Pharmacological and toxicological effects of copper and vanadium using in vitro and in vivo models of Parkinson's Disease

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Pharmacological and toxicological effects of copper and vanadium using *in vitro* and *in vivo* models of Parkinson's Disease

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ABSTRACT

Parkinson's disease (PD) pathology is characterised by distinct types of cellular defects: notably, oxidative damage and mitochondria dysfunction, leading to selective loss of dopaminergic neurons in the substantia nigra pars compacta. Exposure to heavy metals and some environmental toxicants have been associated for many years with this disease pathogenesis. Raised iron levels have been consistently observed in the nigrostriatal pathway in PD cases. This thesis focused on the effects of an endogenous heavy metal micronutrient (copper - Cu) and an exogenous environmental heavy metal (vanadium - Vd), and explored the interplay with iron (Fe), focusing for the first time on sub-toxic effects of these metals upon neuronal cell oxidative and ER stress, differentiation, calcium signalling, motor activity, oxidative stress and lifespan in an *in vitro* (Catecholaminergic a-differentiated (CAD) cells) and *in vivo* (Drosophila *melanogaster)* model of PD respectively.

Undifferentiated CAD cells were more susceptible to vanadium exposure than differentiated cells and this susceptibility was modulated by iron. Both a natural (*Aloysia citrodora)* and synthetic iron chelator, Deferoxamine (DFO), significantly and efficiently protected against chronic sub-toxic Vd-induced mitochondrial oxidative stress in contrast, iron chelation exacerbated the oxidative stress elicited by Cu.

Low dose Cu had no significant effect upon metabolic rate (in both differentiated and differentiating CAD cells) but significantly protected undifferentiated cells, decreased potassium chloride (KCl)-induced depolarisation and positively enhanced the expression of MAP2 in differentiated cells

In vivo exposure of Drosophila *melanogaster (DM)* to sub-toxic doses of Vd had a range of differential biochemical and behavioural effects upon wild-type (WT) and PD Pink-1^{B9} drosophila fly models. In pink-1 flies, exposure to chronic low dose of vanadium exacerbated the existing motor deficits, reduced survival, increased the production of reactive oxygen species (ROS), as well as T-SH and a reduction in survival. In WT, it caused an enhancement in motor activity (like L-dopa), in parallel with a reduction in brain RONS generation and increased total thiol levels (T-SH), with a resulting lifespan extension. Both *Aloysia citrodora* L, and DFO significantly protected against the PD-like phenotypes in both models. The results accrued in this thesis favours the case for iron-chelation therapy as a viable option for the symptomatic treatment of PD.

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List of abbreviations

- OH	Hydroxyl group
Aβ	Amyloid-beta
AD	Alzheimer's Disease
LS	Amyotrophic Lateral Sclerosis
APPA	Amyloid Precursor Protein
APS	Ammonium Persulfate
AR-JP	Autosomal Recessive Juvenile Parkinsonism
Atox1	(Human) Antioxidant protein 1
ATP	Adenosine-triphosphate
ATP13A2	P-Type ATPase
ATP7A	ATPase Cu transporting alpha
ATP7B.-	ATPase Cu transporting beta
ATPase	*Adenosine Triphosphatase*
BBB	Blood-Brain Barrier
BSA	Bovine Serum
C	Carbon
Ca^{2+}	Calcium Ions
Ca	Calcium
$CaCl_2$	Calcium Chloride
CAD	Cath.-a-differentiated
CaMKII	Calmodulin-dependent protein kinase II
CCS	Cu Chaperone For SOD
Cd	Cadmium
Cl	Chlorine
CNS	Central nervous system
CO_2	Carbon Dioxide
Cox11	Cytochrome c oxidase assembly protein COX11 mitochondrial
Cox17	Cytochrome c oxidase Cu chaperone

Cp	Ceruloplasmin
CSF	Cerebrospinal fluid
Cu	Copper
$CuCl_2$	Copper Chloride
D2	Dopamine d2 receptor
DA	Dopamine
DAPI	4′,6-Diamidin-2-phenylindol
DFO	Deferoxamine Mesylate Salt
dH2O	Deionized Distilled H2O
DIV	Days *in vitro*
DJ-1	*Protein deglycase DJ-1,(PARK7)*
DMEM	Dulbecco's Modified Eagle Media
DMSO	Dimethyl sulfoxide
DMT1	Divalent Metal Transporter 1
DNA	Deoxyribonucleic acid
DOPA	Dihydroxyphenylalanine
DTT	Dithiothreitol
E3	Ubiquitin ligase
ER	Endoplasmic reticulum
ERAD	ER-associated degradation
ETC	Electron transport chain
EtOH	ethyl alcohol
FBS	Foetal bovine serum
Fe	Iron
GABA-A	γ-aminobutyric acid
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GSH	Glutathione
GTP	Guanosine-5'-triphosphate
H_2O_2	Hydrogen peroxidase

HD	Huntington's Disease
HEPES	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
Hg	Mercury
HNO3	Nitric Acid
HRP	Horseradish-peroxidase
ICP-MS	Inductively Coupled Plasma Mass Spectrometry
IgG	Immunoglobulin G
IP3	1,4,5-trisphosphate
K	Potassium
K^+	Potassium ion
KCl	Potassium chloride
kDa	kilodaltons
KH_2P0_4	Potassium dihydrogen phosphate
LA	Lens Aperture
LB	Lewy body
LDH	Lactic Dehydrogenase
L-DOPA	l-dihydroxyphenylalanine
LRRK2	Leucine Repeat Rich Kinase 2
MTT	(3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide)
MAO	Monoamine oxidase
MAP	Microtubule Associated Protein
Mb	Molybdenum
$MgCl_2$	Magnesium Chloride
MIBG	Metaiodobenzylguanidine
mM	millimolar
Mn	Manganese
MnSOD	Manganese superoxide dismutase
MPP^+	Methyl-4-Phenylpyridinium
MPTP	Methyl-4-Phenyl-1, 2, 3, 6-Tetrahydropyridine
MRI	Magnetic Resonance Imaging
MS	Multiple Sclerosis
NaCl	Sodium Chloride
NADH CoQ	Complex 1
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
$NaVO_3$	Sodium Metavanadate
NFT	Neurofibrillary Tangles
NH_4Cl	Ammonium Chloride
Ni	Nickel
NMDA	N-Methyl-D-Aspartate
NMDA	N-Methyl-D-Aspartate

NO	Nitric Oxide
NO	Nitric Oxide
NOS	Nitric Oxide Synthase
NOx	Nitric Oxide metabolite
NR	Non-reducing
O_2	Oxygen
^oC	Degree Celsius
OH	Hydroxyl Radical
O/N	Overnight
OPCs	Oligodendrocyte Progenitors
PAM	Peptidyl-Glycine-A-Monooxygenase
Pb	Lead
PBS	Phosphate-buffered saline
PBS++	Phosphate-buffered saline supplemented with calcium & magnesium
PCB	Polychlorinated Biphenyl
PD	PD
PDI	Protein disulphide isomerase
PFA	Paraformaldehyde
PET	Positron Emission Tomography
PFA	Paraformaldehyde
PINK1	PTEN-Induced Kinase 1
PNS	Peripheral Nervous System
PO_4^{3-}	Phosphate
PrP	Prion Protein
PTEN	Phosphatase and Tension Homolog
PTM	Post Translation Modification
R	Reducing
RBR	Retinoblastoma-related protein

ROS	Reactive oxygen species
RPM	Revolution per Minute
RT	Room temperature
SDS	Sodium-dodecyl sulphate
SDS-PAGE	SDS Polyacrylamide gel electrophoresis
Se	Selenium
SFM	Serum-Free Media
SMP	Skim milked powder
SN	Substantia nigra
SNpc	Substantia nigra *pars compacta*
SOD	Super Oxide Dismutase
SP	Senile Plaques
TBS	Tris Buffered Saline
TEMED	N, N, N‘, N‘-Tetramethylethylendiamine
thallium	Tl
T-SH	Total Thiol
UK	United Kingdom
UPR	Unfolded protein response
VTA	Ventral tegmental area
αsyn	α-Synuclein
V	Elementary Vanadium
VO^{2+}	Vanadyl
VO_4^{3-}	Vanadate
WHO	World Health Organisation
XO	Xanthine Oxidase
Zn	zinc

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Chapter 1: Environmental Factors and PD

1.1 Introduction

1.2 The Burden of Neurodegenerative Diseases

Neurodegenerative diseases constitute a global health challenge (World Health Organisation - WHO, 2006; Wichterle and Przedborski, 2010). They account for about 6% of the global burden of disease and it is projected that there will be a two-fold increase in the current number of persons with neurological disorders every 20 years. WHO also projected that by the year 2040, as the populations in the developed world get older, neurodegenerative diseases (such as Alzheimer's), and conditions that affect mainly the motor functions, like PD and Amyotrophic Lateral Sclerosis (ALS) will be the leading causes of death, after cardiovascular disease (Gammon, 2014). Furthermore, neurodegenerative diseases constitute a huge economic burden on individuals, families, caregivers, and society (Weintraub, et al., 2008, Skovronsky, et al., 2006).

Metal dysregulation associated with neurodegenerative disorders is increasingly recognised as a serious, worldwide public health concern (Montgomery, 1995; Caudle, et al., 2012; Shribman, et al., 2013; Benammi, et al., 2015; Bjorklund, et al., 2018). Metal dyshomeostasis has been associated with PD (Myhre, et al.,2013; Goldman, 2014; Dusek, et al., 2015). This has become critical as between 5-10% of reported cases of PD are of genetic origin, whilst 90-95% cases are sporadic with significant linkage with environmental factors. Several PD genes are activated by xenobiotic exposure while the association between PD and exposure to environmental toxicant (such as metals and pesticides) has been demonstrated recently (Bjorklund, et al, 2018).

The aetiology of most neurodegenerative diseases is multifactorial due to the interaction between multiple genes and environmental factors (Cicero, et al., 2017). A host of environmental risk factors have been associated with neurodegenerative disorders. For example, polychlorinated biphenyl (PCB) in the industry (Carpenter, 1998), phalates (Berlinger, 2008), lead (Jusko, et al., 2008), methyl mercury (Oken, et al., 2008), polychlorinated biphenyls (Winneke, 2011), organophosphate pesticides (Eskenazi, et al.,

2007; London, et al., 2012) and organochlorine pesticides (Eskenazi, et al., 2008) endocrine disruptors (Braun, et al., 2011, Miodovnik, et al., 2011), automotive exhaust (Volk, et al., 2011), polycyclic aromatic hydrocarbons (Perera, et al., 2009), brominated flame retardants (Herbstman, et al., 2010), Perfluorinated compounds (Stein and Savitz, 2011) and metals (Neal, et al., 2012) have all been implicated (directly or indirectly) in the pathogenesis of neurodegenerative diseases. Among these, metals are gaining increasing attention because of their intricate roles in biological processes and a growing presence of environmental pollutants by way of industrial and chemical pollution of food, air, and water (Kwok, 2010).

The role of metals in the aetiology of PD is equally of great interest to the scientific world and is a notable public health concern. Several metals are vital to many physiological processes and their homeostasis is tightly regulated and the accumulation or deficiency may have pathophysiological and/or pathogenic consequences (Fraga, 2005). Whilst there is accumulating evidence to substantiate the essential functions of metals in the healthy brain, studies also abound that show that dysregulation or disruption of biometal homeostasis is a key process in the pathogenesis of several neurodegenerative diseases (Devi, et al., 2014; Jomova, et al., 2010).

Chen, et al (2016) observed that the accumulation of metals in the body may result in permanent injuries, including some neurological disorders. In their findings from both epidemiological and clinical studies, they found correlations exist between aberrant metal exposure and several neurological diseases, such as ALS, autism spectrum disorders, Guillain-Barrré disease, Gulf War Syndrome, Huntington's Disease (HD), Multiple Sclerosis (MS) and Wilson's Disease. Similarly, several studies have underscored the detrimental effects of a shift in the physiological balance of metals in biological systems, resulting in neurological disorders such as PD (Devi, et al., 2014; Jomova, et al., 2010; Moriarty, et al., 2014).

