by Becalski, Feng, Lau, and Zhao (2012) once per day and rotenone (2.5mg/kg) once a day for the last seven consecutive days.

Group V received both Zinc (30mg/kg) and Linoleic acid (150µg/kg) orally and subcutaneously once per day for 3 weeks respectively and rotenone (2.5mg/kg) once a day for the last seven consecutive days

Group VI served as a positive control and received Sinemet ® (Levodopa/Carbidopa 100mg/25mg) (6mg/kg) orally once per day according to Shi *et al.* (2015) with rotenone (2.5mg/kg) once a day consecutively for seven days.

**Induction of Parkinsonism by Rotenone**

The rotenone solution was first prepared as a 50X stock in 100% DMSO by dissolving 125mg of rotenone in 1 ml of DMSO. From the stock solution (125 mg/ml), 40µl was then diluted in 1960 µl of olive oil. The solution was vortexed to create an emulsion of the DMSO containing rotenone and Olive oil. Fresh solution was prepared every 2-3times in a week. Rotenone is sensitive to light, so it was stored in small vials and protected from light by wrapping the vials with foil papers and kept in refrigerator. Before administering the rotenone solution to rats, the vials were inverted several times or vortexed to obtain a uniform mixture with the DMSO/Olive oil. Each rat received a volume of 1 ml/kg of Rotenone and normal control animals received the vehicle only (Olive oil/DMSO) (Ojha *et al.*, 2015).

## 3.2 Methodology for Objective One

### 3.2.1 Behavioural Assessment

Behavioural function in rats was measured using a series of tests including; the elevated plus maze which assessed anxiety behaviour, open field test assessed exploration and locomotor ability as well as anxiety. The novel scent and block tests assessed olfactory acuity and olfactory discrimination. Data collection instruments included stop watch timer, video recorder, cages, and vanilla syrup, small blocks, elevated plus maze and wooden open field box.

### 3.2.2 Locomotor Assessment

The Open Field Test provides a means for measuring exploration, locomotion and anxiety in rodents (Walsh and Cummins, 1976). The dimensions of the open field maze were 72 x 72 cm with 36 cm walls. The large open field was used for measuring exploration as well as locomotion.

Rats were exposed to the apparatus for 5 minutes for 2 days consecutively to assess habituation to the new environment. Rats were placed into the centre or at the corners and were allowed to explore the space for 5 minutes. According to Stanford (2007) the behaviours scored include: latency of movement initiation, which indicates the time taken for the rat to initiate mobility

when introduced into the field and is a measure of the rat's exploration; the ambulation frequency, recorded as line crossing, is the number of times the rat crossed each of the lines on the open field with all four limbs. Rearing is the number of times the rat stood on their hind legs in the open field, was used as a measure of movement activity, but is also a measure of exploration and anxiety. Grooming is a displacement behaviour a rodent expresses in a new environment and is measured as the time an animal spends scratching or licking itself. Freezing is the time period in which the rat was completely not in motion.

The ambulation and rearing frequencies were used as measures of locomotor activity, but also measures of exploration and anxiety (Carola, D'Olimpio, Brunamonti, Mangia, and Renzi, 2002; Gould, Dao, and Kovacsics, 2010). Grooming and freezing were used as measures of exploration and anxiety. The horizontal movement (ambulation frequency and freezing) and the vertical movement (rearing frequency) were registered (Paniz *et al.*, 2014; Stanford, 2007).

### 3.2.3. Anxiety Assessment

Anxiety assessment was done using the elevated plus maze (Hogg, 1996). The time spent in the open and closed arms are assessed by this test and are used as measures of anxiety. Behaviour in this task reflects a conflict between the rodent's preference for protected areas and their innate motivation to explore novel environments. The elevated plus maze relies upon the rat's inclination toward dark, enclosed spaces and a natural fear of heights and open spaces (Carola *et al.*, 2002; Hogg, 1996; Holmes, Parmigiani, Ferrari, Palanza, and Rodgers, 2000).

**Figure 2: Showing picture of open field apparatus**

The elevated plus maze (EPM) apparatus was made locally by the researcher according to specifications of Hogg (1996) which consisted of two open arms without walls and two arms closed by walls about 30cm high. The arms are 50 cm long and 10 cm wide. Each rat was placed at the junction of the four arms and number of entries and the total duration of entries into each arm were recorded for 5 minutes. The maze was cleaned with 70% alcohol and dried. The behavioural parameters recorded were: numbers of entries into the open and closed arms; time spent in the open and closed arms. Open arm time was used as an index of anxiety-like behaviour, while number of entries in closed arms was considered as a measure of locomotor activity (Guimarães, Chiaretti, Graeff, and Zuardi, 1990). A rat was considered to have entered an arm when all its four paws were on that arm.

### 3.2.4 Assessment of Olfactory Acuity

Disturbances in sense of olfaction are one of the earliest non-motor symptoms observed in PD patients. This include deficits in odour detection, differentiation, and identification (Tillerson *et al.*, 2006). Olfactory acuity was measured using the novel scent test which is a simple way of quantifying time spent sniffing a new odour (Doty *et al.*, 1984a; Doty, 2012). Each rat was presented small quantities of vanilla and water simultaneously (Taylor *et al.*, 2009a). Time spent sniffing each odour was recorded for a three minute session. Time spent sniffing the vanilla indicated a functional olfactory system to detect new odours. The frequency of sniffing also indicated the exploratory ability to novelty.

### 3.2.5 Assessment of Olfactory Discrimination

The block test was used to evaluate the ability of rats to discriminate between social odours, specifically self and non-self (Taylor *et al.*, 2009b; Tillerson *et al.*, 2006). Each rat was presented with a block smeared with its own bedding and a block smeared with faecal matter of another rat of the same sex. The time spent in contact with each block was recorded for a two minute period (Tillerson *et al.*, 2006). More time spent sniffing the bedding of another rat indicates social attraction, cohesion and novelty, all which points to the rat's ability to discriminate between self as well as social groups. This ability is crucial for feeding, social communication and mating. It is also crucial for the process of learning and memory that are come along with these behaviours.

### 3.3 Methodology for Objective Two

#### 3.3.1 Brain tissue Processing

At the end of the experiment, the rats were fasted overnight and humanely sacrificed by cervical dislocation without anaesthesia. The whole brain of each rat was rapidly dissected, removed and rinsed in ice-cold isotonic saline. The whole brain was weighed and immediately the midbrain region was isolated and this isolated brain section was used for further investigations. Portions of the midbrain were removed to use for histological assessments. The remainder midbrain tissues were homogenized with ice-cold 0.1 M phosphate buffer saline (pH 7.4). Tissue homogenate (10% w/v) was prepared in 50 mM phosphate buffer saline (pH 7.4) using a Symphony type Eperndorff homogenizer. The brain homogenate was centrifuged at 2,000g for 10 min at 4°C. The pellet which contained debris and nuclei was discarded. The supernatant was then again centrifuged at 12,000g for 20 min to obtain post mitochondrial supernatant. The supernatant was kept at −80 °C until determination of the levels and activity of oxidative stress markers (Kaur, Chauhan, and Sandhir, 2011)

### 3.4 Brain Oxidative Stress Marker Assessments

#### 3.4.1 Brain Lipid Peroxidation

The method described by Stocks, Gutteridge, Sharp, and Dormandy (1974) was used to measure lipid peroxidation. A coloured complex called thiobarbituric acid reactive substance is formed from a reaction of malondialdehyde and thiobarbituric acid. The complex was assayed spectrophotometrically at 532 nm and expressed in micro moles.

#### 3.4.2 Brain Total Antioxidant Capacity

The Ferric Reducing Antioxidant Power (FRAP) was used as a method of measuring the total antioxidant capacity of the homogenate (Benzie and Strain, 1996). This method determines the capacity of the homogenate to reduce ferric iron to ferrous iron at a pH of 3.6. A deep blue coloured compound of ferrous tripyridyltriazine (TPTZ-Fe-2+) is formed as a result of the reduction of ferric tripyridyltriazide (TPTZ-Fe3+). The absorbance was measured at 593nm and the results were expressed in micromolar.

#### 3.4.3 Brain Reduced Glutathione

The method of Boyne and Ellman (1972) was used to estimate the level of reduced GSH in the brain homogenate. To precipitate the tissue proteins in the homogenate, it was mixed with 0.1 M

phosphate buffer (pH 7.4) and then added to equal volume of 20 % trichloroacetic acid containing 1 mM EDTA. The mixture stood for 5 min before it was centrifuged for 10 min at 2000 rpm. The supernatant was then transferred to a new set of test tubes, to which was added 1.8 ml of the Ellman's reagent (5, 5'-dithio bis2-nitrobenzoic acid). The reaction mixture was measured at 412 nm against blank.

### 3.4.4 Brain Superoxide Dismutase Activity

The method of Misra and Fridovich (1972) was used to assay the activity of superoxide dismutase. The method is based on the principle that super oxide inhibits the oxidation of adrenaline to adrenochrome. Each 3-ml of the reaction mixture contained 0.1-ml tissue homogenate, 2.8 ml of Potassium phosphate buffer (0.1 M, pH 7.4), and 0.1-ml pyrogallol solution (2.6 mM in 10 mM HCl). The pink product formed, adenochrome was detected in a spectrophotometer at 480 nm. The results were expressed as units/mg protein.

### 3.4.5 Brain Catalase Activity

The method of Sinha (1972) was used to assay catalase activity. A volume of 0.5 ml of the homogenate was added to the reaction mixture which contained 1ml of 0.01 M phosphate buffer (pH 7.0), 0.5 ml of 0.2 M $H_2O_2$, and 0.4 ml $H_2O$. The tubes were all heated at 95 °C for 10 minutes. To terminate the reaction, 2ml of dichromate/acetic acid mixture was added to it. To the control, the homogenate was added after the addition of acid reagent. The absorbance was read at 570 nm, the enzyme activity was expressed as micromoles of $H_2O_2$/ min/mg of protein

### 3.4.6 Brain Glutathione Peroxidase

A colorimetric assay kit was used to assay the activity of glutathione peroxidase (GPx) (Sigma-Aldrich, Germany). This test is based on the principle that reduction of organic peroxide by glutathione peroxidase produces oxidized glutathione which is immediately converted to its reduced form (GSH) at the same time oxidizing NADPH to $NADP^+$. The oxidation of NADPH was monitored spectrophotometrically using as a decrease in absorbance at 340 nm (Galażyn-Sidorczuk, Brzóska, Rogalska, Roszczenko, and Jurczuk, 2012).

### 3.4.7 Total Brain Protein

Quantitative estimation of brain homogenate total protein was carried out according to the Biuret method Gornall, Bardawill, and David (1949). This was done in order to quantify the concentration of proteins in the samples.

### 3.5 Methodology for Objective Three

3.5.1 Light microscopic examination

Small sections of each midbrain from the normal control and the various treated animals were fixed on 10% paraformaldehyde to assess for histological changes. Thin frozen brain sections of 30μm from were obtained using microtome. The tissues were dehydrated in ascending grades of alcohol following fixation, and were then embedded in wax. Approximately 5-7 μm thick paraffin sections were cut and then subjected to hematoxylin–eosin staining as described by Fischer, Jacobson, Rose, and Zeller (2005). Appearances of necrosis, apoptosis, size and quantity of cells were observed. The prepared slides were sent to the histopathology Department of the University of Yaounde, 1 Teaching Hospital for specialist observation and interpretation.

### 3.6 Statistical Analysis

Data of all the results was presented as mean ± SEM in tabular and graphic forms (bar graphs). The analysis of all the studies was done with the help of a one-way analysis of variance (ANOVA) followed by Tukey's test, to test the difference in means. Difference was considered statistically significant at the 95% confidence interval when compared with the rotenone group (Armitage, Berry, and Mathews, 2002). Graph pad Prism 6 software was used for statistical analyses program.

### 3.7 Ethical considerations

The ethical regulations were applied such that the animals suffered minimum pain at any stage of the experiment and the study was carried subject to approval by the Ethics Committees of Kampala International University Western Campus, Uganda and the University Of Yaounde 1 Cameroon. The animals were housed and acclimated in an animal house with room temperature of 25 ± 1°C (Stokes, 2002) with alternating cycles of night and day. To reduce variation and increase power of the experiment animals were housed in cages with fewer animals per cage. They were then randomly assigned to the treatment groups in order to reduce the possibility that one group had a different environment or was treated differently from another group. Animals were identified by their number rather than by their treatment groups (Festing and Altman, 2002).

Handling and restraining of the rats was carried out to ensure that the rats do not associate handling and restrain to stress and pain and thus react aversively. The animals were grasped gently at the base or center of the tail and placed on a surface such as the wire cage top or lid.

The rational was to avoid smooth surfaces as they might behave much more calmly if they have firm footing (Welfare, 2002). Subcutaneous injections were performed while holding the rat in the dorsal recumbent position and inserting the needle in a position below the skin; left or right of the midline, with the angle the needle approximately 45° to the body. Cervical dislocation in unanesthetized rats was used as the method to euthanize the animals and followed laid down guidelines according to AVMA Guidelines on Euthanasia (Leary, Underwood, Anthony, and Cartner, 2013).

### 3.8 Limitations

Financial constraints limited the study to only a few parameters among those known to assess behavioural impairment and oxidative and antioxidant status in PD. Dopaminergic neurodegeneration is best evaluated by using Nissl staining and tyrosine hydroxylase (TH)-positive cells in SNpc using immunohistochemistry, however due to limited resources these were not conducted. The variability in the parameters assessed due to changes in the estrous cycle of the rats could not be accounted and corrected. The study did not confirm the presence or absence of Lewy bodies which is a neuropathological hallmark of Parkinsonism. The inability to determine the blood levels of zinc and linoleic acid before initiating intervention limits the study. The absence of a baseline level or amount of zinc and linoleic acid in the blood and brain tissue of the rats limits objective quantification of the right doses of zinc and linoleic acid to administer to the rats. The amount of zinc and linoleic acid in the rat food would have also influenced the final amount of zinc and linoleic eventually administered.

# CHAPTER FOUR

# RESULTS

## 4.1 Observations of effects of rotenone treatment

### 4.1.1 Motor responses

In this present study, subcutaneous administration of rotenone (2.5 mg/kg) to rats consecutively for 7days produced functional deficits in the form of postural instability and bradykinesia in the open-field test and anxiety-related behaviors in the elevated plus maze. This was accompanied by histological changes and biochemical alterations in the neurons in the midbrain

## 4.2 Percentage Survival

Repeated treatment of rats in this study with rotenone resulted in morbidity and mortality in rats. Death began occurring from the $4^{th}$ day of administration of rotenone. The percentage survival of rats at the end of the experiment was found to reach 83% (5 out of 6). Death of rats occurred in the rotenone (01), zinc and Linoleic (01) and levodopa group (01). The survival of rats was not improved by treatment with Zinc and Linoleic acid as compared to rotenone group. From day 3, some animals showed severe weakness, apparent weight loss and locomotive inability. Animal death could be associated with the cardiotoxic and dysautonimic effects of rotenone (Sherer et al., 2003). Rats that died during the course of the experiment were excluded from statistical analysis.

## 4.3 Behavioral Assessment

The behavioural impairments caused by rotenone administration were improved by treatment with Zinc, Linoleic acid or their combination to varying degrees.

### 4.3.1. Locomotor Assessment

There was a significant decrease in the locomotor activity, expressed by decrease in ambulation and rearing frequencies, in case of rotenone group when compared with normal control (vehicle) group ($P < 0.05$) (Table 1). The findings of each of the indicators of motor function assessed by performing the open field test are presented below.

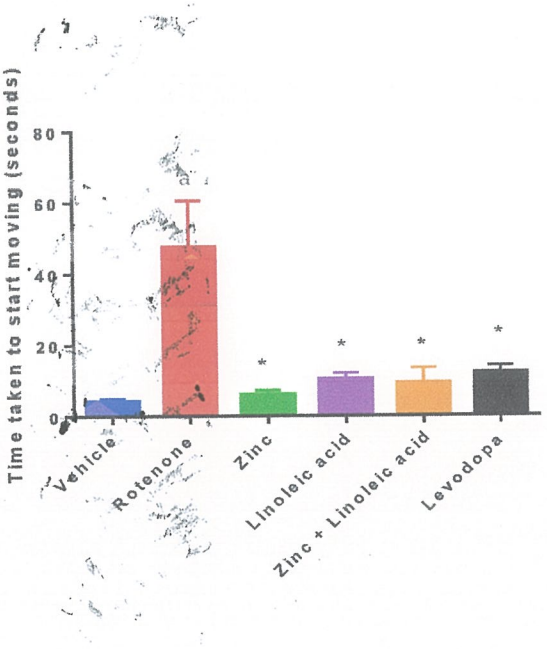
Table 1: **Showing effect of Zinc and Linoleic acid and their combination on parameters of open field test**

| GROUPS | Latency | Ambulation Freq | Freezing duration (seconds) | Grooming Duration (seconds) | Rearing |
|---|---|---|---|---|---|
| Vehicle (N=6) | 4.200 ±0.3742 | 41.80 ±11.25 | 1.400 ±0.8718 | 1.600 ±0.6782 | 8.800 ±2.871 |
| Rotenone (N=5) | 47.60 ±5.802[a] | 12.80 ±4.352[a] | 6.800±0.5831[f] | 7.800 ±1.241[a] | 0.8000 ±0.8000[a] |
| Zinc (N=6) | 6.000 ±0.5477[b] | 71.20 ±3.455* | 0.0 ±0.0* | 1.200 ±0.3742[b] | 20.60± 2.182* |
| Linoleic Acid (N=6) | 10.40 ±0.7483[b] | 35.40± 2.358 | 0.0 ±0.0* | 5.000 ±0.6325 | 12.60± 1.122* |
| Zinc + Linoleic Acid (N=5) | 9.200 ±1.934[b] | 37.00 ±2.098 | 0.0 ±0.0* | 2.200 ±1.020[b] | 4.800 ±1.655 |
| Levodopa (N=5) | 12.00 ±0.9487[b] | 34.60 ±6.997 | 0.6000 ±0.6000* | 1.200 ±0.3742[b] | 2.000 ±0.5477 |

Results are expressed as mean ± SEM. a, $p \leq 0.05$ compared to vehicle group; b, $p \leq 0.05$ compared to rotenone group;* $p \leq 0.0001$ compared to rotenone group; c $p \leq 0.0001$ compared to Zinc + Linoleic acid; d $p \leq 0.0001$ compared to Levodopa; f, $p \leq 0.0001$ compared to vehicle group

#### 4.3.1.1. Latency of movement initiation

Rotenone-treated rats showed increased (4.200 ±0.3742 to 47.60 ±5.802) latency as compared to the normal control rats ($p<0.05$). Zinc, Linoleic acid and their combination reduced the latency time as compared to rotenone-treated group ($p<0.05$). The effect of Linoleic acid was also significantly higher when compared to vehicle group ($p\leq0.05$, Table 1and Figure 3). The effect of zinc, Linoleic acid but not their combination on latency was significant when compared to levodopa. All three interventions were not significant when compared to the normal control rats.

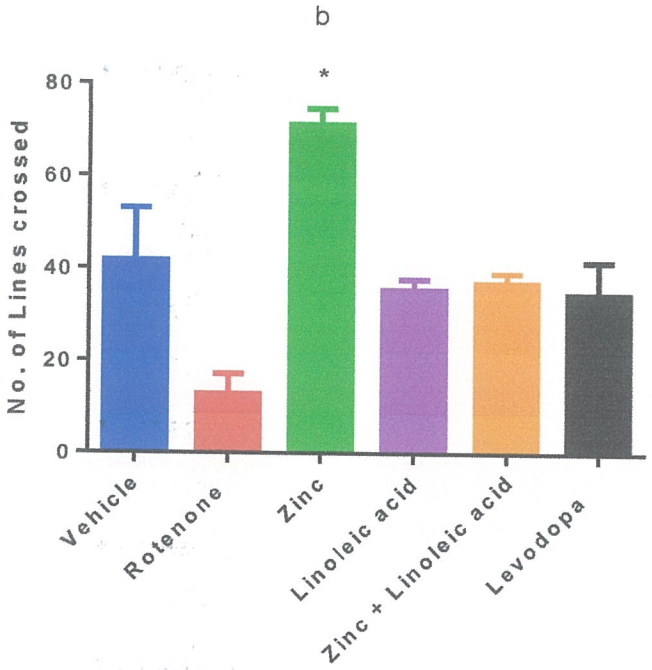



a, $p\leq0.0001$ compared to vehicle group, * $p\leq0.0001$ compared to rotenone group

**Figure 3: Effect of Zinc, Linoleic acid and their combination on the latency for initiation of movement by rats**

#### 4.3.1.2. Ambulation frequency

The present study showed a significant reduction in frequency of ambulation (41.80 ±11.25 to 12.80 ± 4.352) in rotenone group as compared to the normal control group (Figure 4). Zinc, significantly increased the ambulation frequency as compared to rotenone group ($p<0.0001$). Neither Linoleic acid nor the combination of zinc and linoleic acid significantly increased the ambulation frequency compared to the rotenone group ($p>0.05$, Table 1 and figure 4). The effect of zinc was significantly higher when compared to Linoleic acid, the combination and levodopa.

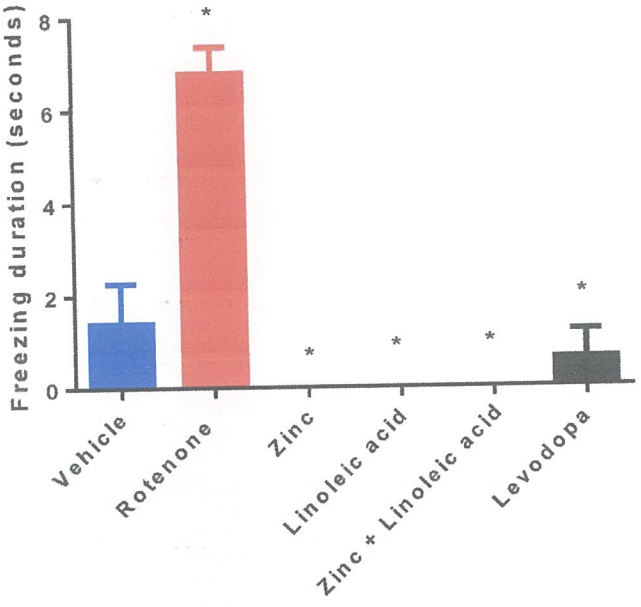



*, $p\leq0.0001$ compared to vehicle group, b $p\leq0.05$ compared to Levodopa

**Figure 4: Effect of Zinc, Linoleic acid and their combination on the ambulation frequency of rats**

#### 4.3.1.3. Freezing Duration

The duration of inactivity recorded as the freezing was significantly higher in rotenone-treated animals as compared to normal control (1.400 ±0.8718 to 6.800±0.5831). Treatment with Zinc and Linoleic acid and their combination significantly reduced the freezing duration in comparison to the rotenone group ($p \leq 0.0001$, Table 1 and Figure 5)

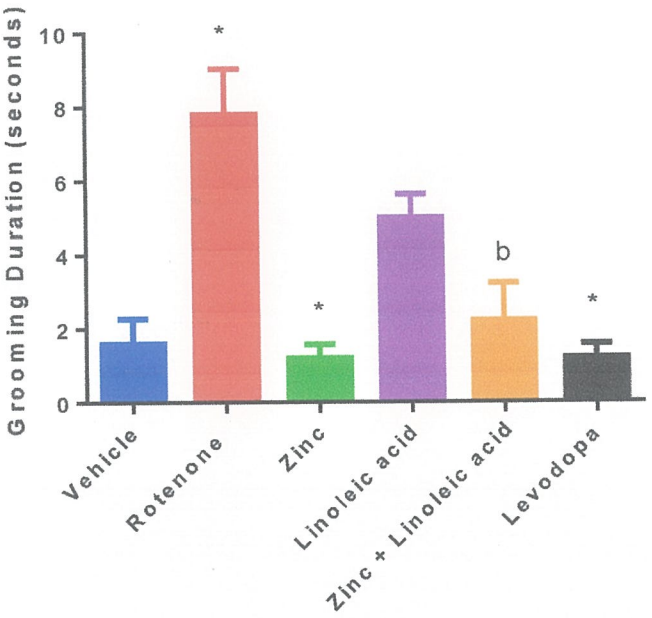



*$p \leq 0.0001$ compared to rotenone group

**Figure 5: Effect of Zinc, Linoleic acid and their combination on inactive sitting duration of rats.**

#### 4.3.1.4 Grooming Duration

Rotenone-treated rats showed increased grooming duration as compared to the normal control group (1.600 ±0.6782 to 7.800 ±1.241). Zinc and their combination but not Linoleic acid alone significantly decreased the grooming duration as compared to rotenone group ($p \leq 0.0001$ and $p < 0.05$ respectively, Table 1 and figure 6).

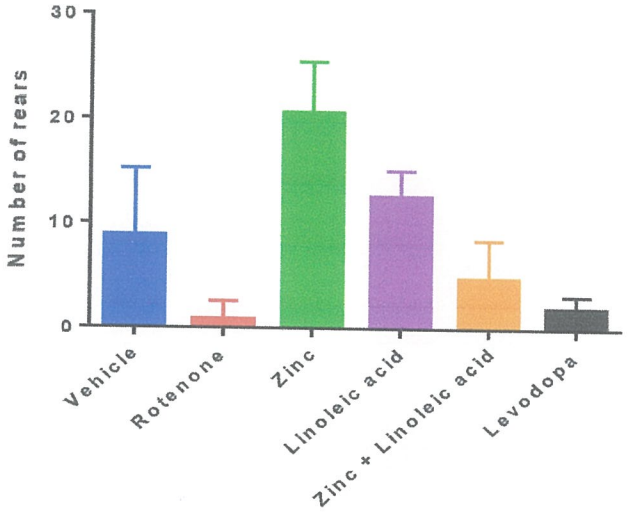



*$p \leq 0.0001$ compared to rotenone group; b<0.05 compared to rotenone group

**Figure 6: Effect of Zinc, Linoleic acid and their combination on the grooming duration of rats.**

#### 4.3.1.5. Rearing frequency

The rotenone group showed significantly lower frequencies in rearing compared to the vehicle group ($p<0.0001$) (Table 1 and figure 7). In addition, the current results indicated that zinc and linoleic alone but not their combination significantly increased the rearing frequency as compared to rotenone group ($p\leq0.0001$ and $p<0.05$ respectively). The effect of zinc ($p<0.0001$) and linoleic acid ($p<0.05$) on the rearing behavior was significantly higher when compared to levodopa.




*, $p\leq0.0001$ compared to rotenone group; $b<0.05$ compared to rotenone group

**Figure 7: Effect of Zinc, Linoleic acid and their combination on rearing frequency of rats.**

### 4.3.2 Anxiety Assessment

In the elevated plus maze, the rotenone-treated rats significantly spent less time in the open arm compared to the vehicle-treated group (52.40 ± 9.626 to 15.00 ± 2.775) ($p<0.05$, Table 2). Zinc but not Linoleic acid or their combination significantly increased the number of entries into the open arms of the maze compared to the rotenone-treated group ($p < 0.0001$). Zinc and its combination with linoleic acid but not linoleic acid alone significantly decreased the number of entries into the close arms of the maze compared to the rotenone-treated group ($p < 0.05$).

**Table 2: Effect of Zinc and Linoleic acid and their combination on parameters of elevated plus maze**

| Groups | Open Arm Time | Close Arm Time | Open Arm Entries | Close Arm Entries |
|---|---|---|---|---|
| | Mean ± SEM | | | |
| Vehicle (6) | 52.40 ± 9.626 | 129.2 ± 27.52 | 4.000 ± 0.5477 | 4.800 ±1.158 |
| Rotenone (5) | 15.00 ± 2.775 | 239.6 ± 21.45[a] | 1.800±0.3742[a] | 3.200 ± 0.6633 |
| Zinc (6) | 180.6 ± 18.50* | 42.60± 2.502* | 7.200±0.3742* | 5.600 ± 0.2449 |
| Linoleic Acid (6) | 32.00 ± 4.680 | 186.4±17.69 | 3.400 ± 0.5099 | 6.000 ± 1.095 |
| Zinc + Linoleic Acid (5) | 56.60 ± 17.44* | 117.6± 32.11* | 3.000 ± 0.7071 | 3.600 ± 0.6782 |
| Levodopa (5) | 50.20 ± 8.558* | 57.80± 7.566* | 4.200± 0.3742[b] | 5.200 ± 0.7348 |

Results are expressed as mean ± SEM. a, $p\leq0.05$ compared to vehicle group; b, $p\leq0.05$ compared to rotenone group; * $p\leq0.0001$ compared to rotenone group

### 4.3.3. Assessment of Olfactory Acuity

Rotenone-treated rats showed decreased sniffing time as compared to the vehicle-treated rats. Zinc and linoleic acid and the combination of the two significantly increased the sniffing duration of vanilla as compared to rotenone group ($p\leq0.0001$ Table 3). The frequency of sniffing of water by the rotenone-treated group was not significant higher ($p> 0.05$, Table 3) as compared to the normal control group. The frequency of sniffing vanilla by the zinc, linoleic acid and the combination group was however significant when compared with the rotenone group.