The role biometals play in the pathogenesis of brain disorders is highlighted by evidence from a plethora of sources, including molecular genetics (Blum, et al., 2001; Lev, et al., 2003; Thomas, 2005; Gasser, 2009); biochemical studies (Cookson, 2005; Riederer, 1983) and biometal imaging (Wang, et al., 2017; Gotz, et al., 2004; White, et al., 2015). Results from

these studies have stimulated a growing interest in understanding the role of metals in brain functions and diseases, as well as interventions that could help restore altered balance in the brain/body.

PD is the first form of chronic neurodegeneration found to be associated with the perturbation of metal homeostasis. This observation dates to about six decades ago (Aizenman, et al., 2015) when Hallgren and Sourander in 1958, in a seminal study, described the observed increase in iron levels in the substantia nigra (SN) of PD patients.

1.3 The epidemiology of PD: influence of environmental factors.

PD is associated with age. It has been noted that the prevalence increases with age and this correlation is astonishingly similar in most countries in Europe (De Rijk et al., 1997; De Rijk et al., 2000; von Campenhausen et al., 2005;). The possible explanation for this remarkable age-specific prevalence across Europe and the observed difference in other regions of the world have been adduced to variations in life expectancy and case ascertainment (Foltynie, et al., 2004). In the UK, age-adjusted prevalence rates are around 150 per 100,000 population per year, with several studies suggesting a mean age of 70 (Hindle, 2010).

Across Europe, the prevalence rates range from between 65.6 per 100,000 to 12,500 per 100,000 (von Campenhausen et al., 2005). The annual incidence rate of PD in Europe is estimated to range from 5 per 100,000 to 346 per 100,000 (von Campenhausen et al., 2005). These data give an indication of the relatively low rate of incidence of PD in some countries in Europe. Whilst investigating the prevalence rate by age and gender, De Rijk (2000) and his colleagues reported a prevalence rate of 1.8 per 100 population in persons 65 years of age and older. This rate, however, increases with age. They noted an increase of 0.6 for those age 65 to 69 years and 2.6 for those 85 to 89 years. They did not observe any sex difference in the prevalence of PD- this underscores the importance of ageing in the progression of this disease (Blin et al., 1991; Fearnely and Lees, 1991; Wüllner et al., 2007; Rajabally and Martey 2013).

In a population study in South-Western Finland, age-adjusted prevalence (with the Finnish general population) was 139 per 100,000 as at 1971 and 166 in 1992 - indicating a change in the epidemiology of PD in Finland (Kuopio et al., 1999). Unlike the findings of De Rijk et

al., (2000) where they reported no gender difference in the rate of prevalence, In an earlier study, Kuopio et al., reported an increase in prevalence from 1.2 in 1971 and 1.7 in 1992 for gender distribution showed. They also reported age-specific prevalence which showed a male preponderance in all age groups (as at 1992) and rural preponderance in age groups over 60 years.

Ageing as a factor in the aetiology of PD and described as the product of a random and predictable process that leads to the accumulation of unrepaired cellular damage, weakening cellular repair and compensatory mechanism (Kirkwood, 2003). However, there are individual variations. These variations are accounted for with lifestyle, environmental factor, and the genes (Kirkwood, 2003). Brian cells are vulnerable to accumulated effects of ageing including mitochondria dysfunction, increased free radical production and oxidative stress. All these lead to genomic instability and DNA mutations (Tai and Schuman, 2008; Best and Coppedè, 2009).

Besides age, exposure to environmental pollutant has been implicated in the pathogenesis of PD. For example, occupational exposure to a high concentration of manganese (Mn) can result in several neurodegenerative disorders similar to PD- such as manganism (Zayed, et al., 1990; Keen and Zidenberg-Cherr, 1994). Populations studies dating back to the last two decades reported that general population living within a mining district displayed poor motor functions (Santos-Burgoa, et al., 2001). In an earlier study of a population in Southwest Quebec exposed to Mn from a variety of sources, a correlation was established between poor performance for coordinated upper limb movement and elevated blood Mn levels (Mergler, et al., 1999).

In a multicentre large case-control study (with biomarker data) Weisskopf et al., (2010) reported cumulative exposure to lead (Pb) in populations across four USA states they investigated and noted an increased odd of PD with increasing cortical bone Pb. Although, no strong association between bone Pb and PD in this study, however, considerable experimental evidence point to the association that Pb disrupts the dopaminergic system (as it acutely increases spontaneous dopamine release, inhibits depolarisation-evoked dopamine release, decrease dopamine neuron spontaneous activity *in vivo* as well as alters dopamine-dependent behaviours)- Minnema, et al., 1986; Cory-Slechta, 1997.

1.4 Importance of metals in the brain

Several important biological functions in the brain require metal ions such as sodium, calcium, potassium, zinc, iron, and Cu (Crichton, et al., 2012). For example, sodium and potassium are involved in the fast transmission of electrical impulses between neurons and along their axons to muscles and endocrine tissues, the maintenance of ionic gradients, and the synthesis of neurotransmitters requires these metal ions. Besides, metals are involved in the regulation of gated sodium and potassium channels, to generate electrochemical gradients across the plasma membranes of neurons, for the transmission of nervous impulses in the central, and the peripheral nervous system.

Furthermore, metals are vital to functional and physicochemical processes, such as electron transport, oxygen transportation, protein modification, neurotransmitter synthesis (Riordan, 1977; Goldstein and Czapski, 1986; Foulkes, 1990; Montgomery, 1995; D'Souza, et al., 2003), redox reaction, immune responses, cell adhesion, and protein and carbohydrate metabolism (Chen, et al., 2014).

Metals in association with proteins are involved in signal transduction. The physiological functions of proteins are dependent on their charge and shape. However, the binding of calcium ions (Ca^{2+}) to proteins (just as the phosphorylation of hydroxyl groups of Ser and Thru residues via a protein kinase) can elicit changes in both the shape and charge of the proteins. For instance, $Ca^{2+,}$ like phosphoryl group, can alter the local electrostatic fields this makes it vital in signal transduction (Xia, et al., 2005) and synaptic plasticity (Hudmon, et al., 2002).

In the nervous system, Ca^{2+} plays a significant role in regulating the range of cellular processes through ligand-gated channels, such as the NMDA receptor; this is activated by N-methyl-D-aspartate or voltage-gated Ca^{2+} channels. Postsynaptically, Ca^{2+}/calmodulin-dependent protein kinase CaMKII is the most abundant protein in the postsynaptic density (Ward, et al., 2015) and plays a central role in Ca^{2+} signal transduction, in the region, where the postsynaptic membrane is in close contact with ion channel which mediates synaptic transmission.

Another notable metal ion that has, to a large extent, been implicated in brain function is Zn^{2+} (Bitanihirwe, et al., 2009; Bitanihirwe, et al., 2009). Zn^{2+} is sequestered by glutamatergic

neurons present in the mammalian forebrain in their synaptic vesicles and released into the synaptic cleft during synaptic transmission. Zinc may also act as a critical neural messenger through its ability to regulate NMDA receptor activity. However, excessive release of zinc into the synaptic cleft and its permissive entry into vulnerable neurons contributes to severe neuronal cell death (Ward, et al., 2015).

Besides Zn, the redox-active metals, Cu, and Iron (Fe) are both essential for normal brain function. Cu is a vital co-factor for two key proteins involved in neurotransmitter synthesis; these are dopamine β-hydroxylase which transforms dopamine to noradrenaline, and peptidyl-α-amidating monooxygenase involved in the amidation of neuropeptide (Lutsenko, et al., 2010). Deficiency of Cu during the foetal and neonatal periods has an adverse effect on both myelin formation and maintenance. Also, excess "free" Cu is equally dangerous because of its capacity (like iron) to participate in redox reactions, thus, generating insidious reactive oxygen and nitrogen species (Cricton, 2009).

Similarly, Fe is essential in several biological processes in the brain, including oxygen transport, DNA synthesis, nitric oxide metabolism, and mitochondrial respiration, as well as several specific neuronal functions in myelin synthesis and neurotransmitter synthesis and metabolism (Crichton, 2009). Iron deficiency has deleterious effects on pre- and postnatal brain development due to its involvement in several nervous system processes (Crichton, 2009). It was reported (Crichton, et al., 2006; Crichton, et al., 2014) that there is an elevation of brain iron in specific brain regions with ageing, such as frontal cortex, caudate nucleus, putamen, substantia nigra and Globus pallidus with no apparent adverse effect. However, aberrant increase in metals (an excessive amount) in the wrong place in specific intracellular compartments or specific regions of the brain could lead to neurodegenerative diseases (Crichton, et al., 2006; Crichton, et al., 2014).

Manganese is another trace element that is necessary for biological processes (Kumar, et al., 2014; Singh, et al., 2020). At low concentration, Mn serves as a co-factor in a variety of metalloproteins, inclusive of MnSOD and arginase. It has also been associated in the function

of a variety of enzymes, including glutamine synthetase, hydrolases and lyases (Chen, et al., 2015).

1.5 Metal-based neurodegeneration

The pathogenetic basis of neurodegeneration due to heavy metals can be categorised into three types: firstly, those diseases resulting from excessive environmental exposure, such as lead poisoning (Erwin, et al., 1995); the second group are disorders associated with aberrant depositions of various metals without excessive environmental exposure. A good example being Wilson's disease, associated with brain Cu. The third group are disorders in which the pathogenesis is due to an augmentation of the naturally occurring presence of metal ions, as seen in increased iron levels in PD. Erwin, et al (1995) suggested that abnormal metal deposition, in neurodegenerative disorders, is greatest in the basal ganglia in contrast to other regions of the brain. This aberrant increase of metals in the basal ganglia has been associated with the onset and progression of PD, and consequently, disruption of basal ganglia function and movement. However, it is unknown if neuronal cell death is a consequence of metal deposition or a secondary effect, arising from other mechanisms that indirectly lead to abnormal metal deposition. The latter is particularly true of Hallervorden-Spatz disease (Erwin, et al., 1995).

Since the advent of neuroimaging techniques, such as magnetic resonance imaging (MRI), with a quantitative analytical method, positron emission tomography (PET), and metaiodobenzylguanidine (MIBG)-cardioscintigraphy among others, the diagnoses of neurodegenerative diseases has become more accurate (Orimo, et al., 1999; Hirata, et al., 2005). Some metals have been associated with the onset and sometimes progression of neurodegenerative diseases such as Cu, Zn and Fe for AD, Fe for PD, Cu and Zn for familial ALS (Crichton, 2006). Table 1 gives a summary/overview of various neurodegenerative disorders and the implicated metal and metalloprotein or enzymes.

Table 1.1: Some neurodegenerative disorders with possible metal-associated pathology (Crichton, 2006)

Disorder	**Implicated Metal**	**Implicated Metalloproteins or enzymes**
PD	Iron	α-synuclein, neuromelanin, lactoferrin, ferritin, melanotransferrin, ceruloplasmin, divalent cation transporter
Alzheimer's disease	Cu, iron, zinc	Aβ, APP
Creutzfeldt-Jakob disease	Cu, Iron	Prion protein
Familial amyotrophic lateral sclerosis	Cu, Zinc deficiency	Superoxide dismutase I
Friedreich's ataxia	Iron	Frataxin, aconitase, mitochondrial proteins
Multiple sclerosis	Iron	Not known
Wilson's disease	Cu	Ceruloplasmin deficiency, Wilson's protein
Hallervorden-Spatz syndrome	Iron	Vitamin B5 metabolism
Huntington's disease	Iron, calcium	Huntingtin

There is regional variation in the distribution of metals in the brain. Functional and metabolic differences may account for some of these variations. For example, Fe has been noted to be higher in brain regions with higher motor activities (Gotz, et al., 2004). In pathological conditions, sometimes moderate to profound differences in regional metal distribution have been reported (Uitti, et al., 1989). Research from patients who had suffered from Parkinsonism (Uitti, et al., 1989) reported higher metal content (for some metals) in certain regions of the brain in PD patients and lower concentrations in PD patients compared to matched control for some metals (see Table 1.2).

The brain regions that are primarily involved in the modulation of motor activity (substantia nigra, caudate nucleus, frontal cortex, and the cerebellum) have been noted to have marked difference in metal content in patients with PD compared with a matched control. These brain regions are components of the extrapyramidal system and lesions therein are known to cause abnormalities in movement (Uitti, et al., 1989). For example, the pre-motor area of the cerebral cortex involved in the production of complex patterned movements and the caudate

nucleus involvement in the inhibition of spontaneous movement, have profuse connections with other areas of the brain concerned with motor control- lesions in the substantia nigra produce parkinsonism. While the cerebellum is in the coordination of voluntary movements required for normal motor action and gait.

Table 1.2: Metals concentrations by brain regions in PD and control brains

	Brain Region (µg/g dry weight.)							
Metal	**Substantia nigra**		**Caudate nucleus**		**Frontal Cortex**		**Cerebellum**	
	PD	Control	PD	Control	PD	Control	PD	Control
Cu	29.3	38.8	25.7	28.6	25.0	26.5	34.1	33.0
Al	10.2	7.7	2.9	10.7	5.0	5.2	1.4	7.9
As	0.2	0.2	0.1	0.1	0.1	0.2	0.1	0.1
Ca	468	623	468	630	506	554	410	618
Fe	653	613	610	651	280	295	268	325
Mg	414	452	471	530	482	539	515	560
Mn	6.8	3.0	6.6	3.6	2.6	1.9	3.0	3.1
Zn	68.8	78.6	72.5	71.0	63.0	51.2	64.8	76.9

-Adapted from Uitti, et al., 1989.

1.6 Metals implicated in the pathogenesis of PD

Metals are crucial in ensuring many cellular processes. Their concentrations within cells must be finely tuned because any aberration may lead to cell death and several diseases. In the brain, some enzymes and chaperones tightly regulate the metal ion content. They do this by controlling metal uptake and delivery to specific domains and by preventing a passive of flux of metals from the circulation. Besides, the blood-brain barrier also regulates the flux of metal from the blood into the brain by constituting a physical/physiological barrier (Outten and O'Halloran, 2001). In a healthy state, the concentrations of free metal ions are maintained at a very low level. Each physiological metal ion is selectively delivered to its site of action to tightly control redox activity. Table 1.3 gives an overview of the metals implicated in the pathogenesis of PD.

Table 1.3: Metals implicated in the pathogenesis of PD

Metals	Levels in PD	Brian regions	Role in disease pathogenesis	Reference
Fe	Elevated	Substantia nigra (SNpc), basal ganglia	Oxidative stress (caused by free Fe), free radical formation and loss of dopaminergic neurons of the SN as well as the deposition of intracellular inclusion bodies (Lewy bodies).	Dexter, et al (1989). Dexter, et al (1991). Montgomery (1995). Barrnham and Bush, (2008). Kozlowski, et al (2012).
	Decrease	Globus pallidus		Dexter, et al (1989). Dexter, et al (1991).
Cu	Decrease	SNpc	Decrease Cu level is accompanied by a loss of copper-dependent enzyme activities, for example, cytochrome *c* oxidase, superoxide dismutase 1, ceruloplasmin.	Barnham and Bush (2008). Binolfi, et al (2012).
Cu	Accumulation (other scholars noted an association between Cu accumulation and PD)	SNpc Basal ganglia nuclei	Leads to dopaminergic neurodegeneration, reduced striatal dopamine, loss of tyrosine hydroxylase projections, loss of neurons in the striatum and substantia nigra respectively- due to generation of neurotoxic reactive species (caused by oxidative stress). It has been shown to accelerate the oligomerization of α-synuclein monomers into neurotoxic fibrils,	Hitoshi, et al (1991). Oder, et al (1993). Gorell, et al (1997). Gorell, et al (1999). Uversky, et al (2001) Barthel, et al (2003). Rossi, et al (2004). Yu, et al (2008). Paris, et al (2009).

			which can impair mitochondria function.	
Zn	Elevated	Substantia nigra (SN), Caudate Nucleus	Though an antioxidant, elevated level in PD patients contributes to neurodegeneration.	Dexter, et al (1989). Forsleff, et al (1999). Barrnham and Bush (2008). Kozlowski, et al (2012).
Mn	Unchanged		Mn increases the rate of dopamine auto-oxidation in the SNpc leading to lipid peroxidation. In excess, it can inhibit mitochondria function, reduce glutathione levels, increase NMDA-mediated neurotoxicity, and alters calcium homeostasis (which culminate in cellular dysfunction). It is a potent mediator of pro-oxidant activities, through the production of (ROS), superoxide, peroxide, and hydroxyl radical.	Montgomery (1995). Caudle, et al (2012). Jenner (2003).
Hg	Not specific	Nigrostriatal dopamine Dopamine Transporters (DAT)	Impacts the normal functioning of the dopamine nigrostriatal dopamine system. Reduces DAT function in striatum. Reduces neurites and of dopaminergic neurons.	Yin, et al (2008). Lin, et al (2011).
Pb	Accumulation	SNpc	Causes a significant modification in the function of the nigrostriatal dopamine system, reduction in the number of dopamine neurons in the SNpc as well as a decrease in the firing rate of dopaminergic neurons. Disrupts Ca uptake through Ca channel.	Coon, et al (2006). Weisskopf, et al (2010). Tavakoli-Nezhad, et al (2001).

1.7 Parkinson's Disease

PD is the second most common form of neurodegeneration, first described, in 1817, by the English surgeon James Parkinson as 'The Shaking Palsy' (Crichton, et al., 2006). It is characterized by a progressive loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc), located in the ventral midbrain as well as Lewy body pathology (Crichton et al., 2006). Clinically, the disorder is characterised by motor symptoms, such as bradykinesia, muscular rigidity, resting tremor, and postural instability (Bonilla-Ramirez, et al., 2013). It is also characterised by non-motor symptoms, including disorders of mood that results in apathy, depression, cognitive dysfunction, hallucinations, as well as complex behavioural disorders (Chaudhuri and Schapira, 2009). Associated with these are common symptoms such as sensory dysfunction with hyposmia or pain (Shulma, et al., 2002; Fasano, et al., 2012; Pfeiffer, 2016).

The Queen Square Brain Bank has developed two steps diagnostic criteria for PD. The first step focuses on the definition of Parkinsonism which requires the presence of bradykinesia (plus at least one of tremor at rest, extrapyramidal rigidity, or postural instability) and certain exclusion and supportive criteria. Table 1.4 describes the clinical classification of PD (Gibb, et al (1988). Table 1.5 outlines the United Kingdom's PD Society Brain Bank Clinical Diagnostic Criteria (Hughes, et al., 1992).

Parkinsonian syndromes may be broadly classified into three categories: idiopathic PD; secondary Parkinsonism; Parkinsonism with neural system degeneration (Parkinson – plus syndromes) (Hughes, et al., 1992).

Table 1.4: Clinical classification of parkinsonian syndromes Gibb, et al., (1988)

Step 1. Diagnosis of a parkinsonian syndrome Bradykinesia and at least one of the following: Muscular rigidity. Rest tremor (4-6 Hz). Postural instability unrelated to primary visual, cerebellar, vestibular or proprioceptive dysfunction.
Step 2. Exclusion criteria History of: Cerebrovascular disease with a stepwise progression. Repeated head injury. Antipsychotic or dopamine-depleting drugs. Definite encephalitis and/or oculogyric crises on no drug treatment. More than one affected relative. Sustained remission. The negative response to large doses of levodopa (if malabsorption excluded). Unilateral features after three years. Other neurological features: supranuclear gaze palsy, cerebellar signs, early severe autonomic involvement. Babinski sign, early severe dementia with disturbances of: language, memory, or praxis. Exposure to a known neurotoxin.
Step 3. Supportive criteria Unilateral onset. Excellent response to levodopa. Rest tremor present. Progressive disorder. Persistent asymmetry, affecting side of onset most. Severe levodopa-induced chorea. Levodopa response for five years plus. The clinical course of 10 years plus.

Table 1.5: United Kingdom PD Society Brain Bank clinical diagnostic criteria.75-Hughes, et al (1992)

1. Idiopathic PD
2. Secondary (symptomatic) parkinsonism Post encephalitic Drug-induced (e.g., antipsychotics, metoclopramide) Arteriosclerotic (small vessel disease of the brain) Toxic (e,g., manganese, carbon monoxide, MPTP) Wilsons disease Post-traumatic Neoplastic Normal-pressure hydrocephalus
3. Parkinsonism in neural system degeneration Multiple system atrophy Striatonigral degeneration Shy-Drager syndrome Olivopontocerebellar atrophy Progressive supranuclear palsy (Steele-Richardson Olszewski) Alzheimer's disease Pallidonigral and corticobasal degenerations Diffuse Lewy body disease

The observed motor deficits are consequences of the loss of dopaminergic neurons and decrease in the amount of the neurotransmitter (dopamine) content in the striatum (Bonilla-Ramirez, et al., 2013), cytoplasmic inclusions of insoluble aggregated proteins (known as Lewy bodies) and elevated levels of iron (Cuervo, et al., 2010; Peng, et al., 2007; Fasano, et al., 2006; Rhodes and Ritz, 2008; Sian-Hulsmann, et al., 2011).

Although the aetiology of the disease is unknown, possible multifactorial factors have been deduced; with input ranging from a variety of genetic (Christine, et al., 2012), exposure to environmental toxins (Goldman, 2014; Sirisha, et al., 2016), gene-environmental interactions (Cannon and Greenamyre, 2013), endotoxin factors (Tufekci, et al., 2010), metals (such as iron) (Mark, et al., 2015) as well as ageing (Subramanian, 2010) nitric oxide metabolism and mitochondria respiration dysregulation (Park, et al.,2018).

It was noted that there is a low risk of developing PD among adults under 60 with no known genetic predisposition (mutations) and no history of prolonged exposures to environmental contaminants, such as heavy metals and pesticides (Abdullahi, et al., 2014). However, the risk of developing idiopathic PD radically increases after the age of 60. This age-related increase may be a result of some factors; the onslaught of endogenous and exogenous cellular insults on neurons over extended periods; accumulation of genetic mutations; a combination of both. Despite the premise that PD arises from polygenic inheritance and a possible consequence of gene-environmental interactions, over 90% of PD cases are sporadic, whilst approximately 5 - 10% are of familial origin, caused by gene mutations which results in the familial forms of the disease (Bonilla-Ramirez, et al., 2013). Fig.1.2 shows how ageing affects the progression of PD.

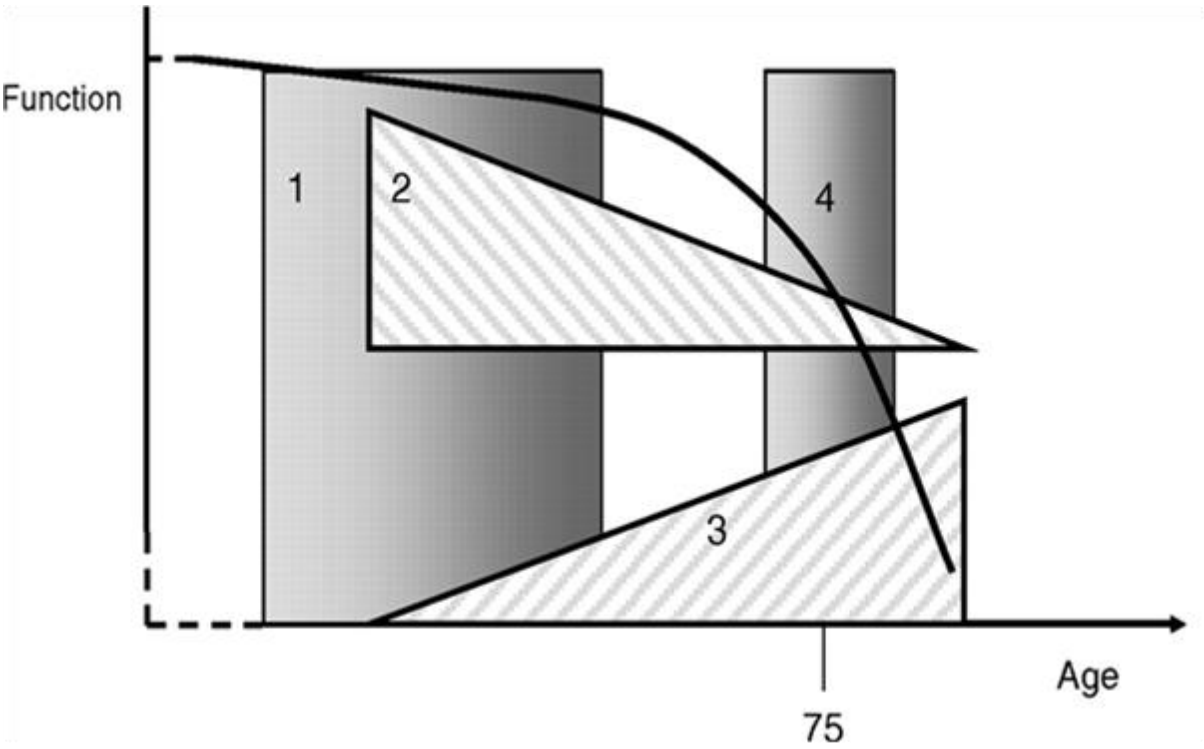


Figure 1.1: A proposed model of Aging and progression of PD; from Ageing, Neurodegeneration and Parkinson's disease– Hindle J, 2010.