Table 3: **Effect of Zinc and Linoleic acid and their combination on parameters of novel scent test**

| Groups | Duration of sniffing water | Duration of sniffing vanilla | Freq. of sniffing water | Freq. of sniffing vanilla |
|---|---|---|---|---|
| | Mean ± SEM | | | |
| Vehicle (6) | 25.00 ±3.536 | 93.20 ±4.576 | 2.000± 0.3162 | 4.200 ±0.3742 |
| Rotenone (5) | 15.00 ±2.588[a] | 7.600 ± 3.203[c] | 3.600± 0.4000 | 0.8000 ±0.3742[a] |
| Zinc (6) | 18.40 ±1.288 | 93.00 ±5.385[*] | 1.800 ±0.3742 | 5.600 ±0.9274[*] |
| Linoleic Acid (6) | 7.600± 1.536[b] | 81.00 ±5.486[*] | 1.800 ±0.3742 | 6.600 ±0.7483[*] |
| Zinc + Linoleic Acid (5) | 9.200 ±2.458 | 94.00 ±7.190[*] | 2.600 ±0.5099 | 7.000 ±1.049[*] |
| Levodopa (5) | 11.00± 0.7071 | 72.00 ±4.626[*] | 2.200 ±0.3742 | 5.800 ±0.7348 |

Results are expressed as mean ± SEM. a, p≤0.05 compared to vehicle group, b, p≤0.05 compared to rotenone group; c p≤0.0001 compared to vehicle group * p≤0.0001 compared to rotenone group

### 4.3.4 Assessment of olfactory discrimination

Rotenone-treated rats showed significant decreased sniffing time of non-self bedding as compared to the vehicle-treated rats (p<0.0001). Zinc and Linoleic acid and the combination of the two significantly increased the sniffing duration of non-self bedding as compared to rotenone group. In addition, Zinc and Linoleic acid and the combination significantly increased the frequency of sniffing non-self bedding as compared to rotenone group (p≤0.0001 Table 4).

Table 4: **Effect of Zinc and Linoleic acid and their combination on parameters of block test**

| Groups | Duration of sniffing self | Duration of sniffing non-self | Freq. of sniffing self | Freq. of sniffing non-self |
|---|---|---|---|---|
| | Mean ± SEM | | | |
| Vehicle (6) | 10.80 ± 1.393 | 70.00 ± 7.583 | 1.600 ±0.2449 | 4.000 ±0.5477 |
| Rotenone (5) | 7.000 ± 1.414 | 1.000 ± 0.6325 | 1.200 ±0.2000[a] | 0.4000±0.2449[a] |
| Zinc (6) | 11.80 ± 2.709[*] | 45.00 ± 5.477[*] | 1.400 ±0.2449 | 4.600 ±0.6782 |
| Linoleic Acid (6) | 13.60 ± 2.064[*] | 45.60 ± 4.226[*] | 1.400 ±0.2449 | 4.600 ±0.5099 |
| Zinc + Linoleic Acid (5) | 10.20 ± .8602[*] | 49.20 ± 3.839[*] | 1.600 ± 0.2449 | 6.600 ± 0.9274 |
| Levodopa (5) | 8.800 ± .3742[b] | 32.40 ± 3.092[b] | 1.600 ± 0.2449 | 2.800 ± 0.3742 |

Results are expressed as mean ± SEM. a, p≤0.0001 compared to vehicle group; b, p≤0.05 compared to rotenone group; * p≤0.0001 compared to rotenone group

## 4.4 Biochemical Measurements

**Table 5: Effect of Zinc and Linoleic acid and their combination on LPO and the antioxidant system enzymes in mid brain of rotenone treated rats.**

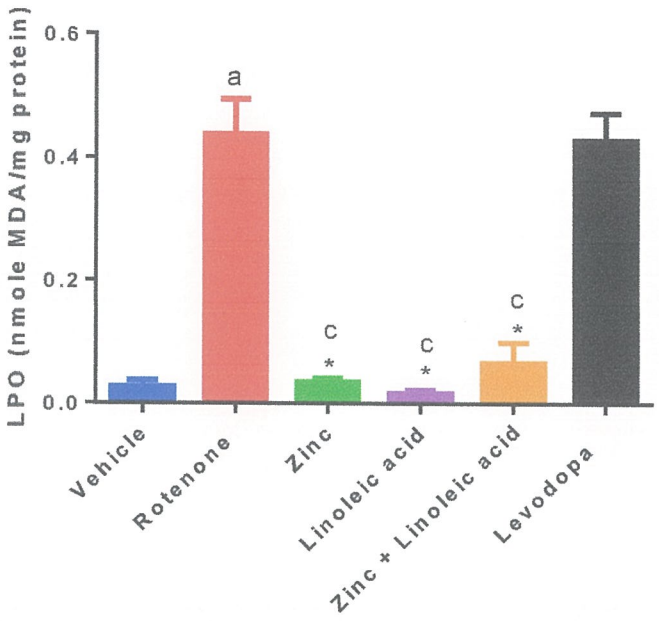
| GROUPS | MDA (µmoles/mg protein) | TAC (µmoles/mg protein) | GSH (µmoles/mg protein) | SOD (UI/mg protein) | CAT (µmole $H_2O_2$/min/mg protein | GPx (UI/ml enzymes) | TP (mg protein) |
|---|---|---|---|---|---|---|---|
| | Mean ± SEM | | | | | | |
| Vehicle (6) | 0.03±0.01 | 699.2 ±3.473 | 4.44±0.43 | 6.68±1 | 0.64±0.01 | 0.0200± 0.003536 | 10.67 ±1.052 |
| Rotenone (5) | 0.44±0.06[a] | 554.6 ±8.921[a] | 0.57±0.18[a] | 1.42±0.63[a] | 0.22±0.03[a] | 0.0062 ±0.002131 | 9.370 ±0.3590 |
| Zinc (6) | 0.03±0.01*[c] | 686.7 ±8.559* | 3.91±0.31* | 5.16±0.24[b] | 0.74±0.03*,[d] | 0.0358 ±0.006778[b] | 10.41 ±0.0651 2 |
| Linoleic Acid (6) | 0.02±0.00*[c] | 702.1 ±4.699* | 3.76±0.49* | 5.34±1.04[b] | 0.74±0.04*,[d] | 0.0752 ±0.004758*,[c] | 10.95 ±0.2067 |
| Zinc + Linoleic Acid (5) | 0.07±0.03*[c] | 630.2 ±33.28[b] | 3.62±0.34* | 7.11±0.73[b] | 0.68±0.05* | 0.0812 ±0.007716*,[c] | 11.30 ±0.6652 |
| Levodopa (5) | 0.43±0.04 | 692.7 ±3.239* | 3.69±0.23* | 5.09±0.55[b] | 0.50±0.08[b] | 0.0170 ±0.0030 | 11.28 ±0.3937 |

Lipid Peroxidation (LPO), Total protein and antioxidant biomarker activity and concentration in the midbrain of the experimental groups. Results are expressed as mean ± SEM a, p≤0.0001 compared to vehicle group; b, p≤0.05 compared to rotenone group; c p≤0.0001 compared to levodopa group, d p≤0.05 compared to levodopa group * p≤0.0001 compared to rotenone group

### 4.4.1 Lipid Peroxidation by MDA measurement

In the estimation of MDA levels, all animals in each group showed variable levels of MDA. There was significant increase in levels of MDA in rotenone treated animals ($p<0.0001$), when compared with normal control animals (0.03±0.01 to 0.44±0.06). There was significant decrease in MDA levels in Zinc treated animals (0.44±0.06 to 0.03±0.01; $P<0.0001$), Linoleic acid (0.44±0.06 to 0.02±0.00; $P<0.0001$), and a combination of both (0.44±0.06 to 0.07±0.03; $P<0.0001$) when compared with rotenone treated animals. The effect of the zinc, Linoleic acid and the combination was significantly greater when compared to the Levodopa group ($p<0.0001$ Table 4). There was however no significant difference in MDA levels of the combination when compared with individual treatments. Zinc lowered the MDA levels to normal control group levels (0.03±0.01 to 0.03±0.01), while Linoleic acid and the combination groups lowered to near normal control levels (0.03±0.01±0.02±0.00 and 0.03±0.01 to 0.07±0.03 respectively. Levodopa did not significantly reduce the MDA levels (0.44±0.06 to 0.43±0.04, $p> 0.05$, Table 5). The

effect of linoleic acid and the combination was significant when compared to levodopa ($p<0.0001$).

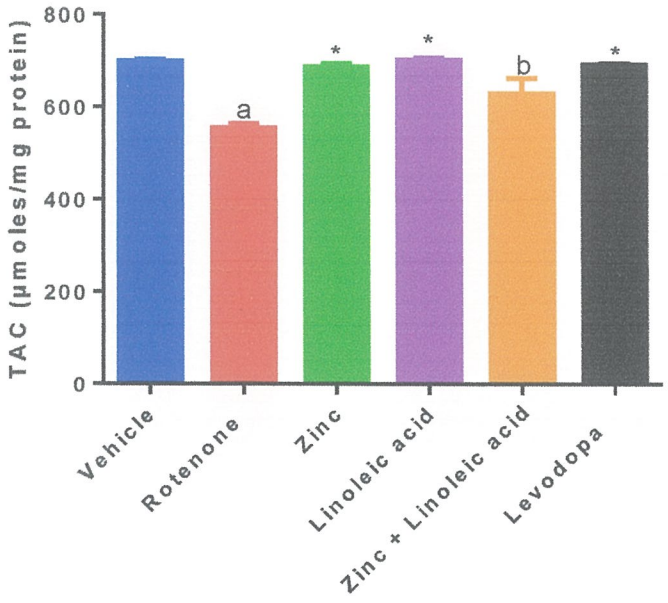



a, $p\leq0.0001$ compared to vehicle group; c $p\leq0.0001$ compared to levodopa group, * $p\leq0.0001$ compared to rotenone group

**Figure 8: Lipid Peroxidation (MDA) levels in the midbrain of experimental groups**

### 4.4.2. Total Antioxidant Capacity

The total antioxidant capacity in rotenone-treated rats was found to decrease compared to vehicle-treated rats (699.2 ±3.473 to 554.6 ±8.921; $p \leq 0.05$, Table 4). The zinc and linoleic acid groups had significantly prevented the decrease in TAC caused by rotenone administration (554.6 ±8.921 to 686.7 ±8.559; 554.6 ±8.921 to 702.1 ±4.699 respectively) ($p < 0.0001$); their combination also prevented this decrease in TAC caused by rotenone administration (554.6 ±8.921 to 630.2 ±33.28) ($p \leq 0.05$, Figure 9). The effect of the combination was not significant when compared to single treatments ($p > 0.05$, Figure 9). Zinc, linoleic acid, the combination and levodopa all increased TAC to near normal control levels.

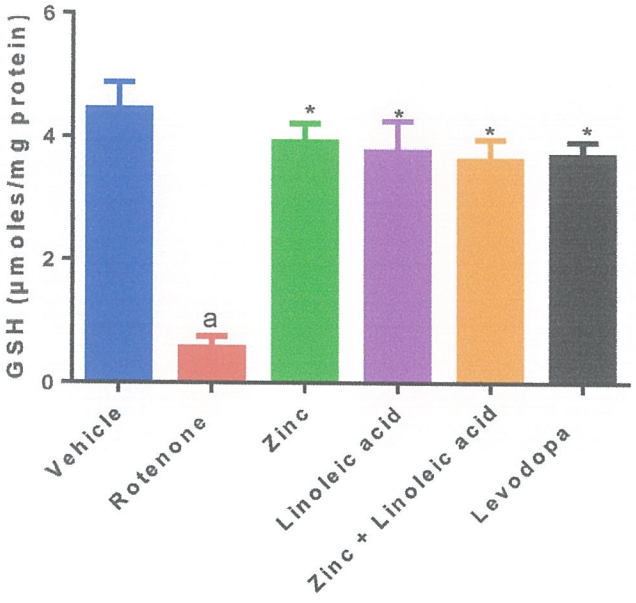



a, $p \leq 0.0001$ compared to vehicle group; b, $p \leq 0.05$ compared to rotenone group, * $p \leq 0.0001$ compared to rotenone group

**Figure 9: Total Antioxidant Capacity (TAC) levels in the midbrain of experimental groups.**

### 4.4.3. Reduced Glutathione Concentration.

There was significant decrease (4.44±0.43 to 0.57±0.18) in brain GSH content in rotenone treated groups as compared to the vehicle group ($p \leq 0.0001$, Table 4, Figure 10). There was significant increase in reduced glutathione levels in zinc treated animals i.e. 0.57±0.18 to 3.91±0.31 ($P<0.0001$), linoleic acid 0.57±0.18 to 3.76±0.49 ($P<0.0001$), and a combination of both i.e. 0.57±0.18 to 3.62±0.34 ($P<0.0001$) when compared with rotenone treated animals i.e 0.57±0.18. The combination of zinc and linoleic acid did not significantly increase GSH levels when compared to individual treatment ($p > 0.05$, Table 4, Figure 10). Levodopa significantly increased brain GSH levels compared to the rotenone group (0.57±0.18 to 3.69±0.23). The effect of zinc, linoleic acid, their combination and levodopa was not significant compared to the normal control group

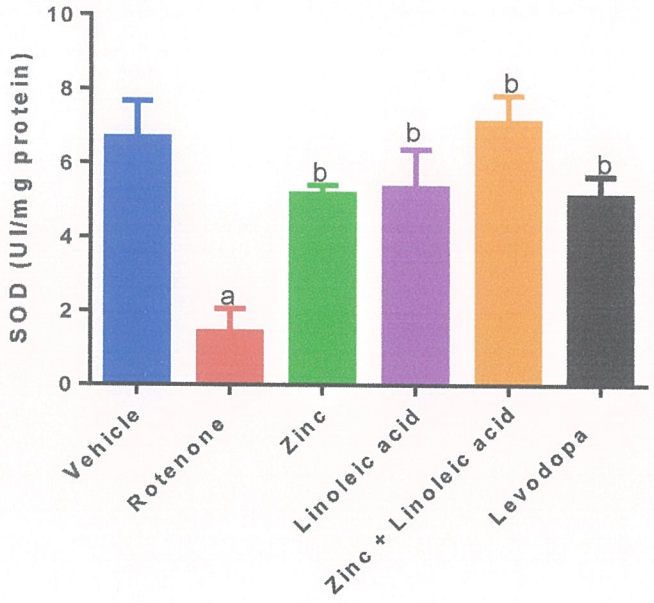



a, $p \leq 0.05$ compared to vehicle group. *, $p \leq 0.0001$ compared to rotenone group.

**Figure 10: Reduced Glutathione levels in the midbrain of experimental groups.**

### 4.4.4 Superoxide dismutase.

There was a significant decrease in SOD activity in rotenone-treated rats as compared to vehicle-treated rats (6.68±1 to 1.42±0.63; $p \leq 0.05$, Table 4). The zinc group had a significant increase in SOD activity as compared to rotenone group (1.42±0.63 to 5.16±0.24, $p < 0.05$). The linoleic acid group showed a similar effect (1.42±0.63 to 5.34±1.04, $p < 0.05$), as did their combination enhanced SOD activity as compared to rotenone group (1.42±0.63 to 7.11±0.73, $p \leq 0.05$, Figure 11). The combination of zinc and linoleic acid also significantly increased SOD activity when compared to individual treatment ($p < 0.05$, Figure 11). The effect of zinc, linoleic acid, their combination and levodopa was not significant when compared to the normal control group. Also the effect of zinc, linoleic acid and their combination was not significant when compared to levodopa ($p > 0.05$).

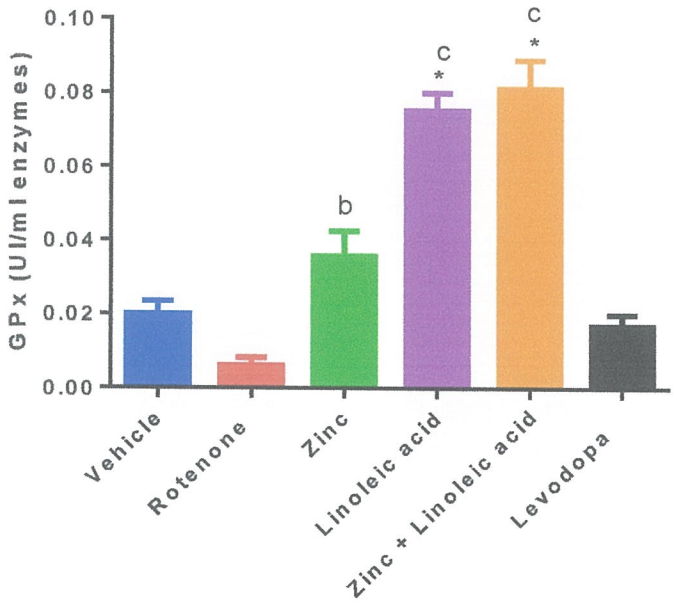



a, $p \leq 0.05$ compared to vehicle group. b, $p \leq 0.05$ compared to rotenone group.

**Figure 11: Superoxide Dismutase level in the midbrain of the experimental groups.**

### 4.4.5 Glutathione Peroxidase

The glutathione peroxidase activity was not significantly decreased in rotenone-treated rats as compared to vehicle-treated rats (0.0200± 0.003536 to 0.0062 ±0.002131 $p>0.05$, Table 5). The linoleic acid and the combination group had significant increase in GPx activity as compared to rotenone group (0.0062 ±0.002131 to 0.0752 ±0.004758 and 0.0062 ±0.002131 to 0.0812 ±0.007716 respectively, $p<0.0001$, Table 4); the zinc group also showed a significant effect (0.0062 ±0.002131 to 0.0358 ±0.006778, $p\leq0.05$, Figure 12). The effects of zinc and linoleic acid on increasing GPx activity were significant in comparison to the normal control group (0.0200± 0.003536 to 0.0358 ±0.006778 and 0.0200± 0.003536 to 0.0752 ±0.004758 respectively, $p<0.0001$). The effects of linoleic acid and the combination was significant when compared to levodopa ($p<0.0001$) while zinc effect was not significant ($p>0.05$).

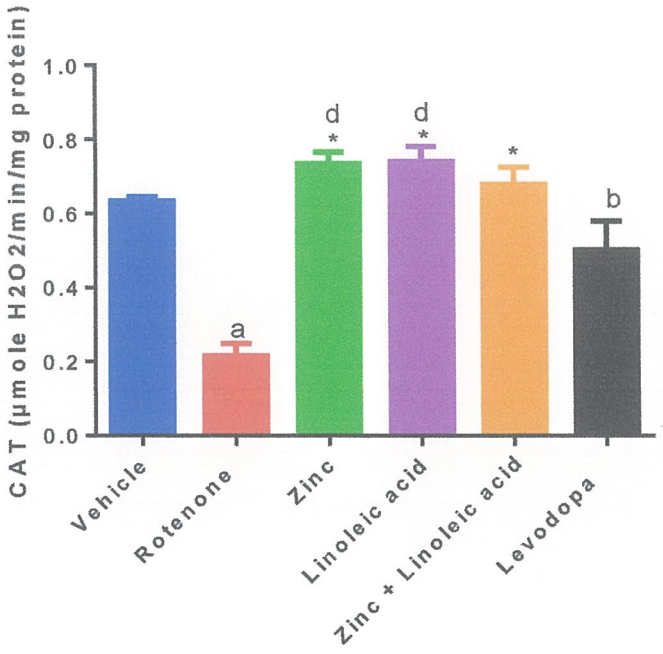



b, $p\leq0.05$ compared to rotenone group, c $p\leq0.0001$ compared to levodopa group, * $p\leq0.0001$ compared to rotenone group

**Figure 12: Glutathione Peroxidase activity in the midbrain of experimental groups.**

### 4.4.6. Catalase

Catalase activity in the vehicle treated group was found to be 0.64±0.01 µM of $H_2O_2$ used/min/mg protein. Rotenone treatment resulted in a significant decrease in CAT level in the midbrain as compared to the vehicle-treated group (0.64±0.01 to 0.22±0.03, $p<0.0001$). The zinc group (0.22±0.03 to 0.74±0.03), linoleic acid group (0.22±0.03 to 0.74±0.04) and their combination (0.22±0.03 to 0.68±0.05) had a significant increase in CAT activity as compared to rotenone group; ($p\leq0.0001$, Figure 13). The combination of zinc and linoleic acid however did not significantly increase catalase activity when compared to individual treatment ($p > 0.05$, Table 4, Figure 13). The CAT activity increasing effects of zinc, Linoleic acid and their combination was not significant when compared to the normal control and levodopa groups.




a, $p\leq0.0001$ compared to vehicle group; b, $p\leq0.05$ compared to rotenone group; d $p\leq0.05$ compared to levodopa group * $p\leq0.0001$ compared to rotenone group

**Figure 13: Catalase activity in the midbrain of experimental groups.**

### 4.5 Histopathology

The vehicle-treated group animals showed normal neuronal density and normal cellularity of neurons with no atypical cells, no visible cell death. In rotenone treated group there was neuronal cell death, slight structural damage to the midbrain. The zinc group showed midbrain structure with slight hyperplasia of cells, presence of micro vacuolization and augmented number of cells reflecting cellular injury however with small blood vessels. There was no visible cell death in the linoleic acid group. The combination group showed normal neuron cell population, with some cells appearing multinucleated. The levodopa group showed slight cellularity of the midbrain region with multinucleated cells. There was no apparent cell death.

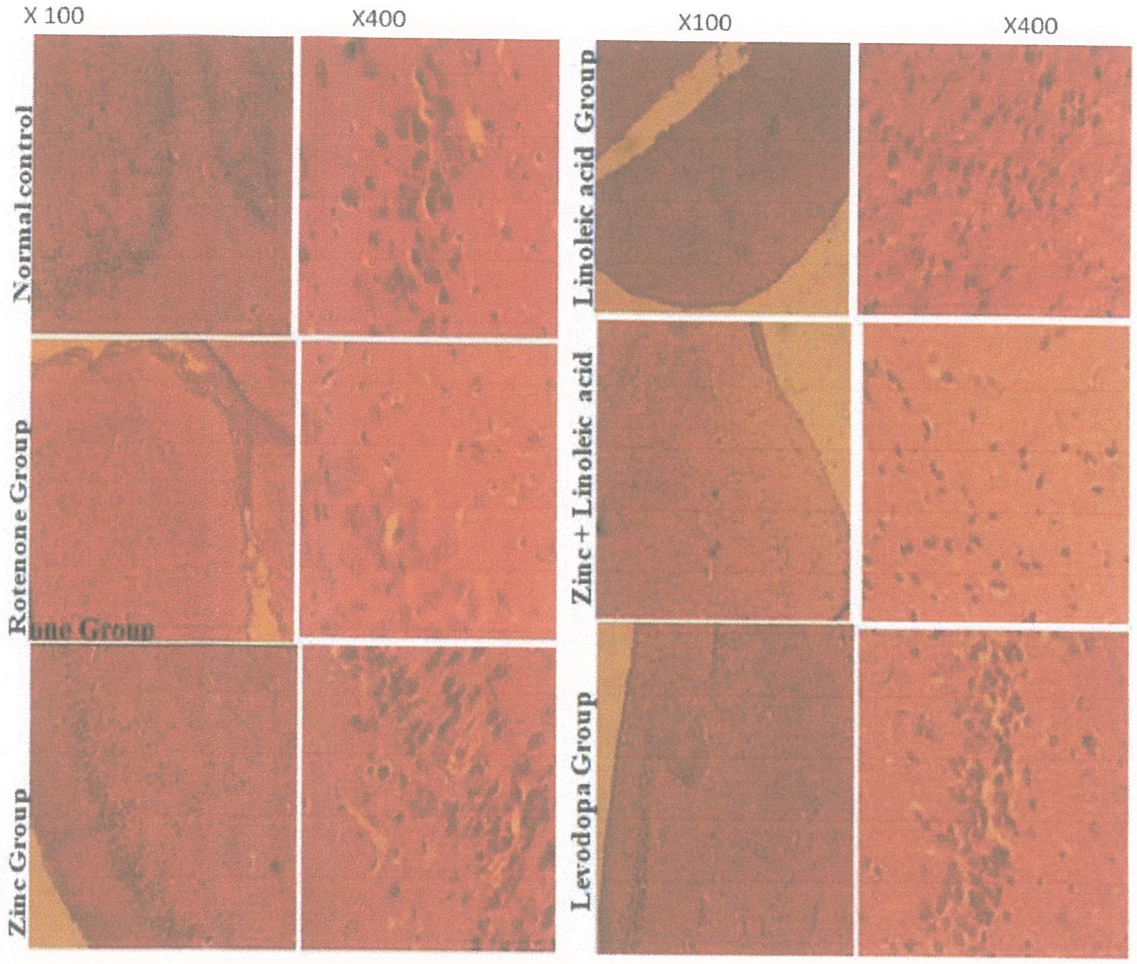



Figure 14: Histopathological changes in the mid brain of rats stained with haematoxylin and eosin.

Histopathological changes in the mid brain of rats stained with haematoxylin and eosin ×100 and ×400: Vehicle section showing normal histoarchitecture. Rats treated with rotenone (2.5mg/kg) with decrease neuron size and density. Rats treated with rotenone and interventions showing increased cellularity compared to rotenone group alone

# CHAPTER FIVE

# DISCUSSION, CONCLUSION AND RECOMMENDATIONS

## 5.1Discussions

The present study was done to evaluate the role of zinc and linoleic acid and their combination, as neuroprotective agents against rotenone-induced parkinsonism. As shown previously by Alam and Schmidt (2002), intraperitoneal injection of rotenone over seven days precipitate both motor and non-motor impairments including difficulties in the locomotor activity, decreased rearing behaviour, decreased anxiety and olfactory disturbances. Interestingly, pre-treatment of rats with zinc or linoleic acid improved several behavioural parameters. These improvements correlated with the enhancement of the total brain antioxidant capacity and activities of antioxidant enzymes in this study.

In animal model studies, rotenone (an alkaloidal pesticide), a specific inhibitor of complex I is employed to increase oxidative stress-mediated neuropathology (Tanner, 2011a). More specifically, it is used to generate toxin-based rodent models of Parkinson's disease (Sherer *et al.*, 2003). Because of its mechanism of action involving oxidative stress, exposure of rodents to Rotenone provides a valuable model for studying both mechanisms of oxidative stress and neuroprotection by antioxidant agents (Perfeito, Cunha-Oliveira, and Rego; 2012). Pathologically, Rotenone induces the degeneration of the nigrostriatal dopaminergic pathway which is one of the cardinal pathological markers of Parkinson's disease (Alam and Schmidt, 2002; Tanner, 2011b). As shown in this study, at the behavioural level, Rotenone precipitates behavioural alterations including both motor and several non-motor deficits (Inden *et al.*, 2011). These alterations have been shown to correlate with the extent of striatal lesions of the Rotenone-treated rats (Mulcahy, Walsh, Paucard, Rea, and Dowd, 2011).

Motor performances in all the experimental groups were assessed using the Open Field Test (Stanford, 2007). Remarkably, pretreatment of rats with zinc protected the animals from the effect of Rotenone. In fact, rats treated with zinc showed a significant increase in the frequency of ambulation and the numbers of entries in the elevated plus maze. zinc also reduced significantly the freezing and latency periods similar to the works of Joshi, Akhtar, Najmi, Khuroo, and Goswami (2012) and Cunha, Machado, Bettio, Capra, and Rodrigues (2008).This enhancement of locomotor activity in the zinc group could be due to the relatively higher dose of the zinc (30mg/kg) used in our study corroborating the already observed dose-dependent effect

of zinc on locomotion as assessed by the EPM (Partyka *et al.*, 2010). Regarding linoleic acid, no significant effect on motor activity was observed. This result corroborates with the studies of Bourre *et al.* (1989) in which diet rich in alpha-linoleic acid improved radial maze learning performance without affecting motor activity. Rearing is an important item of the behavioral repertoire rodents use to explore the environment (Fleming *et al.*, 2004). In the open field test, rearing as a vertical movement is more sensitive to nigral DAergic degeneration relative to horizontal movements (Gould *et al.*, 2010). Like its effect on locomotor activity, zinc prevented the deterioration of the rearing behavior induced by rotenone treatment. Similar to zinc pre-treatment, Linoleic acid protected the rearing behavior of rats against the impairment effect of rotenone treatment. In a study on the role of omega-3 fatty acid status on rat adaptation to chronic restraint stress, Linoleic acid-enriched diet was able to abolish the stress-induced reduction in locomotor activity and rearing in the open field test (Calon and Cole, 2007).