(1)More rapid turnover of dopamine- more likelihood of dyskinesia (2) Reducing compensatory mechanisms (3) Increasing cellular dysfunction and accumulation of other pathologies (4) Aggregation of complications/more rapid progression.

Presently, no curative treatment is available for either the sporadic or genetic forms of Parkinsonism. Current treatment regimens are aimed at ameliorating the symptoms by replacing deficient dopamine – which improves motor activity. There is a great drive towards improving the quality of life of PD patients. As a result, translational clinicians/scientists are working hard to develop innovative treatment approaches for AR-JP patients based on our growing understanding of the genetics and related pathophysiology (Jankovic and Aguilar, 2008). In the quest to elucidate the underlying cause of PD, protein misfolding has been unravelled in the pathogenesis of PD.

In idiopathic PD, aggregation of heavily ubiquitinated WT (WT) α–synuclein is found in Lewy bodies within the dopaminergic neurons, axons, and synapses of the substantia nigra (Crichton, et al., 2006). The Synucleins are a family of small highly charged proteins, which are expressed in neural tissues (George, 2002). Two forms of these proteins, α-synuclein and β-synuclein are found in brain tissues, chiefly in the neocortex, hippocampus, striatum, thalamus and cerebellum; whilst the gamma-synuclein is found predominantly in the peripheral nervous system, that is, in motor neurons, sympathetic neurons and primary sensory neurons Perez, et al., (2004).

Alpha-synuclein plays a vital functional role in the modulation of dopamine release (as a regulatory protein) by binding to and inhibiting tyrosine hydrolase. This enzyme is crucial in the biosynthesis of dopamine- this enzyme is delineated as a rate-limiting enzyme. Eriksen, et al (2003) observed that the dopaminergic neurons are peculiarly and selectively vulnerable to the toxic effects of α-synuclein. Excessive production of WT α-synuclein exhibits a triplication of the α-synuclein gene, leading to an autosomal dominant PD (Singleton, et al., 2003). The presence of Lewy bodies in the affected regions of the brain indicates the misfolding of these proteins (α-synuclein).

Dauer, et al (2002) highlighted the relevance of α-synuclein in the development of PD. They exemplified this in a knock-out and knock-in mice for the gene. They demonstrated in their studies that mice lacking this gene have decreased striatal dopamine function, a reduction in motor response to amphetamine and an increase in the release of dopamine following a paired-

pulse stimulus. Increased levels of α-synuclein in transfected human culture foetal dopaminergic neurons lead to apoptotic cell death. It was observed that inhibition of dopamine synthesis (in these cells), prevented apoptosis. This may be due to a reaction between α-synuclein and endogenous levels of dopamine in the generation of ROS (Dauer, et al., 2002).

An association/link between α-synuclein and Fe was demonstrated *in vitro*. It was observed that Fe enhanced intracellular aggregation of α-synuclein (Ostrerova, et al., 1999; Golts, et al.,2002; Hasegawa, et al., 2016). *In vitro* studies lead to the formation of advanced glycation end products (Uversky, et al., 2001; Munch, et al., 2000) while α-synuclein liberated hydroxyl radicals when incubated with Fe^{2+} (Tabner, et al., 2001).

Iron is a key driver in the oxidative process leading to the pathogenesis of PD. As an unavoidable consequence of ageing, there is an increase in the levels of brain Fe in certain regions of the brain such as in the putamen, prefrontal cortex, motor cortex, sensory cortex, and thalamus (Crichton, et al., 2006). It is localised within the H- and L-ferritin in the substantia nigra (Zecca, et al., 2001; 2004) and neuromelanin (Zecca, et al., 2004) with no apparent adverse effects. There is significant variation in the distribution and concentration of Fe in different brain regions.

Areas associated with motor function tend to have more Fe than non-motor related regions. The main differences in the brains of PD patients and those of normal subjects are that there are clearly defined 2-fold elevation of Fe in the substantia nigra and the lateral Globus pallidus, in comparison with age-matched controls (Gotz, et al., 2004). Metal dyshomeostasis is strongly implicated in the pathogenesis of PD and it was one of the earliest forms of chronic neurodegenerative diseases associated with dysregulation of metal homeostasis.

The earliest research on the possible effect of metal perturbation and PD dates to the works of Hallgren and Sourander 1958 (Editorial, Neurobiology of Disease, 2015), where they describe the increased levels of iron in the substantia nigra of PD patients. Later studies showed alternations in the levels of metals in the brain of PD patients, post-mortem compared to non-

PD match controls of similar age (Wenstrup, et al., 1990). Kumudini and his colleagues (2014) demonstrated that elevated iron levels stimulated oxidative stress in PD and metal-induced oxidative stress is implicated in the pathogenesis of PD (Kumudini, et al., 2014).

A further role of metals in the pathological process of the disease was elucidated based on observational studies which evaluated occupational and environmental exposures as well as levels of metals in biological fluids employing case-control study designs (Cicero, et al., 2017). Taba, et al (2002) observed that the prevalence rates of PD in Europe (using Estonia as a case study) are not significantly higher in urban areas than rural ones. In Africa, a case-control study on risk factors for PD in southwestern Nigeria found blacksmithing (OR 8.0 95% CI 1.3-50.7)[6] to be significantly associated with PD (Falope, et al., 1992; Akinyemi, 2012; Akinyemi and Okubadejo, 2010).

Environmental link in the aetiology of PD was underscored by the landmark epidemiological study of about 20,000 pairs of twins. The findings in this study show no definite genetic cause was identified to explain the occurrence of PD. This finding made the researchers conclude that PD was an environmentally influenced disorder (Tanner, et al., 1999).

Several environmental risk factors have been associated with PD. Lauretti, et al (2017) posited that circadian rhythm dysfunction is a risk factor for developing PD. Besides, a notable shift towards environmental risk factors was accentuated when illicit drug users inadvertently generated a product of narcotic synthesis, MPTP (1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine). which was reported to have resulted in Parkinsonism, among users.

Furthermore, exposures to different pesticides (such as rotenone, paraquat i.e. N, N'-dimethyl-4, 4'—bipyridine dichloride), maneb, and manganese, ethylene-1, 2-bisdithiocarbamate, polymer among others have been associated with an increased risk of PD (Bjorklund, 1995; Bjorklund, 1997; Goldman, 2014; Bjorklund, 2018; Bjorklund, 2018). In addition to pesticides, some commonly encountered environmental toxicants have been implicated in PD for

examples of heavy metals, solvents, and several other pollutants (Bjorklund, 1997; Goldman, 2014).

Chronic environmental exposure to metals is another risk factor implicated in PD. Exposure to a low dose of certain metals, cumulatively, results in accumulation of these metals in the brain. For example, studies in a rat showed that there is an interaction between Mn and Fe from the plasma to the brain and other organs (Gorell, et al., 1999). Gorell and his colleagues (1999) demonstrated that these metal transfer across the blood-brain barrier (BBB) is synergistic rather than competitive. Thus, suggesting that excessive intake of Fe alongside Mn will accentuate the risk of tissue damage in the brain than by one metal alone. Chronic exposure of rats to Mn alters Fe homeostasis by causing a unidirectional influx of Fe from systemic circulation across the BBB (Zheng, et al., 1999).

Earlier studies by Olanow, et al (1996) showed the deleterious effect of chronic exposure of metals. In their investigation, they observed L-DOPA resistance Parkinsonian syndrome in Rhesus monkeys exposed to Mn and a focal mineral deposit in the Globus pallidus and substantia nigra pars reticularis, consisting primarily, of iron and aluminium. Zn and Mn influence the neurotransmitter concentrations in the synaptic cleft by modulating neurotransmitter receptors and transporters as well as iron channels (Takeda, 2004). Specifically, Zn is seen to act as an inhibitory neuromodulator to glutamate release in the hippocampus while manganese induces both functional and toxic effects in the synapse (Takeda, 2004).

In vitro Mn has been shown to induce cell death in dopaminergic cells such as SH-SY5Y and CATH.a cells, causing an increase in the endoplasmic reticulum (ER) stress-associated genes, inclusive of parkin (Higashi, et al., 2006). Concomitant exposure of these cell lines to Cu and Mn shows enhanced cytotoxicity and clastogenicity of levodopa and dopamine (Snyder and Friedman, 1998).

1.8 Mitochondrial dysfunction and PD

Several lines of evidence strongly suggest mitochondrial dysfunction as a major causative factor in PD. There is ongoing intensive research to unravel the molecular mechanism. At the cellular level, mitochondria play a key role, both as regulators of ATP production via oxidative phosphorylation and as regulators of intracellular calcium homeostasis and production of reactive oxygen species (Crichton, 2006). Excitotoxicity is associated with the mitochondria. This is due to increased mitochondrial calcium overload which results in the generation of ROS leading to the release of pro-apoptotic mitochondrial proteins; culminating in the demise of the cell by either apoptosis and/or necrosis (Rego and Oliveira, 2003).

Besides, mitochondria play a unique role in regulating the factors that either suppress or promote protein misfolding, such as molecular chaperones, the protein degradation system, and free radical production. Mitochondria defects can lead to the aggregation of α-synuclein. The translocation of the misfolded proteins to the mitochondria membrane plays an important role in activating or perpetuating neurodegeneration by causing changes in membrane permeability and the release of cytochrome c (Crichton, 2006).

In the striatum of PD patients, there is a defect in mitochondria oxidative phosphorylation. This defect results in a reduction in the activity of NADH CoQ (known as complex 1). The reduced activity of complex 1 is specifically found in the SNpc, and not in other parts of the brain. Such specificity plays a significant role in the degeneration of nigrostriatal dopaminergic neurons (Ebadi, et al., 2001). Further investigations suggest that the inhibition of complex 1 may be the central cause of sporadic PD and iron accumulation in the mitochondria, both *in vitro* and *in vivo,* adversely alter complex 1 (Crichton, 2006). Besides, disorders in complex 1 cause aggregation of α-synuclein and this contributes to the demise of dopaminergic neurons.

Other mitochondria complexes (II and IV) are also implicated. For example, the production of Nitric oxide (NO) - either from the mitochondria or inducible NOS within the cell cytosol inhibits components of the mitochondria respiratory chain, complexes I, II and IV (precisely in a reduced state of GSH). This cause a cellular energy-deficient state (Brown, 1999; Brown, 2001; Brown and Borutaite, 2002; Brown and Borutaite, 2007).

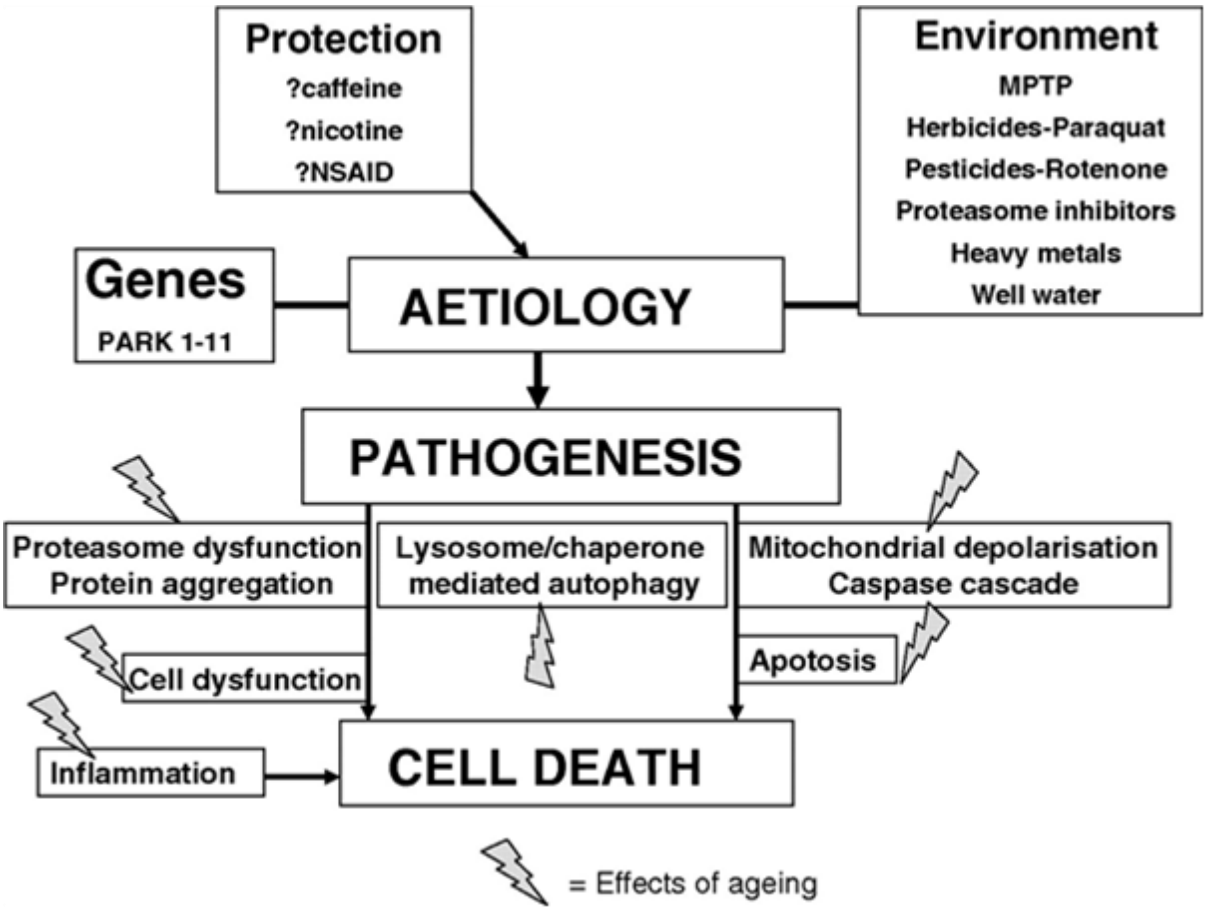


Figure 1.2: Overview of the aetiology and pathogenesis of Parkinson's disease- Hind J, (2010).

The pathogenesis of PD is multifactorial, and this is orchestrated by a stream of events (see Fig. 1.4) which ultimately leads to neuronal cell death. These include oxidative stress, impaired mitochondrial function, excitotoxicity via glutamate pathways, protein misfolding and aggregation due to ubiquitin proteasomal system dysfunction, impaired lysosome, and chaperone-mediated autophagy (Hindle, 2010). Associated with these is the development of cytoplasmic inclusion bodies called Lewy bodies that contain neurofilament proteins and ubiquitinated α-synuclein. Besides, inflammation and humoral immune reactions have been noted to contribute to the processes linked to cell death through apoptosis (McNaught, 2002). Most of these mechanisms align with the changes in ageing. Many environmental agents may be inhibitors of proteasomal function and, in rat models, proteasomal inhibition alone can reproduce the key features of PD.

1.9 Role of Dopamine in PD

In the healthy state, dopamine is produced in the SN and transmitted to the putamen and caudate nucleus with a resultant inhibitory effect on movement. In contrast, acetylcholine, which is synthesised in the basal ganglia, has an excitatory effect on movement. A fine balance between these two systems (dopaminergic and cholinergic) must be maintained to ensure a fine gait and movement. The degeneration of dopaminergic neurons in the SNpc (as seen in PD) results in an imbalance between acetylcholine and dopamine - the shift of balance towards cholinergic activity produce the characteristic symptoms of rigidity, tremor, and bradykinesia.

'Oxidative stress hypothesis' has been proposed to explain the role of oxidative stress vis-a-vis the degeneration of dopaminergic neurons (Kim, et al., 2002). During a normal life span (70-80 years), there is about 3-5% decrease of dopaminergic neurons every decade. However, at the time of diagnosis of PD, there is a loss of about 60-80% of dopamine-containing neurons. This hypothesis insinuates the initiation of nigral dopaminergic neuron loss with the depletion of glutathione, implying PD may be a consequence of aberrant oxidation of dopamine.

Cellular metabolism of dopamine generates H_2O_2 which is reduced to hydroxyl radicals in the presence of iron thereby propagating neuronal damage (Kim, et al., 2009). The antioxidant, glutathione (GSH) helps to maintain a healthy milieu in the SN neuronal cells against oxidative stress. A shift in the balance between form prooxidant to antioxidant result in the death of the nigral dopaminergic neuronal cells, by apoptosis (See Fig. 2).

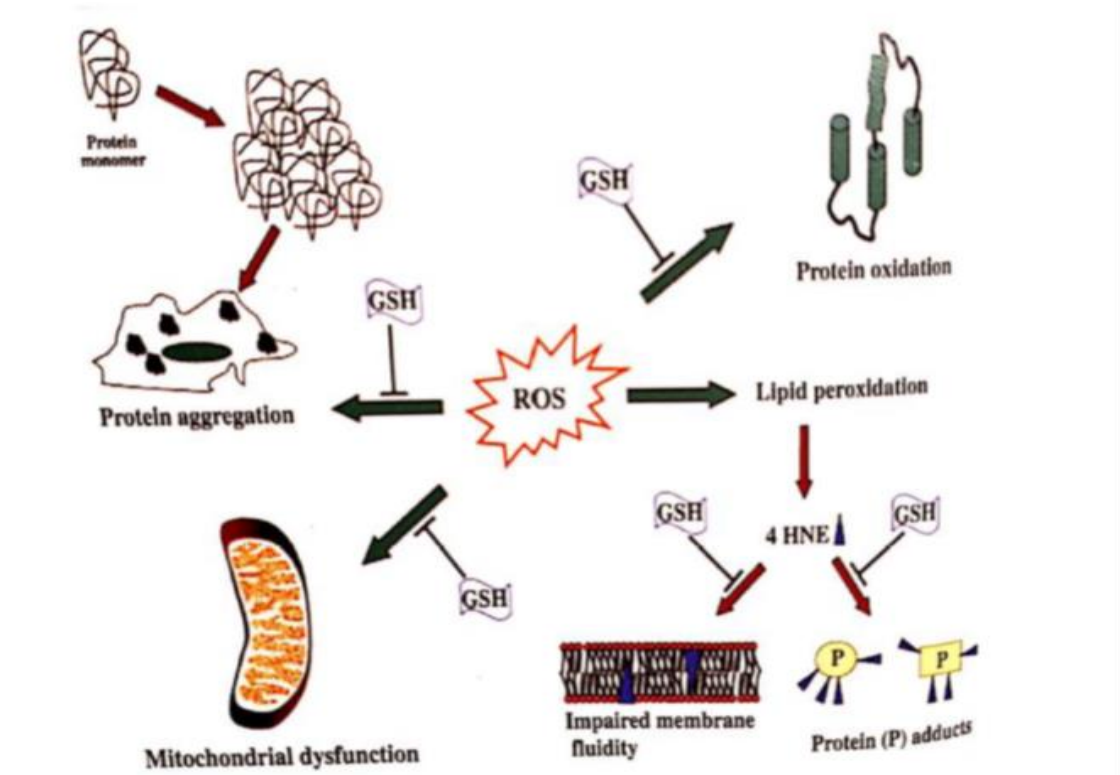


Figure 1.3: The antioxidant properties of GSH as relevant to SN dopaminergic neuronal cells in PD – Crichton, et al., 2006.

Metal Dysregulation and PD

Heavy metals play a critical role in many neurological diseases. Several epidemiological studies have shown an association between PD and exposure to metals, some of which include mercury, lead, manganese, Cu, iron, aluminium. Metal accumulation in the nervous system inevitably leads to a toxic environment, resulting in a predicted pattern often associated with metal toxicity; oxidative stress, mitochondria dysfunction and protein misfolding (6-13). A damaged neuron requires a greater amount of energy to synthesize neurotransmitters, as well as maintain homeostasis. The resulting burden combined with neurotoxicity may lead to neuronal death. Given that the nervous system regenerates poorly, the resultant impairment and the associated neurodegeneration become progressive with age, which is typical of PD.

The contribution of metals in the aetiology of PD is very crucial; attracting attention, great interest to both neurotoxicologist and medicinal chemist (Montgomery, 1995; Chin-Chan, et al., 2015; McAllum, et al., 2016; Schuh, 2016; Lucchini, et al., 2017). As previously observed, PD falls within the group of disorders in which the pathogenesis may be due to the abnormal function of a normally present metal. The role of metals may either be as metallic toxicants or through depletion of metals that are essential for biological activities and maintenance of health (Bjorklund, et al., 2018).

1.9.1 PD and Iron

Iron is an essential element which is crucial for physiological processes in several organs, including the brain. Its role in the pathophysiology of PD has been extensively studied and its neurotoxic effect has been well reported (Obeso, et al.,2000; Double, et al., 2003; Castellanos et al., 2005; Moore, et al., 2005). Iron deficiency impairs health functions in both the central and peripheral nervous system and has been implicated in PD with restless leg syndrome because it results in depletion of brain dopamine and 5-HT levels (Piao, et al., 2017).

Double, et al., (2003) demonstrated that microinjection of Fe in the SN has a neurotoxic effect on tyrosine hydroxylase immunopositive neurons. Accumulation of iron in deep grey matter nuclei in the basal ganglia and midbrain have been associated with PD pathology as well (Xuan, et al., 2016; Xuan, et al., 2017). Uneven distribution of Fe in the brain has been reported

(Dexter, et al., 1989; Riederer, et al., 1992; Behnke, et al., 2003; Oakley, et al., 2007). Examination of postmortem PD brains have shown increased levels of total Fe in the SNpc, caudate nucleus, putamen, or cerebral cortex, but not in the cerebellum, - an index to the fact that the underlying mechanisms for Fe accumulation may be specific for the SNpc (Behnke et al., 2003; Dexter, et al., 1989; Oakley, et al., 2007; Riederer, et al., 1989). In contrast, there is a reduced level of this metal in the Globus pallidus compared to control values (Dexter, et al., 1989).

There is ambiguity about the presence of increased Fe and the onset of neurodegeneration. Riederer, et al (1989) in their investigation, noted the insignificant difference in Fe content in brain tissues showing moderate neurodegeneration, implying that Fe accumulation in PD may be a possible consequence of underlying mechanisms of neuronal death or else its levels should be expected to increase in the early stages of the disorder. Some underlying mechanisms may initiate neuronal death at the early onset of the disorder leading to Fe accumulation which, in turn, may potentiate oxidative damage (See Fig. 1.3).