Zinc and linoleic acid exhibited anxiolytic-like effects in rats, as they both increased the time spent on open arms and the number of entries into the open arms. Although Samardzic *et al.* (2013) reported an anxiogenic effect of zinc (30mg/kg), the anti-anxiety effect of Zinc shown in our study is consistent with previous studies (Erreger and Traynelis, 2008; Wlaź *et al.*, 2011). The route of administration could account for these conflicting results. The bioavailability of zinc has been shown to be variable depending on the route of administration (Bray and Bettger, 1990). Mechanistically, the anxiolytic effect of zinc has been linked with the inhibition of glutamate NMDA/AMPA receptors and an increase BDNF gene expression in the hippocampus (Brocardo *et al.*, 2007; Szewczyk *et al.*, 2008). Moreover, the reported modulation of the serotonin neurotransmission by zinc could underlie its effect on anxiety-like behaviour (Partyka *et al.*, 2011; Samardzic *et al.*, 2013), Linoleic acid has been suggested to act as an anxiolytic agent by preventing cellular inflammation as well as improving the antioxidant capacity of cells (Dyall and Michael-Titus, 2008; Ross, 2009; Seki, Tani, and Arita, 2009). Omega-3 PUFAs have been shown to prevent the development of stress-related disorders such as anxiety and depression by protecting glutaminergic neurotransmission in stress induced damage (Denis, Potier, Vancassel, Heberden, and Lavialle, 2013). In a study to investigate the beneficial effect of fish oil on anxiety, depression and cognitive behavior in olfactory bulbectomised rats, Pudell and colleagues suggested that the anti-anxiety effect of omega-3 PUFAs could be due to its ability to increase serotonin levels in the brain (Pudell *et al.*, 2014)

Impaired odour detection, differentiation, and identification has been correlated positively with an increased risk of developing PD, suggesting that behavioural testing of olfaction can facilitate

an earlier detection of the disease (Doty, Shaman, Kimmelman, and Dann, 1984b; Henderson, Lu, Wang, Cartwright, and Halliday, 2003). These changes are not responsive to dopaminergic therapies (Tillerson *et al.*, 2006). The rotenone group showed significant decrease in sniffing and exploration times. Zinc and linoleic acid significantly increased the frequency and duration of sniffing the novel scent indicating that they improved the olfactory senses in comparison to the rotenone group in which this was highly impaired. Zinc is believed to play an important role in the regeneration of receptor cells not only on taste buds, but also on the olfactory epithelium (Slotnick, Sanguino, Husband, Marquino, and Silberberg, 2007). Therefore, Zinc deficiency is thought to produce olfactory disorder (Duncan-Lewis, Lukman, and Banks, 2011; Jafek, Linschoten, and Murrow, 2004) although details of the mechanism by which it does so are still unclear. The mechanism by which linoleic acid improves olfactory function is not also clear but has been linked to its antioxidant role in the olfactory epithelium as well as neural development (Karr, Alexander, and Winningham, 2011).

A number of studies have shown that the dopaminergic neurons in PD exists in a state of constant oxidative stress, due in part largely to the generation of $H_2O_2$ (Jenner and Olanow, 1996). Lipid peroxidation measured by MDA levels was observed to be elevated in rotenone treated animals than controls. This agrees with other studies which suggest that oxidative damage is involved in the neuronal abnormalities in PD (Liu, Wang, Pan, Ding, and Lu, 2013; Tsang and Chung, 2009). However, treatment with zinc and linoleic acid or their combination reduced MDA levels and therefore lipid peroxidation and brought it to control levels. The reduction of MDA levels and thus lipid peroxidation by zinc is in line with the study of Ozturk *et al.* (2003) who investigated the effects of zinc deficiency and supplementation on malondialdehyde and glutathione levels in blood and tissues of rats performing swimming exercise, and showed that zinc supplemented rats had increased reduced glutathione and decreased MDA levels. The lowered MDA levels due to linoleic acid corroborates with the studies of Yang and colleagues who showed reduced MDA levels in the hippocampus of diabetic rats on administration of omega-3 PUFA (Yang *et al.*, 2012). The mechanism involved in lowering lipid peroxidation by linoleic acid appears to involve detoxification of peroxy radicals and other ROS species (Gladine *et al.*, 2014).

Decreased levels of GSH might play an important role in inducing oxidative stress in brain (Zeevalk *et al.*, 2008). Such levels have been detected in remaining neurons and in SNpc of PD patients as compared to controls of similar age (Tamilselvam *et al.*, 2013). In this present study, GSH levels were lower in rotenone treated animals. Studies have shown decreased levels of

GSH leads to oxidative damage to DNA, protein and lipids in PD (Chinta and Andersen, 2008; Surapaneni and Venkataramana, 2007; Vinish *et al.*, 2011). Oral administration of zinc and subcutaneous administration of Linoleic acid increased GSH levels. Zinc has been suggested to exert this GSH-increased level effect through the actions of metallothionein (Prasad, 2014). On the other hand the GSH activity-increasing effect of linoleic acid corroborates with the study of Ahmad and Beg (2016), who evaluated the therapeutic effect of omega-6 linoleic acid and thymoquinone enriched extracts from Nigella sativa oil in the mitigation of lipidemic oxidative stress in rats. The effects of linoleic acid on GSH may be due to their direct antioxidant effects or the prevention of GSH oxidation.

The rotenone treated group showed a significant decrease in glutathione peroxidase activity. This agrees with the studies of Testa, Sherer, and Greenamyre (2005). Zinc pre-treatment was able to increase glutathione peroxidase activity. This corroborates with the study of the effect of zinc supplementation on glutathione peroxidase activity and selenium concentration in the serum, liver and kidney of rats chronically exposed to cadmium by (Galażyn-Sidorczuk *et al.*, 2012), showed that zinc significantly increased glutathione peroxidase levels. Linoleic acid in the study of Ahmad and Beg (2016) also protected glutathione peroxidase activity by 90%.

SOD activity in the group treated with rotenone was found to be reduced. Complex-I of the mitochondrial respiratory chain is a major source of superoxide free radicals. The loss in SOD activity might contribute to increase in oxidative stress in rotenone treated animals (Fridovich, 1995; Liochev and Fridovich, 2007). A lowered SOD activity would be detrimental in cases when superoxide radical production is increased. The decrease in SOD activity following rotenone treatment might be due to inactivation of SOD by ROS (Fridovich, 1995). Administration of zinc was beneficial in restoring the SOD activity. This agrees with the studies of Paz Matias *et al.* (2015) who investigated the effect of zinc supplementation on superoxide dismutase activity in patients with ulcerative rectocolitis. In the study of Ahmad and Beg (2016), omega-6 linoleic acid supplementation also increased SOD activity.

The present study showed reduced CAT activity in rotenone-treated rats as compared to vehicle-treated rats. This agrees with the studies of Liu *et al.* (2013); Prema, Janakiraman, Manivasagam, and Arokiasamy (2015) and Soczynska *et al.* (2008). A study by Sharma and Bafna (2012) showed a non-significant increase in the activity of CAT in rotenone treated rats compared to vehicle-treated rats. Zinc and linoleic acid increased the activity of CAT compared

to the rotenone treated group. Zinc effect on Catalase activity agrees on the study of (Parashuramulu, Nagalakshmi, Rao, Kumar, and Swain, 2015) who investigated the effect of zinc supplementation on antioxidant status and immune response in buffalo calves and showed that zinc supplementation increased Catalase activity. The protective effects of linoleic acid might reflect its ability to improve energy metabolism and repair damaged layers of lipids, hence suppressing the exudation of free electrons from the mitochondrial electron transport system, which is a prerequisite reaction to generate free radicals (Liu, Killilea, and Ames, 2002).

The antioxidant effect of Zinc has been suggested to work via metallothionein by regulating the secretion of pro-inflammatory cytokines and these metallothionein's are strong scavengers of free radicals (Hijova, 2004; Maret, 2013). Linoleic acid has been suggested to act as an anxiolytic agent by preventing cellular inflammation as well as improving the antioxidant capacity of cells (Simopoulos, 2002). Linoleic acid with efficient free radical scavenging capacity could be involved in lowering MDA and slowing the degradation of the antioxidant enzymes CAT, SOD and GPx on its administration (Eckert, Lipka, and Muller, 2013; Innis, 2008), thus improving the total antioxidant capacity. The combination of zinc and linoleic acid showed remarkable antioxidant effect by lowering MDA levels and increasing GSH, SOD above the levels of individual interventions.

In histopathological findings visible neuronal cell loss in the midbrain was identified that indicates the damage of neurons at this region in rotenone treated animals. Findings from earlier studies have shown that the substantia nigra is more vulnerable to damage by rotenone (Liu *et al.*, 2013). This may be attributed to higher susceptibility of the neurons in this region to free radicals. Histological findings in the midbrain were more correlated with impaired motor coordination responses along with biochemical evidences (Testa *et al.*, 2005). This may indicate that the midbrain area responds highly to oxidative stress caused by rotenone. This vulnerability of the midbrain to rotenone-induced oxidative stress has also been demonstrated in other studies (Sherer *et al.*, 2003). Zinc and linoleic acid and their combination improved the neuronal loss in the midbrain caused by rotenone administration. The decreases in necrotic cells observed in the zinc treated group agrees with the studies of Galvo *et al.* (2015) who also observed a decrease in the number of necrotic cells in hippocampus as well as cortex of zinc supplemented group for both male and female pups, in a study investigating the effect of "Prenatal zinc in adult rat offspring exposed to lipopolysaccharide during gestation". Linoleic acid effect on the midbrain corroborates with the studies of Beltz, Tlusty, Benton, and Sandeman (2007), who investigated

the effect of Linoleic acid on hippocampal neurogenesis. They advanced suggestions that linoleic acid plays this role via increasing expression of brain derived neurotrophic factor.

### 5.2 Conclusion

Zinc, Linoleic acid and their combination showed neuroprotective and behavioural effect in rat model of Parkinsonism induced with Rotenone.

Zinc prevented development of behavioural deficits in rats compared to Linoleic acid and their combination caused by rotenone treatment.

Zinc, Linoleic acid and their combination prevented the increase in MDA levels and decrease in brain antioxidant status induced by rotenone treatment.

Cell death and reduction in neuron size induced by rotenone was prevented by treatment with zinc, linoleic acid and their combination.

### 5.3 Recommendations

Further studies are needed to explore mechanisms involved in the neuroprotective effect of zinc and linoleic acid in the experimental models of PD.

A longer duration of study will be also be needed to understand the point of initiation and course of progression of PD in rodent models to note the onset of non-motor and motor deficits and how zinc and linoleic acid interfere with the progression.

It is recommended that the blood levels and brain tissue amounts of zinc and linoleic acid be established before pre-treatment to determine the amount of exogenous zinc and linoleic acid administered.

It will also be required to use different doses of zinc and linoleic acid to determine the dose at which maximum effective is obtained.

## REFERENCES


Abdullah H. Baqui, Robert E. Black, Christa L. Fischer Walker, Shams Arifeen, Khalequz Zaman, Mohammad Yunus, M. A. W. and L. E. C. (2006). Zinc supplementation and serum zinc during diarrhea. *Indian Journal of Pediatrics*, *73*(6), 493–7.

Ahmad, S., and Beg, Z. H. (2016). Evaluation of therapeutic effect of omega-6 linoleic acid and thymoquinone enriched extracts from Nigella sativa oil in the mitigation of lipidemic oxidative stress in rats. *Nutrition*, *32*(6), 649–655. https://doi.org/10.1016/j.nut.2015.12.003

Alam, M., and Schmidt, W. J. (2002). Rotenone destroys dopaminergic neurons and induces parkinsonian symptoms in rats. *Behavioural Brain Research*, *136*(1), 317–324. https://doi.org/10.1016/S0166-4328(02)00180-8

Ames, B. N., Shigenaga, M. K., and Hagen, T. M. (1993). Oxidants, antioxidants, and the degenerative diseases of aging. *Proceedings of the National Academy of Sciences of the United States of America*, *90*(17), 7915–7922. https://doi.org/10.1073/pnas.90.17.7915

Armitage, P., Berry, G., and Mathews, J. N. . (2002). *Statistical Methods in Medical Research. Statistical Methods in Medical Research* (Vol. 49). Retrieved from

Bastianetto, S., and Quirion, R. (2004). Natural antioxidants and neurodegenerative diseases. *Frontiers in Bioscience*, *9*, 3447–3452.