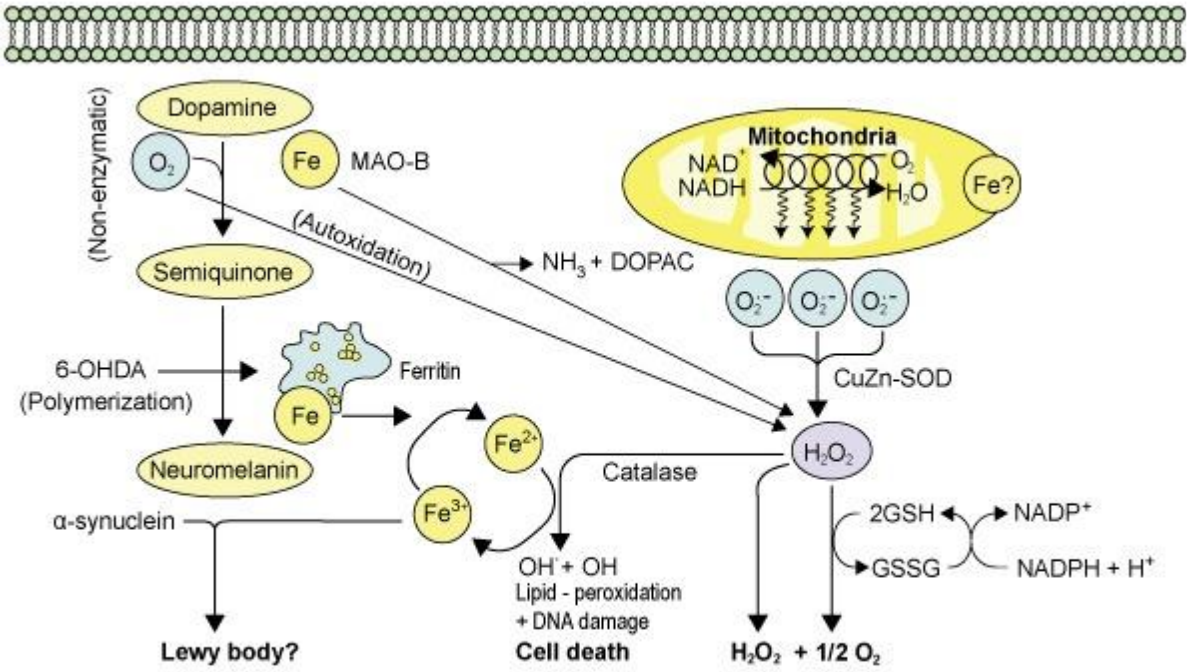


Figure 1.4: Fe induces oxidative stress in PD, resulting in the death of the nigral dopaminergic neuronal cells by a process of Adapted apoptosis (Crichton, et al., 2006)

Behnke, et al (2003) in their studies found ferritin-reactive microglia around degenerating neurons. This is interesting, since ferritin is the main Fe-binding protein, and the result suggests that Fe accumulates within microglia cells. This has invariably thrown up some hypotheses. On one hand, is the speculation that microglia release Fe that could be toxic to the surrounding neurons. On the other hand, that Fe may accumulate in both neurons and microglia but due to the greater sensitivity of neurons to the toxic effect of this metal, which possibly explains why only, the surviving ferritin-positive microglia may be found in post-mortem brain tissues surrounding degenerating neurons (Rivera, et al., 2010). This, therefore, underpins the need for an investigation of the role of microglia in brain Fe accumulation.

Advancements in neuroimaging techniques have made it possible to quantify and establish the distribution of metals in the brain. Through neuroimaging, the content of Fe is seen to increase in PD in the SNpc in living patients. For instance, several investigators have been able to establish, by transcranial sonography, that the SN in PD patients is associated with a high ultrasound echo (referred to as "hyperechogenic"). This reflects the deposition of metal in this region of the brain (Behnke, et al., 2003; Becker, et al., 2001; Rivera-Mancia, 2010; Martinez-Hernandez, et al., 2011).

In their investigation of vulnerability factors for PDs, Behnke, et al (2003) made the following observations: firstly, that hyperechogenicity may be present in up to 90% of PD patients and may also be found in healthy subjects, but in the latter, it is a reflection of nigrostriatal dysfunction, because of its association with decreased [18F] DOPA uptake (Berg, et al., 2002; Behnke, et al., 2003; Wegrzynowicz, 2018). Secondly, they also observed that hyperechogenicity in healthy elderly subjects was associated with motor alterations, such as hypokinesia (Behnke, et al., 2003). This increase response during ultrasound examination of the brain, they suggested may likely be due to Fe accumulation, since the echogenicity with post-mortem human brain tissue, correlates well with Fe content, but not so with Cu, magnesium, Zn or calcium (Behnke, et al., 2003; Berg, et al., 2002). Other imaging findings by Vymazal, et al., (1999) and Brar, et al., (2009) further support Fe accumulation in SNpc in PD and its role in nigrostriatal dysfunction in this disorder. Taken together, all these findings strongly implicate Fe as a major player in the aetiology of PD.

It is very vital to note also that Fe content in the brain increase during normal ageing and it is often associated with a decrease in motor performance (Thomas, et al., 1993; Bartzokis, et al., 1994; Bartzokis, et al., 2007). However, when compared with those of PD patients, it has been established that Fe levels are higher than expected compared to the normal ageing process (Focht, et al., 1997; Wayne, et al., 2004).

Several investigators have made some attempts to elucidate the possible mechanism underlying the increase in brain Fe content in PD. One significant observation is based on the fact that excitotoxicity might be involved in PD, which invariably leads to Fe accumulation. Since Fe uptake is enhanced by N-Methyl-D-Aspartate (NMDA) receptor activation, via nitric oxide (NO) signalling (Munoz, et al., 2011). Glutamate and NO are involved in excitotoxic death (Chen, et al., 1995), which has been proposed to occur in PD (Wang and Qin, 2010; Dong, et al., 2009; Koutsillieri and Riederer, 2007). This may imply that Fe accumulation in PD may be associated with neuronal death through glutamate receptors (Dugan, et al., 1994; Jellinger, 1999; Lau and Tymianski, 2010). Cheah et al., (2002) in their investigations supported this neurotoxic pathway, by establishing the fact that Fe chelation reduces NMDA-induced excitotoxicity.

Further evidence implicating Fe in the possible aetiology of PD is the effect of the Cu protein, Ceruloplasmin (Cp) on the oxidation state of Fe. Cp and hephaestin oxidize Fe^{2+} to $Fe3^{+}$ to facilitate iron removal (Loeffler, et al., 1996; Qian and Ke, 2001; Popescu and Nichol, 2011). Jiang, et al., (2015) demonstrated this in a double knockout mouse lacking both cap and hephaestin, by iron overload and neurodegeneration. Furthermore, Jiang, et al (2015) and Wang, et al (2015) have explored the role of these two proteins in PD. In their investigations, using a model of 6-hydroxydopamine (6-OHDA), they observed a decreased expression of hephaestin (Wang, et al., 2007); while Hochstrasser, et al (2004) found an association between mutations of Cp genes and PD. These suggest that iron overload may be in part a consequence of altered oxidation of Fe, preventing its extrusion from neurons.

Evidence implicating Fe involvement in the pathophysiology of PD was demonstrated by Hare, et al (2013). They found an upregulation of divalent metal transporter 1(DMT1) in the SNpc of PD patients as well as the SNpc of mice exposed to 1-methyl-4-phenyl-1, 2, 3, 6 tetrahydropyridines (MPTP) - an established neurotoxin known to induce several features of PD. Zhang, et al (2009) verified these findings when they treated dopaminergic cell lines (MES23.5) with an active metabolite of MPTP- 1-methyl-4-phenylpyridinium (MPP^+). They observed an upregulation of DMT1 which was correlated with Fe accumulation. Besides, it was observed that rodents that carry a mutation that impairs DMT1 Fe transport were protected from injury caused by both MPTP and 6-OHDA. Fe as divalent metal, therefore, plays a critical role in the pathogenesis of PD. Once Fe accumulates in PD, it enhances neuronal death, through oxidative stress.

Fe also induces lipid peroxidation (Muller, et al., 1985; Minotti and Aust, 1992; Oteiza, 1994) and increases ROS production by the autoxidation of 6-OHDA (Mendez-Alvarez, et al., 2001). Also, Fe stimulates the formation of intracellular aggregates of α-synuclein and favours oxidative damage. It has been established (Narhi, et al., 1999; Lee, et al., 2002; Li, et al., 2005) that familial PD is associated with mutations in α-synuclein which enhances the aggregation of the protein (Narhi, et al., 1999). This, therefore, implies persons with mutations in α-synuclein could be more susceptible to oxidative damage by Fe. The Fe-induced oxidative damage is mediated through the Fenton reaction, in which hydrogen peroxide (H_2O_2) (Meneghini, et al., 1993). The source of the H_2O_2 in this reaction, among several sources, maybe from monoamine oxidase activity and from Fe-induced oxidation of dopamine (Campos, et al., 2015). This leads to the enhancement of this damaging mechanism. Aracena, et al (2006) reported an increase in glutathione biosynthesis in survival cells after Fe overload-implicating oxidative stress as a direct precursor Fe neurotoxicity.

1.9.2 PD and Cu

As an essential trace element, Cu acts as a co-factor of several enzymes, such as cytochrome c oxidase and SODs (Banci, 2013; Desai, et al., 2008), and plays a vital role in electron transport, oxygen transport, protein modification and neurotransmitter synthesis (Banci, 2013; Desai, et al., 2008). Various studies have shown that Cu may lead to both toxic and protective effects under certain experimental conditions. In peripheral tissue, Dexter, et al (1989) and Popescu,

et al (2009) reported incidence of toxicity with Cu and at the same time noted some protective effects in cases of Cu deficiency.

According to Rivera-Mancia et al (2010), cases of toxicity arising from a high concentration of Cu were not directly associated with PD, whilst decreased levels of Cu has been reported in PD (Dexter, et al., 1989; Popescu, et al., 2009). The protective role of Cu becomes relevant in cases of Cu-deficiency in PD (Rivera-Mancia, et al., 2010). Excessive Cu has been associated with increased generation of ROS, DNA, and mitochondrial dysfunction. Cu has been shown to enhance the self-aggregation of the β-amyloid peptide (20), increased level of Cu in the Cytochrome. In patients with PD disorders, the concentration of Cu is altered in both the CSF and brain (56,117) and this has been implicated in the pathophysiology of this disorder. Free Cu has been implicated in oxidative stress mechanisms, alpha-synuclein oligomerization and Lewy body formation, as well as GABA-A and NMDA receptor neurotransmission modulation. Cu can influence iron content in the brain through ferroxidase ceruloplasmin activity (Montes, et al., 2014).

1.9.3 PD and Manganese

At higher concentrations, Manganese (Mn) has been shown to exert neurotoxic effects resulting in impairment of dopaminergic, glutamatergic, and GABAergic transmission, as well as mitochondrial dysfunction, oxidative stress and marked neuroinflammation (Cicero, et al., 2017). The principal anatomic target of manganese neuronal accumulation is the Globus pallidus in the basal ganglia, leading to Parkinsonism (Tuschl, et al., 2013). A pathognomonic sign of such accumulation from Magnetic Resonance Imaging (MRI) is a T1-weighted image, showing an appearance of hyperintense basal ganglia (Tuschl, et al., 2013).

Manganism may either be congenital or acquired. Quadri, et al (2012) reported an autosomal recessively inherited disordered of manganese metabolism which is caused by a mutation in the SLCC30A10 gene. Acquired causes of manganism maybe due to intravenous use of methcathinone, excessive nutritional intake, or impaired hepatic excretion of manganese, which may lead to hepatocerebral degeneration (Ferrara and Jankovic, 2009).

Several epidemiological studies have investigated the possible link between chronic manganese exposure as an environmental factor and PD. Results from such studies show that occupational exposure for a duration greater than 30 years (Lai, et al., 2002; Sayed, et al., 1990), 20 years (Gorell, et al., 1997) and 10 years (Recite, et al., 2017) were consistent and showed that progression of Parkinsonism increased with cumulative manganese exposure. Persons with manganese-induced Parkinsonism have a clinical resemblance to idiopathic PD, though there is a preservation of dopamine synthesis in manganism (Tuschl, et al., 2013). In case-control studies that evaluated blood levels of manganese in PD subjects compared to controls, the outcomes reveal a positive association between high manganese levels and PD (Ahmed and Santosh, 2010; Hegde, et al., 2004; Kumudini, et al., 2014; Squitti, et al., 2007a). However, less consistent results were obtained from CSF analysis in which higher levels of manganese was reported in a study (Hozumi, et al.,2011). Six other investigators reported no substantial difference between PD subjects and match controls (Alimonti, et al., 2007; Bocca, et al., 2006; Forte, et al., 2004; Gazzanigga, et al., 1992; Jimenez, et al., 1998; Pall, et al., 1987).