Becalski, A., Feng, S. Y., Lau, B. P.-Y., and Zhao, T. (2012). Glycidyl fatty acid esters in food by LC-MS/MS: method development. *Analytical and Bioanalytical Chemistry*, *403*(10), 2933–42. https://doi.org/10.1007/s00216-012-5932-8

Beltz, B. S., Tlusty, M. F., Benton, J. L., and Sandeman, D. C. (2007). Omega-3 fatty acids upregulate adult neurogenesis. *Neuroscience Letters*, *415*(2), 154–158. https://doi.org/10.1016/j.neulet.2007.01.010

Benzie, I. ., and Strain, J. . (1996). The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. *Analytical Biochemistry*. https://doi.org/10.1006/abio.1996.0292

Blanckenberg, J., Bardien, S., Glanzmann, B., Okubadejo, N. U., and Carr, J. A. (2013). The prevalence and genetics of Parkinson's disease in sub-Saharan Africans. *Journal of the Neurological Sciences*, *335*(1–2), 22–25. https://doi.org/10.1016/j.jns.2013.09.010

Blesa, J., Trigo-Damas, I., Quiroga-Varela, A., and Jackson-Lewis, V. R. (2015). Oxidative stress and Parkinson's disease. *Frontiers in Neuroanatomy*, *9*(July), 91.

https://doi.org/10.3389/fnana.2015.00091

Bosco, D. a, Fowler, D. M., Zhang, Q., Nieva, J., Powers, E. T., Wentworth, P., ... Kelly, J. W. (2006). Elevated levels of oxidized cholesterol metabolites in Lewy body disease brains accelerate α-synuclein fibrilization. *Nature Chemical Biology*, *2*(5), 249–253. https://doi.org/10.1038/nchembio782

Bourre, J. M., Francois, M., Youyou, A., Dumont, O., Piciotti, M., Pascal, G., and Durand, G. (1989). The effects of dietary alpha-linolenic acid on the composition of nerve membranes, enzymatic activity, amplitude of electrophysiological parameters, resistance to poisons and performance of learning tasks in rats. *Journal of Nutrition*, *119*(12), 1880–92. https://doi.org/0022-3166/89

Boyne, A. F., and Ellman, G. L. (1972). A methodology for analysis of tissue sulfhydryl components. *Analytical Biochemistry*, *46*(2), 639–653. https://doi.org/10.1016/0003-2697(72)90335-1

Braak, H., and Del Tredici, K. (2009). Neuroanatomy and pathology of sporadic Parkinson's disease. *Advances in Anatomy, Embryology, and Cell Biology*. https://doi.org/10.1007/978-3-540-79850-7_1

Braak, H., Ghebremedhin, E., Rüb, U., Bratzke, H., and Del Tredici, K. (2004). Stages in the development of Parkinson's disease-related pathology. *Cell and Tissue Research*. https://doi.org/10.1007/s00441-004-0956-9

Bray, T. M., and Bettger, W. J. (1990). The physiological role of zinc as an antioxidant. *Free Radical Biology and Medicine*, *8*(3), 281–291. https://doi.org/10.1016/0891-5849(90)90076-U

Brocardo, P. S., Assini, F., Franco, J. L., Pandolfo, P., Miller, Y. M. R., Takahashi, R. N., ... Rodrigues, A. L. S. (2007). Zinc attenuates malathion-induced depressant-like behavior and confers neuroprotection in the rat brain. *Toxicological Sciences*, *97*(1), 140–148. https://doi.org/10.1093/toxsci/kfm024

Calon, F., and Cole, G. (2007). Neuroprotective action of omega-3 polyunsaturated fatty acids against neurodegenerative diseases: Evidence from animal studies. *Prostaglandins Leukotrienes and Essential Fatty Acids*, *77*(5–6), 287–293. https://doi.org/10.1016/j.plefa.2007.10.019

Cannon, J. R., Tapias, V., Na, H. M., Honick, A. S., Drolet, R. E., and Greenamyre, J. T. (2009). A highly reproducible rotenone model of Parkinson's disease. *Neurobiology of Disease*,

*34*(2), 279–290. https://doi.org/10.1016/j.nbd.2009.01.016

Carola, V., D'Olimpio, F., Brunamonti, E., Mangia, F., and Renzi, P. (2002). Evaluation of the elevated plus-maze and open-field tests for the assessment of anxiety-related behaviour in inbred mice. *Behavioural Brain Research*, *134*(1–2), 49–57. https://doi.org/10.1016/S0166-4328(01)00452-1

Chaudhuri, K. R., and Schapira, A. H. V. (2009). Non-motor symptoms of Parkinson's disease: dopaminergic pathophysiology and treatment. *Lancet Neurology*, *8*(5), 464–74. https://doi.org/10.1016/S1474-4422(09)70068-7

Checkoway, H., and Nelson, L. M. (1999). Epidemiologic approaches to the study of Parkinson's disease etiology. *Epidemiology*, *10*(3), 327–336. https://doi.org/00001648-199905000-00020 [pii]

Chinta, S. J., and Andersen, J. K. (2008). Redox imbalance in Parkinson's disease. *Biochimica et Biophysica Acta - General Subjects*. https://doi.org/10.1016/j.bbagen.2008.02.005

Clark, L. N., Ross, B. M., Wang, Y., Mejia-Santana, H., Harris, J., Louis, E. D., … Marder, K. (2007). Mutations in the glucocerebrosidase gene are associated with early-onset Parkinson disease. *Neurology*, *69*(12), 1270–1277. https://doi.org/10.1212/01.wnl.0000276989.17578.02

Cummings, J. L. (1992). Depression and Parkinson's disease: A review. *The American Journal of Psychiatry*, *144*, 53–54.

Cunha, M. P., Machado, D. G., Bettio, L. E. B., Capra, J. C., and Rodrigues, A. L. S. (2008). Interaction of zinc with antidepressants in the tail suspension test. *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, *32*(8), 1913–1920. https://doi.org/10.1016/j.pnpbp.2008.09.006

Czernecki, V., Pillon, B., Houeto, J. L., Pochon, J. B., Levy, R., and Dubois, B. (2002). Motivation, reward, and Parkinson's disease: Influences of dopatherapy. *Neuropsychologia*, *40*(13), 2257–2267. https://doi.org/10.1016/S0028-3932(02)00108-2

Dauer, W., and Przedborski, S. (2003). Parkinson ' s Disease : Mechanisms and Models. *Neuron*, *39*, 889–909. https://doi.org/10.1007/s11910-013-0409-5

Dawson, T. M., and Dawson, V. L. (2003). Molecular pathways of neurodegeneration in Parkinson's disease. *Science (New York, N.Y.)*, *302*(5646), 819–822. https://doi.org/10.1126/science.1087753

Delion, S., Chalon, S., Hérault, J., Guilloteau, D., Besnard, J. C., and Durand, G. (1994). Chronic dietary alpha-linolenic acid deficiency alters dopaminergic and serotoninergic neurotransmission in rats. *The Journal of Nutrition*, *124*(12), 2466–2476.

Denis, I., Potier, B., Vancassel, S., Heberden, C., and Lavialle, M. (2013). Omega-3 fatty acids and brain resistance to ageing and stress: Body of evidence and possible mechanisms. *Ageing Research Reviews*. https://doi.org/10.1016/j.arr.2013.01.007

Doty, R. L. (2012). Olfactory dysfunction in Parkinson disease. *Nature Reviews. Neurology*, *8*(6), 329–339. https://doi.org/10.1038/nrneurol.2012.80

Doty, R. L., Shaman, P., Kimmelman, C. P., and Dann, M. S. (1984a). University of Pennsylvania Smell Identification Test: a rapid quantitative olfactory function test for the clinic. *Laryngoscope*, *94*(2 Pt 1), 176–178.

Doty, R. L., Shaman, P., Kimmelman, C. P., and Dann, M. S. (1984b). University of Pennsylvania Smell Identification Test: a rapid quantitative olfactory function test for the clinic. *The Laryngoscope*, *94*(2 Pt 1), 176–8. https://doi.org/10.1288/00005537-198402000-00004

Duncan-Lewis, C. A., Lukman, R. L., and Banks, R. K. (2011). Effects of zinc gluconate and 2 other divalent cationic compounds on olfactory function in mice. *Comparative Medicine*, *61*(4), 361–365.

Dyall, S. C., and Michael-Titus, A. T. (2008). Neurological benefits of omega-3 fatty acids. *NeuroMolecular Medicine*. https://doi.org/10.1007/s12017-008-8036-z

Eckert, G. P., Lipka, U., and Muller, W. E. (2013). Omega-3 fatty acids in neurodegenerative diseases: Focus on mitochondria. *Prostaglandins Leukotrienes and Essential Fatty Acids*, *88*(1), 105–114. https://doi.org/10.1016/j.plefa.2012.05.006

El-Tawil, A. M. (2003). Zinc deficiency, in men with Crohn's disease may contribute to poor sperm function and male infertility. *Andrologia*, *35*(6), 337–341. https://doi.org/10.1046/j.0303-4569.2003.00588.x

Elbaz, A., and Tranchant, C. (2007). Epidemiologic studies of environmental exposures in Parkinson's disease. *J Neurol Sci*, *262*(1–2), 37–44. https://doi.org/10.1016/j.jns.2007.06.024

Erreger, K., and Traynelis, S. F. (2008). Zinc inhibition of rat NR1/NR2A N-methyl-D-aspartate receptors. *The Journal of Physiology*, *586*(3), 763–78. https://doi.org/10.1113/jphysiol.2007.143941

Erro, R., Bhatia, K. P., and Tinazzi, M. (2015). Parkinsonism following neuroleptic exposure: A double-hit hypothesis? *Movement Disorders*, *30*(6), 780–785. https://doi.org/10.1002/mds.26209

Festing, M. F. W., and Altman, D. G. (2002). Guidelines for the design and statistical analysis of experiments using laboratory animals. *ILAR Journal / National Research Council, Institute of Laboratory Animal Resources*, *43*(4), 244–258. https://doi.org/10.1093/ilar.43.4.244

File, S. E., Baldwin, H. A, Johnston, A. L., and Wilks, L. J. (1988). Behavioral effects of acute and chronic administration of caffeine in the rat. *Pharmacology, Biochemistry, and Behavior*, *30*(4), 809–15. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/3227034

Fischer, A. H., Jacobson, K. A, Rose, J., and Zeller, R. (2005). Hematoxylin and Eosin ( H and E ) staining. *CSH Protocols*, *2008*(4), pdb.prot4986. https://doi.org/10.1016/0016-5085(95)23032-7

Fleming, S. M., Tetreault, N. A., Mulligan, C. K., Hutson, C. B., Masliah, E., and Chesselet, M. F. (2008). Olfactory deficits in mice overexpressing human wildtype α-synuclein. *European Journal of Neuroscience*, *28*(2), 247–256. https://doi.org/10.1111/j.1460-9568.2008.06346.x

Fleming, S. M., Zhu, C., Fernagut, P. O., Mehta, A., DiCarlo, C. D., Seaman, R. L., and Chesselet, M. F. (2004). Behavioral and immunohistochemical effects of chronic intravenous and subcutaneous infusions of varying doses of rotenone. *Experimental Neurology*, *187*(2), 418–429. https://doi.org/10.1016/j.expneurol.2004.01.023

Fox, S. H., and Brotchie, J. M. (2014). *Levodopa-induced dyskinesia in Parkinson's disease. Levodopa-Induced Dyskinesia in Parkinson's Disease*. https://doi.org/10.1007/978-1-4471-6503-3

Fridovich, I. (1995). Superoxide Radical and Superoxide Dismutases. *Annual Review of Biochemistry*, *64*(III), 97–112. https://doi.org/10.1146/annurev.biochem.64.1.97

Fujikawa, T., Kanada, N., Shimada, A., Ogata, M., Suzuki, I., Hayashi, I., and Nakashima, K. (2005). Effect of sesamin in Acanthopanax senticosus HARMS on behavioral dysfunction in rotenone-induced parkinsonian rats. *Biological and Pharmaceutical Bulletin*, *28*(1), 169–72. https://doi.org/10.1248/bpb.28.169

Galażyn-Sidorczuk, M., Brzóska, M. M., Rogalska, J., Roszczenko, A., and Jurczuk, M. (2012). Effect of zinc supplementation on glutathione peroxidase activity and selenium concentration in the serum, liver and kidney of rats chronically exposed to cadmium.

*Journal of Trace Elements in Medicine and Biology : Organ of the Society for Minerals and Trace Elements (GMS)*, *26*(1), 46–52. https://doi.org/10.1016/j.jtemb.2011.10.002

Galvo, M. C., Chaves-Kirsten, G. P., Queiroz-Hazarbassanov, N., Carvalho, V. M., Bernardi, M. M., and Kirsten, T. B. (2015). Prenatal zinc reduces stress response in adult rat offspring exposed to lipopolysaccharide during gestation. *Life Sciences*, *120*, 54–60. https://doi.org/10.1016/j.lfs.2014.10.019

Gjerstad, M. D., Alves, G., Wentzel-Larsen, T., Aarsland, D., and Larsen, J. P. (2006). Excessive daytime sleepiness in Parkinson disease: Is it the drugs or the disease? *Neurology*, *67*(5), 853–858. https://doi.org/10.1212/01.wnl.0000233980.25978.9d

Gladine, C., Newman, J. W., Durand, T., Pedersen, T. L., Galano, J. M., Demougeot, C., … Comte, B. (2014). Lipid profiling following intake of the omega 3 fatty acid DHA identifies the peroxidized metabolites F4-neuroprostanes as the best predictors of atherosclerosis prevention. *PLoS ONE*, *9*(2). https://doi.org/10.1371/journal.pone.0089393

Goldman, J. G., and Postuma, R. (2014a). Premotor and nonmotor features of Parkinson's disease. *Current Opinion in Neurology*, *27*(4), 434–41. https://doi.org/10.1097/WCO.0000000000000112

Goldman, J. G., and Postuma, R. (2014b). Premotor and nonmotor features of Parkinson's disease. *Current Opinion in Neurology*, *27*(4), 434–41. https://doi.org/10.1097/WCO.0000000000000112

GORNALL, A. G., BARDAWILL, C. J., and DAVID, M. M. (1949). Determination of serum proteins by means of the biuret reaction. *The Journal of Biological Chemistry*, *177*(2), 751–766.

Gould, T. D., Dao, D. T., and Kovacsics, C. E. (2010). *The Open Field Test. NeuroMethods* (Vol. 42). https://doi.org/10.1007/978-1-60761-303-9-1

Grazioli, V., Schiavo, R., Casari, E., Marzatico, F., Rodriguez Y Baena, R., and Gaetani, P. (1998). Antioxidant enzymatic activities and lipid peroxidation in cultured human chondrocytes from vertebral plate cartilage. *FEBS Letters*, *431*(2), 149–153. https://doi.org/10.1016/S0014-5793(98)00731-5

Greenamyre, J. T., Betarbet, R., and Sherer, T. B. (2003). The rotenone model of Parkinson's disease: genes, environment and mitochondria. *Parkinsonism and Related Disorders*, *9*, 59–64. https://doi.org/10.1016/S1353-8020(03)00023-3

Guimarães, F. S., Chiaretti, T. M., Graeff, F. G., and Zuardi, A. W. (1990). Antianxiety effect of cannabidiol in the elevated plus-maze. *Psychopharmacology*, *100*(4), 558–559. https://doi.org/10.1007/BF02244012

Gupta, M., Gupta, B. K., Thomas, R., Bruemmer, V., Sladek, J. R., and Felten, D. L. (1986). Aged mice are more sensitive to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine treatment than young adults. *Neuroscience Letters*, *70*(3), 326–331. https://doi.org/10.1016/0304-3940(86)90573-2

Hallett, M. (2014). Tremor: Pathophysiology. *Parkinsonism and Related Disorders*, *20*(SUPPL.1). https://doi.org/10.1016/S1353-8020(13)70029-4

Halliday, G. M., Del Tredici, K., Braak, H., Tredici, K. Del, and Braak, H. (2006). Critical appraisal of brain pathology staging related to presymptomatic and symptomatic cases of sporadic Parkinson's disease. *Journal of Neural Transmission. Supplementum*, (70), 99–103. https://doi.org/10.1016/S0197-4580(02)00065-9

Hawkins, P. (2002). Recognizing and assessing pain, suffering and distress in laboratory animals: a survey of current practice in the UK with recommendations. *Laboratory Animals*, *36*, 378–395. https://doi.org/10.1258/002367702320389044

Henderson, J. M., Lu, Y., Wang, S., Cartwright, H., and Halliday, G. M. (2003). Olfactory deficits and sleep disturbances in Parkinson's disease: a case-control survey. *Journal of Neurology, Neurosurgery, and Psychiatry*, *74*(7), 956–8. https://doi.org/10.1136/jnnp.74.7.956

Hijova, E. (2004). Metallothioneins and zinc: their functions and interactions. *Bratislavske Lekarske Listy*.

Hirose, G. (2006). Drug induced parkinsonism. *Journal of Neurology*, *253*(S3), iii22-iii24. https://doi.org/10.1007/s00415-006-3004-8

Hogg, S. (1996). A review of the validity and variability of the elevated plus-maze as an animal model of anxiety. In *Pharmacology Biochemistry and Behavior* (Vol. 54, pp. 21–30). https://doi.org/10.1016/0091-3057(95)02126-4

Holmes, A., Parmigiani, S., Ferrari, P. F., Palanza, P., and Rodgers, R. J. (2000). Behavioral profile of wild mice in the elevated plus-maze test for anxiety. *Physiology and Behavior*, *71*(5), 509–516. https://doi.org/10.1016/S0031-9384(00)00373-5

Hwang, O. (2013). Role of oxidative stress in Parkinson's disease. *Experimental Neurobiology*, *22*(1), 11–7. https://doi.org/10.5607/en.2013.22.1.11

Inden, M., Kitamura, Y., Abe, M., Tamaki, A., Takata, K., and Taniguchi, T. (2011). Parkinsonian rotenone mouse model: reevaluation of long-term administration of rotenone in C57BL/6 mice. *Biological and Pharmaceutical Bulletin*, *34*(1), 92–96. https://doi.org/10.1248/bpb.34.92

Innis, S. M. (2008). Dietary omega 3 fatty acids and the developing brain. *Brain Research*. https://doi.org/10.1016/j.brainres.2008.08.078

Iso, H., Sato, S., Umemura, U., Kudo, M., Koike, K., Kitamura, A., … Shimamoto, T. (2002). Linoleic Acid, Other Fatty Acids, and the Risk of Stroke. *Stroke*, *33*(8), 2086–2093. https://doi.org/10.1161/01.STR.0000023890.25066.50

Jafek, B. W., Linschoten, M. R., and Murrow, B. W. (2004). Anosmia after intranasal zinc gluconate use. *American Journal of Rhinology*, *18*(3), 137–41.

Jenner, P., and Olanow, C. W. (1996). Oxidative stress and the pathogenesis of Parkinson's disease. *Neurology*, *47*(6 Suppl 3), S161–S170. https://doi.org/10.1212/WNL.47.6_Suppl_3.161S

Jenner, P., and Olanow, C. W. (2006). The pathogenesis of cell death in Parkinson's disease. *Neurology*, *66*(10 Suppl 4), S24-36. https://doi.org/10.1002/mds.21675

Joshi, M., Akhtar, M., Najmi, A. K., Khuroo, A. H., and Goswami, D. (2012). Effect of zinc in animal models of anxiety, depression and psychosis. *Hum Exp Toxicol*, *31*(12), 1237–1243. https://doi.org/10.1177/0960327112444938

Kalia, L. V., and Lang, A. E. (2015). Parkinson's disease. *The Lancet*, *386*(9996), 896–912. https://doi.org/10.1016/S0140-6736(14)61393-3

Karr, J. E., Alexander, J. E., and Winningham, R. G. (2011). Omega-3 polyunsaturated fatty acids and cognition throughout the lifespan: A review. *Nutritional Neuroscience*, *14*(5), 216–225. https://doi.org/10.1179/1476830511Y.0000Q00012

Kaur, H., Chauhan, S., and Sandhir, R. (2011). Protective effect of lycopene on oxidative stress and cognitive decline in rotenone induced model of Parkinson's disease. *Neurochemical Research*, *36*(8), 1435–1443. https://doi.org/10.1007/s11064-011-0469-3

Leary, S., Underwood, W., Anthony, R., and Cartner, S. (2013). *AVMA Guidelines for the Euthanasia of Animals: 2013 Edition. American Veterinary Medical Association.*

Lesage, S., and Brice, A. (2009). Parkinson's disease: From monogenic forms to genetic susceptibility factors. *Human Molecular Genetics*. https://doi.org/10.1093/hmg/ddp012

Liochev, S. I., and Fridovich, I. (2007). The effects of superoxide dismutase on H2O2 formation. *Free Radical Biology and Medicine*. https://doi.org/10.1016/j.freeradbiomed.2007.02.015

Liu, C.-B., Wang, R., Pan, H.-B., Ding, Q.-F., and Lu, F.-B. (2013). [Effect of lycopene on oxidative stress and behavioral deficits in rotenone induced model of Parkinson's disease]. *Zhongguo Ying Yong Sheng Li Xue Za Zhi = Zhongguo Yingyong Shenglixue Zazhi = Chinese Journal of Applied Physiology*, *29*(4), 380–384. Retrieved from

Liu, J., Killilea, D. W., and Ames, B. N. (2002). Age-associated mitochondrial oxidative decay: improvement of carnitine acetyltransferase substrate-binding affinity and activity in brain by feeding old rats acetyl-L- carnitine and/or R-alpha -lipoic acid. *Proceedings of the National Academy of Sciences of the United States of America*, *99*(4), 1876–1881. https://doi.org/10.1073/pnas.261709098

Liu, Z., Li, T., Yang, D., and W. Smith, W. (2013). Curcumin protects against rotenone-induced neurotoxicity in cell and drosophila models of Parkinson's disease. *Advances in Parkinson's Disease*, *2*(1), 18–27. https://doi.org/10.4236/apd.2013.21004

Luchtman, D. W., Meng, Q., and Song, C. (2012). Ethyl-eicosapentaenoate (E-EPA) attenuates motor impairments and inflammation in the MPTP-probenecid mouse model of Parkinson's disease. *Behavioural Brain Research*, *226*(2), 386–396. https://doi.org/10.1016/j.bbr.2011.09.033

Mackay-Sim, A., and Dreosti, I. E. (1989). Olfactory function in zinc-deficient adult mice. *Experimental Brain Research*, *76*(1), 207–212. https://doi.org/10.1007/BF00253638

Madegowda, R. H., Kishore, A, and Anand, A. (2005). Mutational screening of the parkin gene among South Indians with early onset Parkinson's disease. *Journal of Neurology, Neurosurgery, and Psychiatry*, *76*(11), 1588–1590. https://doi.org/10.1136/jnnp.2004.046888

Maret, W. (2013). Zinc and human disease. *Metal Ions in Life Sciences*, *13*, 389–414. https://doi.org/10.1007/978-94-007-7500-8-12

Martinu, K., and Monchi, O. (2013). Cortico-basal ganglia and cortico-cerebellar circuits in Parkinson's disease: pathophysiology or compensation? *Behavioral Neuroscience*, *127*(2), 222–36. https://doi.org/10.1037/a0031226

Meredith, G., and Rademacher, D. (2011). MPTP mouse models of Parkinson's disease: an update. *Journal of Parkinson's Disease*, *1*, 19–33. https://doi.org/10.3233/JPD-2011-11023

Misra, H. P., and Fridovich, I. (1972). The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. *Journal of Biological Chemistry*, *247*(10), 3170–3175. https://doi.org/4623845

Mocchegiani, E., Malavolta, M., Marcellini, F., and Pawelec, G. (2006). Zinc, oxidative stress, genetic background and immunosenescence: implications for healthy ageing. *Immunity and Ageing : I and A*, *3*, 6. https://doi.org/10.1186/1742-4933-3-6

Morley, J. F., Pawlowski, S. M., Kesari, A., Maina, I., Pantelyat, A., and Duda, J. E. (2014). Motor and non-motor features of Parkinson's disease that predict persistent drug-induced Parkinsonism. *Parkinsonism and Related Disorders*, *20*(7), 738–742. https://doi.org/10.1016/j.parkreldis.2014.03.024

Muangpaisan, W., Hori, H., and Brayne, C. (2009). Systematic Review of the Prevalence and Incidence of Parkinson's Disease in Asia. *Journal of Epidemiology*. https://doi.org/10.2188/jea.JE20081034

Mulcahy, P., Walsh, S., Paucard, A., Rea, K., and Dowd, E. (2011). Characterisation of a novel model of Parkinson's disease by intra-striatal infusion of the pesticide rotenone. *Neuroscience*, *181*, 234–242. https://doi.org/10.1016/j.neuroscience.2011.01.038

Nathan, B. P., Yost, J., Litherland, M. T., Struble, R. G., and Switzer, P. V. (2004). Olfactory function in apoE knockout mice. *Behavioural Brain Research*, *150*(1–2), 1–7. https://doi.org/10.1016/S0166-4328(03)00219-5

Nuti, A., Ceravolo, R., Dell'Agnello, G., Gambaccini, G., Bellini, G., Kiferle, L., ... Bonuccelli, U. (2004). Environmental factors and Parkinson's disease: a case-control study in the Tuscany region of Italy. *Parkinsonism and Related Disorders*, *10*(8), 481–5. https://doi.org/10.1016/j.parkreldis.2004.01.008

Ojha, S., Javed, H., Azimullah, S., Khair, S. B. A., and Haque, M. E. (2015). Neuroprotective potential of ferulic acid in the rotenone model of Parkinson's disease. *Drug Design, Development and Therapy*, *9*, 5499–5510. https://doi.org/10.2147/DDDT.S90616

Ozturk, A., Baltaci, A. K., Mogulkoc, R., Oztekin, E., Sivrikaya, A., Kurtoglu, E., and Kul, A. (2003). Effects of zinc deficiency and supplementation on malondialdehyde and glutathione levels in blood and tissues of rats performing swimming exercise. *Biological Trace Element Research*, *94*(2), 157–66. https://doi.org/10.1385/BTER:94:2:157

Paine, T. a, Jackman, S. L., and Olmstead, M. C. (2002). Cocaine-induced anxiety: alleviation by diazepam, but not buspirone, dimenhydrinate or diphenhydramine. *Behavioural*

*Pharmacology*, *13*(7), 511–23.

Paniz, L. G., Calcagnotto, M. E., Pandolfo, P., Machado, D. G., Santos, G. F., Hansel, G., … Souza, D. O. (2014). Neuroprotective effects of guanosine administration on behavioral, brain activity, neurochemical and redox parameters in a rat model of chronic hepatic encephalopathy. *Metabolic Brain Disease*, *29*(3), 645–654. https://doi.org/10.1007/s11011-014-9548-x

Parashuramulu, S., Nagalakshmi, D., Rao, D. S., Kumar, M. K., and Swain, P. S. (2015). Effect of zinc supplementation on antioxidant status and immune response in buffalo calves. *Animal Nutrition and Feed Technology*, *15*(2), 179–188. https://doi.org/10.5958/0974-181X.2015.00020.7

Parkinson, J. (2002). An essay on the shaking palsy. 1817. *The Journal of Neuropsychiatry and Clinical Neurosciences*, *14*(2), 223–236; discussion 222. https://doi.org/10.1176/appi.neuropsych.14.2.223

Parron, T., Requena, M., Hernandez, A. F., and Alarcon, R. (2011). Association between environmental exposure to pesticides and neurodegenerative diseases. *Toxicol Appl Pharmacol*, *256*(3), 379–385. https://doi.org/S0041-008X(11)00175-X [pii]\r10.1016/j.taap.2011.05.006

Partyka, A., Jastrzebska-Wiesek, M., and Nowak, G. (2010). Evaluation of anxiolytic-like activity of zinc. *17th International Congress of the Polish Pharmacological Society Krynica Zdroj Poland*, *62*, 57–58.

Partyka, A., Jastrzębska-Więsek, M., Szewczyk, B., Stachowicz, K., Sałwińska, A., Poleszak, E., … Nowak, G. (2011). Anxiolytic-like activity of zinc in rodent tests. *Pharmacological Reports*, *63*(4), 1050–1055. https://doi.org/10.1016/S1734-1140(11)70621-1

Patil, S. P., Jain, P. D., Ghumatkar, P. J., Tambe, R., and Sathaye, S. (2014a). Neuroprotective effect of metformin in MPTP-induced Parkinson's disease in mice. *Neuroscience*, *277*, 747–754. https://doi.org/10.1016/j.neuroscience.2014.07.046

Patil, S. P., Jain, P. D., Ghumatkar, P. J., Tambe, R., and Sathaye, S. (2014b). Neuroprotective effect of metformin in MPTP-induced Parkinson's disease in mice. *Neuroscience*, *277*, 747–754. https://doi.org/10.1016/j.neuroscience.2014.07.046

Paz Matias, J., Costa e Silva, D. M., Climaco Cruz, K. J., Gomes da Silva, K., Monte Feitosa, M., Oliveira Medeiros, L. G., … do Nascimento Nogueira, N. (2015). Effect of zinc supplementation on superoxide dismutase activity in patients with ulcerative rectocolitis.