1.9.4 PD and Selenium

Selenium (Se) is an essential trace element. It is specifically incorporated into selenoproteins, in the form of selenocysteine. The human selenoproteome consists of 25 proteins and it is classified into housekeeping and stress-related proteins. Se exerts a very vital antioxidant activity through its oxidoreductase functions, such as the glutathione peroxidase which protects against hydrogen peroxide and lipid peroxides, which are influenced by dietary selenium levels (Cicero, et al., 2017). Dysregulation of Se may result in an imbalance which may increase oxidative stress implicated in neurodegeneration (Ellwanger, et al., 2016).

Data from case-control studies reported ambiguous results, while some studies suggested higher blood levels of Se in PD as compared to controls (Quareshi, et al., 2006; Zhao, et al., 2013), others suggested lower levels (Ahmed and Santosh, 2010; Hegde, et al., 2004). A larger proportion of investigators on this subject reported no relevant difference between groups (Baillet, et al., 2010; Fukushima, et al., 2010; Gellein, et al., 2008; Takahashi, et al., 1994; Younes-Mhenni, et al., 2013).

1.9.5 PD and Zinc

Zinc is essential for most physiological functions and its status in the human body is affected among others, by factors that affect homeostasis (Maret, 2013; Sandstead, 1991). Unlike other transition elements, Zn is redox neutral and readily binds to proteins with the appropriate amino acid motifs- and there are well over 3000 proteins known to have such signatures (Andreini, et al., 2006). This explains the ubiquity of zinc in several physiological functions, ranging from enzymes and transcription factors to signalling proteins (inclusive of storage proteins, proteins with structural metal sites, as well as those involved in DNA repair, replication, and translation).

Zn plays a vital role in PD neurodegenerative process by impinging on lysosomal functions. A compromised autophagy-lysosomal pathway is increasingly implicated in the pathogenesis of neurodegenerative diseases, including PD (Cicero, et al.,2017). In an earlier study, Dehay, et al (2013) in their investigations, observed a link between PD and mutations in lysosomal-related genes (such as glucocerebrosidase) and lysosomal type 5 ATPase. For example, mutations in PARK9 results in Kufor-Rakeb syndrome, which is characterised by juvenile-onset Parkinsonism, pyramidal signs, and dementia (Cicero, et al., 2017).

Tsunemi and Krainc (2014) reported that the loss of PARK9 results in the dyshomeostasis intracellular zinc levels, which contributes to lysosomal dysfunction and, subsequently, the accumulation of alpha-synuclein. Despite the plethora of laboratory investigations implicating Zn in the aetiology of the PD neurodegenerative process available case-control studies, evaluating environmental/occupational exposure to Zn have failed to demonstrate any significant association with PD (Gorell, et al., 1997; Lai, et al., 2002; Seidler, et al., 1996). However, results from studies on blood Zn levels in PD and matched controls support a possible inverse association (Ahmed and Santosh, 2010; Hegde, et al., 2004; Squitti, et al., 2007a; Zhao, et al. ,2013).

1.9.6 PD and Mercury

Elevated levels of mercury (Hg) has been linked with the incidence of PD (Ohlson, et al., 1981). Also, a parallel has been drawn between the effects of Hg exposure (or ingestion) and the associated effects of PD (Bjorklund, et al., 2018). It has been shown that exposure to Hg results in loss of dopamine receptors, tubulin degeneration, axon degeneration and glutathione depletion (Bjorklund, et al., 2018). Besides, an increase in glutamate level is seen in persons exposed to Hg over a long period as well as tau phosphorylation, mitochondria dysfunction and increase in amyloid-β level (which promotes α-synuclein aggregation) (Bjorklund, et al., 2018). All these observed effects of Hg exposure have corresponding similarities in symptoms of idiopathic cases of PD.

Studies have shown that there is a six-fold frequency of detectable blood Hg levels in persons with PD compared with match controls (Dantzig, 2006). Exposure to Hg has a deleterious effect and it is implicated as a significant risk factor in the aetiology of PD. Ngim, et al (1989) in their epidemiological studies, where they examined the association between body burden and mercury level and idiopathic PD, observed an 8-fold increase in the risk of developing PD. Occupational exposure is a great source of Hg toxicity and in most industrialised countries, the use of dental amalgams has been directly linked with elevated mortality of PD (as well as dementia) has been well documented (Bjorklund, et al., 2018).

1.9.7 PD and Other Metals

Some other metals with neurotoxic effects have been associated with secondary Parkinsonism. Although data showing a possible association between PD and other metals are poorly represented in literature, some have shown a positive association, such as nickel (Ahmed and Santosh, 2010), cadmium (Chen, et al., 2016) and Lead (Chen, et al., 2016) and thallium (Galvan-Arzate, et al., 1998). Notable among these are cadmium and lead. Cadmium (Cd) is a transition heavy metal with no known biological activity and it is known to be carcinogenic (Bjorklund, et al., 2018). It can easily be absorbed through the nasal mucosa or the olfactory bulb, thereby, destroying the Blood-Brain Barrier (Wang, et al., 2013).

In vitro studies have revealed that Cd can induce oxidative stress, suppresses gene expression, and inhibits DNA damage repair and apoptosis. Wang, et al (2013) while investigating the neurotoxic effect of cadmium, observed that Cd may interfere with the normal functions of the nervous system and that infants and children were more susceptible than adults. Chronic exposure to Cd has been linked as a possible aetiological factor of neurodegenerative disease, including PD (Okuda, et al.,1997), presenting with symptoms such as headache, olfactory dysfunction, slowing of vasomotor functioning, decreased equilibrium and PD-like symptoms (Wang, et al., 2013).

Just like Cd, lead (Pb) is a non-essential heavy metal and a ubiquitously distributed pollutant in the ecosystem. The major route of entry into the body is by inhalation and oral ingestion (Chen, et al., 2016). Lead exposure has been shown to result in oxidative stress, mitochondria dysfunction, and disruption of Ca^{2+} homeostasis. The principal target of Pb-induced toxicity is the nervous system (Chen, et al., 2016). Another naturally occurring trace element that is extremely toxic is thallium (Cvjetko, et al., 2010). Exposure to thallium (Tl) interferes with several K^{+}-dependent processes because of the similarities in size between K and Tl, as well as the univalent nature of both ions. One of the affected processes is the generation of ATP (Galvan-Arzate, et al., 1998; Ibrahim, et al., 2006; Eskandari, et al., 2010). At the cellular level, elevated levels of Tl have been shown to cause a decrease in ATP production, increase in ROS formation, glutathione oxidation and decrease in dopamine levels in the brain (Budtz-Jorgensen, et al., 2007; Eskandari, et al., 2010).

1.10 Synergistic Toxicity

The toxicity of metals can be accentuated with combined exposure. Metal synergistic effects were seen between metals and PD with combined exposures of iron-Cu, lead-Cu and lead-iron when compared to the effects of single metals (Monte, et al., 2002; Bjorklund, et al., 2018; Bjorklund, et al., 2018). The synergistic effect of the metal combination is very pronounced with Hg. When combined with other metals such as aluminium, manganese, zinc, and cadmium. It significantly exacerbates Hg toxicity even at low non-toxic doses (Blanusa, et al., 2005; Blaurock-Busch, et al., 2012). Similar synergistic effects are seen with metals combination with pesticides (Pape-Lindstrom and Lydy, 1997; Singh, et al., 2017; Uwizeyimana, et al., 2017).

In animal studies designed to investigate the acute effect of low levels of Pb, Hg and Mn individually and in combination on the central and peripheral nervous activities, Papp, et al (2006) observed a pronounced synergistic effect. Also, Haley (2005) demonstrated accentuated metal toxicity arising from its synergistic effect by combining Hg with safe levels of aluminium hydroxide or the antibiotic neomycin. This resulted in a significant increase in neuronal mortality. Similarly, Zn also exacerbated the toxicity of Hg, by increasing cytotoxicity and the inhibition of tubulin (Boyd, 2007; Zahir, et al., 2005). Interestingly, a PD-associated protein (DJ-1protein) with protective functions, can bind both Hg and Pb. The genetic variant of DJ-1 protein does not exert any protective effect on Hg toxicity and therefore increases the risk for PD (Migliore and Coppede, 2009; Dias, et al., 2013).

Some epidemiological studies have shown a strong association between pesticide exposure and PD (Parron, et al., 2011; Dick, 2006; Gorell, et al., 1998; Freire and Koifman, 2012). Whilst this may be true, pesticides and metals promote the aggregation of α-synuclein (a presynaptic protein) with neurodegenerative effects. Its aggregation is an important step in the aetiology of PD (Uversky, et al., 2001; Uversky, et al., 2002; Uversky, et al., 2010; Rokad, et al., 2017). For instance, ions of Cu (II) is efficient in the aggregation of α-synuclein and related physiological content without changing the resulting fibrillary construction (Uversky, et al., 2010; Uversky, et al., 2007).

Binofil, et al (2006), observed that some metals selectively bind to the N & C-terminal of α-synuclein. For instance, some divalent metals such as Mn and Fe bind with C-terminus of α-synuclein with low-affinity (non-specific binding interface) whereas Cu, on the other hand, interacts at the N-terminal region of α-synuclein at high affinity, being the most potent metal in the aggregation of α-synuclein filament assembly (Binofil, et al., 2012). Metals synergistically or in combination with other chemicals can have a deleterious effect on the physiological functions of the human body. Fe combines with the herbicide paraquat synergistically to accelerate the age-related loss of nigral dopaminergic neurons (Peng, et al., 2007).

1.11 Effect of metal exposure on dopaminergic neurons

The dopaminergic neurons in the substantia nigra have long axons that consist of tubulin molecules (Castel, et al., 1991; Lie, et al., 2002). Low doses of Hg inhibit the formation of tubulin; whilst other ATP- or GTP-binding proteins are not affected (Vogel, et al., 1985; Leong, et al., 2001). There are about 14 sulfhydryl groups (SH-) in tubulin, Hg binds to sulfhydryl with greater affinity. In contrast, other metals, such as aluminium, zinc, iron and lead are incapable of inhibiting the binding of tubulin to GTP (Pendergrass, et al., 1997; Mutter, et al., 2004).

Several investigators (Hirsch, et al., 1991; Mann, et al., 1994; He, et al., 1996; Berg, et al., 1999) have demonstrated, from analysis of brain tissues from PD patients, that there are high levels of aluminium, iron, and zinc in the substantia nigra compared to those of match controls. Of significant interest is the fact that there is that the accumulation of iron in the substantia nigra is twice as much as those of matched controls (Dexter, et al., 1987; Dexter, et al., 1989; Youdim, et al., 1993; Sofic, et al., 1998). An elevated proportion of trivalent iron was found in Lewy bodies and dopaminergic neurons of the substantia nigra of PD patients.

Unilateral injection of Fe(III) chloride into the substantia nigra, in adult rats, resulted in a selective decrease in striatal dopamine (about 95%) as well as impairment of dopamine-related behavioural responses, suggesting (that elevated) iron may be implicated in the initiation of the loss of dopaminergic neurons in PD (Bharath, et al., 2002; Kaur, et al., 2004; Drechsel and Patel, 2008; Tanaka, et al., 1991). Besides, Fe exposure to manganese oxide have been also associated with the loss of dopaminergic neurons. In their investigations, (Ulin, et al., 1989; Tedroff, et al., 1992; Racette, et al., 2012) reported a reduction of 11C-nomifensine (a potential ligand for the evaluation of monoamine re-uptake sites) at the presynaptic dopaminergic terminals, within the striatum following subcutaneous injections of manganese oxide.