*Nutricion Hospitalaria*, *31*(3), 1434–1437. https://doi.org/10.3305/nh.2015.31.3.8402

Perfeito, R., Cunha-Oliveira, T., and Rego, A. C. (2012). Revisiting oxidative stress and mitochondrial dysfunction in the pathogenesis of Parkinson disease--resemblance to the effect of amphetamine drugs of abuse. *Free Radical Biology and Medicine*, *53*(9), 1791–806. https://doi.org/10.1016/j.freeradbiomed.2012.08.569

Poewe, W. (2008). Non-motor symptoms in Parkinson's disease. *European Journal of Neurology*. https://doi.org/10.1111/j.1468-1331.2008.02056.x

Politis, M., and Niccolini, F. (2015). Serotonin in Parkinson's disease. *Behavioural Brain Research*. https://doi.org/10.1016/j.bbr.2014.07.037

Prasad, A. S. (2012a). Discovery of human zinc deficiency: 50 years later. *Journal of Trace Elements in Medicine and Biology*, *26*(2–3), 66–69. https://doi.org/10.1016/j.jtemb.2012.04.004

Prasad, A. S. (2012b). Impact of the discovery of human zinc deficiency. *Kuwait Medical Journal*, *44*(3), 180–182. https://doi.org/10.1016/j.jtemb.2014.09.002

Prasad, A. S. (2014). Zinc: An antioxidant and anti-inflammatory agent: Role of zinc in degenerative disorders of aging. *Journal of Trace Elements in Medicine and Biology*. https://doi.org/10.1016/j.jtemb.2014.07.019

Prema, A., Janakiraman, U., Manivasagam, T., and Arokiasamy, J. T. (2015). Neuroprotective effect of lycopene against MPTP induced experimental Parkinson's disease in mice. *Neuroscience Letters*, *599*, 12–19. https://doi.org/10.1016/j.neulet.2015.05.024

Pudell, C., Vicente, B. A., Delattre, A. M., Carabelli, B., Mori, M. A., Suchecki, D., … Ferraz, A. C. (2014). Fish oil improves anxiety-like, depressive-like and cognitive behaviors in olfactory bulbectomised rats. *European Journal of Neuroscience*, *39*(2), 266–274. https://doi.org/10.1111/ejn.12406

Rikans, L., and Hornbrook, K. (1997). Lipid peroxidation, antioxidant protection and aging. *Biochimica et Biophysica Acta*, *1362*(2–3), 116–127. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9540842%5Cnhttp://ukpmc.ac.uk/abstract/MED/9540842

Ross, B. M. (2009). Omega-3 polyunsaturated fatty acids and anxiety disorders. *Prostaglandins Leukotrienes and Essential Fatty Acids*, *81*(5–6), 309–312. https://doi.org/10.1016/j.plefa.2009.10.004

Samardzic, J., Savic, K., Stefanovic, N., Matunovic, R., Baltezarevic, D., Obradovic, M., ... Obradovic, D. (2013). Anxiolytic and antidepressant effect of zinc on rats and its impact on general behavioural parameters. *Military Medical and Pharmaceutical Journal of Serbia*, *70*(4), 391–395. https://doi.org/10.2298/VSP111129036S

Sazawal, S., Black, R. E., Jalla, S., Mazumdar, S., Sinha, A, and Bhan, M. K. (1998). Zinc supplementation reduces the incidence of acute lower respiratory infections in infants and preschool children: a double-blind, controlled trial. *Pediatrics*. https://doi.org/10.1542/peds.102.1.1

Seki, H., Tani, Y., and Arita, M. (2009). Omega-3 PUFA derived anti-inflammatory lipid mediator resolvin E1. *Prostaglandins and Other Lipid Mediators*. https://doi.org/10.1016/j.prostaglandins.2009.03.002

Sharma, A., Kaur, P., Kumar, B., Prabhakar, S., and Gill, K. D. (2008). Plasma lipid peroxidation and antioxidant status of Parkinson's disease patients in the Indian population. *Parkinsonism and Related Disorders*, *14*(1), 52–57. https://doi.org/10.1016/j.parkreldis.2007.06.009

Sharma, N., and Bafna, P. (2012). Effect of Cynodon dactylon on rotenone induced Parkinson's disease. *Oriental Pharmacy and Experimental Medicine*, *12*(3), 167–175. https://doi.org/10.1007/s13596-012-0075-1

Sherer, T. B., Betarbet, R., and Greenamyre, J. T. (2005). Rotenone rat and other neurotoxin models of parkinson disease. In *Movement Disorders* (pp. 161–172). https://doi.org/10.1016/B978-012088382-0/50013-X

Sherer, T. B., Betarbet, R., Testa, C. M., Seo, B. B., Richardson, J. R., Kim, J. H., ... Greenamyre, J. T. (2003). Mechanism of toxicity in rotenone models of Parkinson's disease. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *23*(34), 10756–10764. https://doi.org/23/34/10756 [pii]

Shi, H., Yang, X., Zhao, H., Zhang, S., Zu, J., Zhang, W., ... Yan, C. (2015). Ranitidine reduced levodopa-induced dyskinesia by remodeling neurochemical changes in hemiparkinsonian model of rats. *Neuropsychiatric Disease and Treatment*, *11*, 1331–1337. https://doi.org/10.2147/NDT.S80174

Shin, H. W., and Chung, S. J. (2012). Drug-Induced parkinsonism. *Journal of Clinical Neurology (Korea)*, *8*(1), 15–21. https://doi.org/10.3988/jcn.2012.8.1.15

Sigalov, A. B., and Stern, L. J. (1998). Enzymatic repair of oxidative damage to human apolipoprotein A-I. *FEBS Letters*, *433*(3), 196–200. https://doi.org/10.1016/S0014-5793(98)00908-9

Simonian, N. A., and Coyle, J. T. (1996). Oxidative stress in neurodegenerative diseases. *Annual Review of Pharmacology and Toxicology*, *36*, 83–106. https://doi.org/10.1146/annurev.pa.36.040196.000503

Simopoulos, A. (2002). Omega-3 fatty acids in inflammation and autoimmune diseases. *Journal of the American College of Nutrition*, *21*(6), 495–505. https://doi.org/10.1080/07315724.2002.10719248

Sinha, A. K. (1972). Colorimetric assay of catalase. *Analytical Biochemistry*, *47*(2), 389–394. https://doi.org/10.1016/0003-2697(72)90132-7

Slotnick, B. M., Sanguino, A., Husband, S., Marquino, G., and Silberberg, A. (2007). Olfaction and olfactory epithelium in mice treated with zinc gluconate. *The Laryngoscope*, *117*(4), 743–9. https://doi.org/10.1097/MLG.0b013e318033006b

Soczynska, J. K., Kennedy, S. H., Chow, C. S., Woldeyohannes, H. O., Konarski, J. Z., and McIntyre, R. S. (2008). Acetyl-carnitine and α-lipoic acid: possible neurotherapeutic agents for mood disorders? *Expert Opinion on Investigational Drugs*, *17*(6), 827–843. https://doi.org/10.1517/13543784.17.6.827

Stanford, S. C. (2007). The Open Field Test: reinventing the wheel. *Journal of Psychopharmacology (Oxford, England)*, *21*(2), 134–135. https://doi.org/10.1177/0269881107073199

Stocks, J., Gutteridge, J. M. C., Sharp, R. J., and Dormandy, T. L. (1974). The inhibition of lipid autoxidation by human serum and its relation to serum proteins and α tocopherol. *Clinical Science and Molecular Medicine*, *47*(3), 223–233.

Stokes, W. S. (2002). Humane endpoints for laboratory animals used in regulatory testing. *ILAR Journal / National Research Council, Institute of Laboratory Animal Resources*, *43 Suppl*, S31-8. https://doi.org/10.1093/ilar.41.2.72

Sun, E., Xu, H., Liu, Q., Zhou, J., Zuo, P., and Wang, J. (1995). The mechanism for the effect of selenium supplementation on immunity. *Biol Trace Elem Res*, *48*(3), 231–238. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9398943

Surapaneni, K., and Venkataramana, G. (2007). Status of lipid peroxidation, glutathione, ascorbic acid, vitamin E and antioxidant enzymes in patients with osteoarthritis. *Indian*

*Journal of Medical Sciences*. https://doi.org/10.4103/0019-5359.29592

Suttrup, I., and Warnecke, T. (2016). Dysphagia in Parkinson's Disease. *Dysphagia*. https://doi.org/10.1007/s00455-015-9671-9

Sydenham, E., Dangour, A. D., and Lim, W.-S. (2012). Omega 3 fatty acid for the prevention of cognitive decline and dementia. *The Cochrane Database of Systematic Reviews*, *6*(6), CD005379. https://doi.org/10.1002/14651858.CD005379.pub3

Szewczyk, B., Poleszak, E., Sowa-Kućna, M., Siwek, M., Dudek, D., Ryszewska-Pokraśniewicz, B., ... Nowak, G. (2008). Antidepressant activity of zinc and magnesium in view of the current hypotheses of antidepressant action. *Pharmacological Reports*. https://doi.org/10.1016/j.ultrasmedbio.2011.05.163

Tamilselvam, K., Braidy, N., Manivasagam, T., Essa, M. M., Prasad, N. R., Karthikeyan, S., ... Guillemin, G. J. (2013). Neuroprotective effects of hesperidin, a plant flavanone, on rotenone-induced oxidative stress and apoptosis in a cellular model for Parkinson's disease. *Oxidative Medicine and Cellular Longevity*. https://doi.org/10.1155/2013/102741

Tanner, et al. (2011a). Rotenone, Paraquat, and Parkinson's Disease. *Environmental Health Perspectives*, *119*, Vol. 119 N° 6 866-872.

Taylor, T. N., Caudle, W. M., Shepherd, K. R., Norrian, A., Jackson, C. R., Iuvone, P. M., ... Miller, G. W. (2009a). Nonmotor symptoms of Parkinson's disease revealed in an animal model with reduced monoamine storage capacity. *Journal of Neuroscience*, *29*(25), 8103–13. https://doi.org/10.1523/JNEUROSCI.1495-09.2009

Teixeira, H. D., Schumacher, R. I., and Meneghini, R. (1998). Lower intracellular hydrogen peroxide levels in cells overexpressing CuZn-superoxide dismutase. *Proceedings of the National Academy of Sciences of the United States of America*, *95*(14), 7872–5. https://doi.org/10.1073/pnas.95.14.7872

Testa, C. M., Sherer, T. B., and Greenamyre, J. T. (2005). Rotenone induces oxidative stress and dopaminergic neuron damage in organotypic substantia nigra cultures. *Molecular Brain Research*, *134*(1), 109–118. https://doi.org/10.1016/j.molbrainres.2004.11.007

Tillerson, J. L., Caudle, W. M., Parent, J. M., Gong, C., Schallert, T., and Miller, G. W. (2006). Olfactory discrimination deficits in mice lacking the dopamine transporter or the D2 dopamine receptor. *Behavioural Brain Research*, *172*(1), 97–105. https://doi.org/10.1016/j.bbr.2006.04.025

Tissingh, G., Booij, J., Bergmans, P., Winogrodzka, A., Janssen, A. G., van Royen, E. A., ...

Wolters, E. C. (1998). Iodine-123-N-omega-fluoropropyl-2beta-carbomethoxy-3beta-(4-iod ophenyl)tropane SPECT in healthy controls and early-stage, drug-naive Parkinson's disease. *Journal of Nuclear Medicine : Official Publication, Society of Nuclear Medicine*, *39*(7), 1143–8.

Tsang, A. H. K., and Chung, K. K. K. (2009). Oxidative and nitrosative stress in Parkinson's disease. *Biochimica et Biophysica Acta*, *1792*(7), 643–50. https://doi.org/10.1016/j.bbadis.2008.12.006

Veeriah, S., Taylor, B. S., Meng, S., Fang, F., Yilmaz, E., Vivanco, I., … Chan, T. a. (2010). Somatic mutations of the Parkinson's disease-associated gene PARK2 in glioblastoma and other human malignancies. *Nature Genetics*, *42*(1), 77–82. https://doi.org/10.1038/ng.491

Vijayakumar, D., and Jankovic, J. (2016). Drug-Induced Dyskinesia, Part 2: Treatment of Tardive Dyskinesia. *Drugs*. https://doi.org/10.1007/s40265-016-0568-1

Vinish, M., Anand, A., and Prabhakar, S. (2011). Altered oxidative stress levels in Indian Parkinson's disease patients with PARK2 mutations. *Acta Biochimica Polonica*, *58*(2), 165–169.

Walsh, R. N., and Cummins, R. a. (1976). The Open-Field Test: a critical review. *Psychological Bulletin*, *83*(3), 482–504. https://doi.org/10.1037/0033-2909.83.3.482

Welfare, N. I. H. O. of L. A. (2002). Public health service policy on humane care and use of laboratory animals. *Http://grants.nih.gov*.

Wlaź, P., Kasperek, R., Wlaź, A., Szumiło, M., Wróbel, A., Nowak, G., and Poleszak, E. (2011). NMDA and AMPA receptors are involved in the antidepressant-like activity of tianeptine in the forced swim test in mice. *Pharmacological Reports*, *63*(6), 1526–1532. https://doi.org/10.1016/S1734-1140(11)70716-2

Wright, J. W., and Harding, J. W. (2012). Importance of the brain angiotensin system in Parkinson's disease. *Parkinson's Disease*. https://doi.org/10.1155/2012/860923

Yang, R.-H., Wang, F., Hou, X.-H., Cao, Z.-P., Wang, B., Xu, X.-N., and Hu, S.-J. (2012). Dietary ω-3 polyunsaturated fatty acids improves learning performance of diabetic rats by regulating the neuron excitability. *Neuroscience*, *212*, 93–103. https://doi.org/10.1016/j.neuroscience.2012.04.005

Zawada, W. M., Banninger, G. P., Thornton, J., Marriott, B., Cantu, D., Rachubinski, A. L., … Jones, S. M. (2011). Generation of reactive oxygen species in 1-methyl-4-phenylpyridinium (MPP+) treated dopaminergic neurons occurs as an NADPH oxidase-dependent two-wave

cascade. *J Neuroinflammation*, *8*(1), 129. https://doi.org/10.1186/1742-2094-8-129

Zeevalk, G. D., Razmpour, R., and Bernard, L. P. (2008). Glutathione and Parkinson's disease: Is this the elephant in the room? *Biomedicine and Pharmacotherapy*, *62*(4), 236–249. https://doi.org/10.1016/j.biopha.2008.01.017

Zimmer, L., Delpal, S., Guilloteau, D., Aïoun, J., Durand, G., and Chalon, S. (2000). Chronic n-3 polyunsaturated fatty acid deficiency alters dopamine vesicle density in the rat frontal cortex. *Neuroscience Letters*, *284*(1–2), 25–28. https://doi.org/10.1016/S0304-3940(00)00950-2