Exposure to metals with a high affinity for sulfhydryl groups such as Hg, Cd, Cu and Zn also leads to a reduction in D2 dopamine receptor sites. (Scheuhammer and Cherian, 1985). Scheuhammer and Cherian (1985) in their investigations of the effects of heavy metals cations, sulfhydryl reagents and other chemicals agents on striatal D2 dopamine receptors. They

demonstrated that low concentrations of Hg were capable of completely abolishing D2 dopamine receptors; whilst the administration of 3mM of Cu or cadmium only resulted in a 40-60% reduction in dopamine receptors.

The lethality of Hg mercury is premised on the fact that Hg targets the areas of the brain in which its detoxification is impracticable (Rouleau, et al., 1999; Mieiro, et al., 2010; Mieiro, et al., 2011; Pereira, et al., 2014). Even at the lowest levels, inorganic mercury results in the destruction of intracellular microtubules and the degeneration of axons (Choi, et al., 2011). This interesting neurodegenerative cascade is unique to mercury and not reported in metals like cadmium, aluminium, lead, or manganese. Hg depletes glutathione (Richardson and Murphy, 1975; Gstraunthaler, et al., 1983; Graff et al., 1999) and causes the impairment of mitochondrial functions (Southard and Nitisewojo, 1973; Lund, et al., 1991; Carocci, et al., 2014).

1.12 Oxidative stress in PD

In the pathogenesis of PD, a prime cause is an oxidative stress (Jenner, et al., 1992; Ebadi, et al., 1996; Zhang, et al., 2000; Jenner, et al., 2003; Hwang, 2013; Tsang, et al., 2009; Dias, et al., 2013; Blesa, et al., 2015). Oxidative stress opens a cascade of biochemical and metabolic events. It causes mitochondrial dysfunction (Lin and Beal, 2006). There is an upregulation of ROS production in PD patient, oxidative stress (Hartley, et al., 1993) and impairment of mitochondria functionality (Guo, et al., 2013). The increased level of oxidative stress in PD patients mirrors an elevated level of iron levels (Bharath, et al., 2002; Jenner, et al., 2003), nucleic acid oxidation (Kikuchi, et al., 2002; Zhang, et al.,1999), and elevated lipid peroxidation (Dexter, et al., 1989; Fahn, et al.,1992), as well as low contents of the antioxidant glutathione (GSH) in the dopaminergic regions of the brain (Perry, et al., 1982; Perry and Wong, 1986; Venkateshappa, et al., 2012).

Elevated levels of nitrated and oxidized proteins are found within the substantia nigra of PD patients (Danielson and Andersen, 2008). *In vivo* studies as well as post-mortem studies reveals nigral cells degeneration because of oxidative stress (Hirsch, 1993; Foley and Riederer, 2000). Danielson et a (2008) demonstrated that oxidative stress has a significant impact on neurodegeneration in animal models of PD. Fe also induces oxidant and oxidative stress to the

dopaminergic nigrostriatal system, which underscores an important contribution to the pathogenesis of PD (Rauhala, et al., 1998; Lin, et al., 1999). The post-translation of α-synuclein and other neuronal proteins could be due to redox metal ions and oxidative stress (Norris, et al., 2003; Miotto, et al., 2014).

1.13 Risk Factors

It has been established that some metals with neurotoxic effects are potent risk factors in the aetiology of PD. Specifically, metals with neurotoxic effects have been associated with secondary Parkinsonism (Taba, et al., 2017). Heavy metals are significant environmental pollutants and their toxicity is of increasing significance for ecological, nutritional and environmental reasons (Jaishankar et al., 2014). The global atmospheric, soil and surface water concentration of metals have been on the rise (Hope, 1994; Fortoul, et al., 2002). There is an increase in metal accumulation in tissue samples (Fortoul, et al., 2002), plants (Jaishankar, et al., 2013) and marine life (Nagajyoti, et al., 2010) - at an alarming concern to public health practitioners and environmentalist.

There has been great debate as to what constitutes a 'heavy metal' and which element should be classified as heavy metal (Adal, 2015). Some authors base their definition on atomic weight (Tchounwou, et al., 2012), delimiting heavy metal as a group of metals with a specific density greater than 4.0g/cm3 or greater than 5.0g/cm^3 and which adversely affect the environment and living organism (Jarrup, 2003; Castro-Gonzalez, et al., 2008). Some of these metals are very useful in maintaining various biochemical and physiological functions in living organisms. Commonly found heavy metals in wastewater are arsenic, cadmium, chromium, Cu, lead, nickel and zinc- all of which cause great risk to human health and the environment (Lambert, et al., 2000) others include manganese, mercury and Vd.

Some of these metals play a crucial biological function in both plants and animals, their unique chemical and oxidation-reduction properties confer on them additional benefits that help them to maintain homeostasis, transport, compartmentalization and binding to designated cell constituents (Jashangar, et al., 2014). An issue of greater concern is the ability of these metals to bind with proteins sites which are not made for them, by displacing original metals from

their natural binding sites causing cellular dysfunction. This insidious capacity makes heavy metal poisoning of great interest to the toxicologist. Environmental exposure to heavy metal is a prime agent associated with cognitive and neurological deficits (Neal, et al., 2012). They play a significant role in neurodegeneration because these disorders incorporate different pathological conditions, that share similar critical metabolic processes, such as protein aggregation and oxidative stress - both of which are associated with the involvement of metal ions (Gaeta, et al., 2005).

Gaeta, et al., (2005) identified two key components/ pathways that define the aetiology of neurodegeneration. They pointed out that these two key components – extrinsic and intrinsic components (among others) are linked to metal ions. They identified the extrinsic component which is environment-related as extrinsic neurotoxins. For example, metal and infective damage, and the excitotoxicity – which is related to intrinsic neurotoxins, such as metals and excitatory amino acids.

The neuroscience community paid little attention to the neurometabolic of metals until about two decades ago (Zatta, et al., 2003). In the recent past, the neurobiology of heavy metals is now receiving growing interest and has been linked to major neurodegenerative diseases including ALS (Trojsi, et al., 2013); PD (Cotzias, et al., 1976, Taylor, et al., 2000) and AD (Uversky, et al., 2001; Mates, et al., 2010; Notarachille, et al., 2014).

Vd is one heavy metal that may have laid below the radar of metal biologist, largely because it has no known biological function. However, Todorich, et al (2011) observed that Vd disrupts iron homeostasis in oligodendrocyte progenitors (OPCs). In their investigation, they posited that exposure of gestating rats (at 2nd post-natal week) to Vd produces hypo-myelination with a variety of related neurobehavioural phenotypes. The severity of some metal toxicity depends on the concentration and the duration of exposure.

Adal (2015) reported a link between chronic exposure to metal dust and pneumoconiosis, neuropathies, hepatorenal degeneration and a certain variety of cancers. Whilst the duration of

exposure and the concentration of redox-active metal exacerbate the toxicity associated with heavy metals, the irreversible nature of some of these metals in biological systems and their non-biodegradability make them important environmental hazards. This is largely due to the fact that the elimination of metals from the environment is difficult, if not impossible because they cannot be decomposed (Jamal, et al., 2013); this makes research into the health implications of this heavy metal globally imperative. Trojsi et al (2013) identified cyanobacteria, heavy metals and pesticides as potential risk factors associated with the aetiologies of ALS. Several scholars have highlighted some notable health impact of some heavy metal at the cellular and functional levels, elucidating their physiological and biochemical pathways.

1.14 Pathophysiology of Metal Toxicity

There are several investigators from existing literature that have explored the role of redox-active metals in cellular dysfunction and homeostatic imbalance. Smith, et al (1994) and Sayre, et al (2002) implicated aluminium, lead, mercury, zinc, Cu, and iron in AD pathogenesis. They further argued that disruption in the homeostasis of Cu and iron is particularly significant when considered against the backdrop of oxidative stress parameters, such as lipid peroxidation and the oxidative damage to neurofibrillary tangles (NFT), senile plaques and nucleic acid (Nunomora, et al., 1999). Heavy metals bind to oxygen, nitrogen and sulfhydryl groups in proteins resulting in the alteration of enzymatic activity. The affinity of metal ions for the sulfhydryl group serves as a protective role in heavy metal homeostasis as well.

Marcus, et al (1998), Nunomura, et al (1999), Sayre, et al (1994), Smith, et al (1994, 1996, and 1997) all emphasised the role of oxidative stress in the aetiology of idiopathic AD. However, there is a degree of uncertainty whether the presence of the enhanced oxidative event is the cause or the result of the diseases. Busciglio, et al (1995), Nunomura (1999) and Odetti, et al (1998) observed that in AD, oxidative stress is a pre-existing condition that subsequently leads to neuronal changes associated with the disease process. However, in Down's syndrome, evidence of oxidative stress is found long before neuropathological changes, even in the foetal stage. Lovell, et al (1998) found out that multivalent transition metals such as Cu and manganese are essential in most biological reactions such as the synthesis of DNA and proteins. They also observed that the levels of Cu and iron are increased in senile plaques (SP) and their

presence in SP and neurofibrillary tangles induces hydrogen peroxide dependent oxidation. This indicates that Cu plays a role in modulating oxidative events, which results in neurodegeneration. Some metals, like lead, cadmium and Vd, however, have no known biological activity.

Other metals have been implicated in oxidative stress and neurodegeneration. Iron implicated in redox transitions consequently generates oxygen free radicals. The cellular damage is mitigated by the production of lactoferrin, which protects against severe inflammation. Lactoferrin level is increased in patients with neurodegenerative disease (Fillebeen, et al., 1999). Brown, et al., (1998, 2004) reported that a transmembrane glycoprotein (Prion protein) incorporates Cu and has SOD. They suggested that PrP may play a role in protecting cells from oxidative damage. The misfolding of this protein is responsible for spongiform encephalopathies (Prion disease).

Vd has been shown to cause a sustained decrease in blood glucose levels in insulin-deficient rats (Shecter, 1990; Brichard, et al., 1991; Shecter, et al., 1993 and Fantus, et al., 1991). It was reported by Zaporowska and Wasilewski (1992) that Vd reduces the deformation of erythrocyte and produces peroxidative changes in erythrocytes membrane, leading to haemolyses. Vd has also been associated with hepatotoxicity (Youves, et al., 1991a and 1991b), reproductive and developmental toxicity (Liobet, et al., 1986) and demyelination (Mustapha, et al., 2014).

1.15 Metalloproteins and functional relevance

Proteins contain metal-binding sites. These metal-binding sites are responsible for catalyzing important biological processes such as photosynthesis, respiration, water oxidation, molecular oxygen reduction and nitrogen fixation (Lu, et al.,2009). Most biologically active metals possess characteristics that are linked to their chemical properties (Wright, et al., 2007). The neurotoxic actions of aluminium, zinc and lead are associated with the induction of oxidative stress through their capacity to interact with reactive oxygen species (thus, increasing their oxidant activity (Oteiza, et al., 2004). Half of the metals must associate with an enzyme to function (Waldron, et al., 2009). These metalloproteins are rich in thiol ligand which allows

high-affinity binding with some heavy metals such as cadmium, Cu, silver, and zinc among others (Adal, 2015).

Other proteins involved in both heavy metal transport and excretion through the formation of protein-ligand complexes are ferritin, transferrin, albumin, and haemoglobin. Schwarz, et al., (2009) in their review highlighted the importance of trace element molybdenum (Mb) as an important co-factor in wide varieties of enzymes. They pointed out that Mb is essential in most organisms and forms the centre of a large valence of an enzyme, such as nitrogenase, nitrate reductase, sulphite oxidase and xanthine oxidoreductase; two scaffolds hold Mb in situ- the Fe-Mb co-factor and protein-based co-factor.

1.16 Experimental Models of PD

There are several animal PD models used in research, however, the most popular are the pharmacological and the genetic models. The pharmacological animal models include the 6-hydroxydopamine (6-OHDA), 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine(MPTP), rotenone, and paraquat. The genetic models include those with mutations in the α–synuclein, PINK1 (based on loss of function), Parkin, or LRRK2 genes (Smith, et al., 2005; Nuytemans, et al., 2010). The 6-OHDA animal model was the first model of PD to be associated with dopaminergic neuronal death within the SNpc. This model is generated by injecting the toxin directly into the SNpc, medial forebrain bundle or striatum (Blandini, et al., 2008; Ren, et al., 2013). The MPTP animal model uses MPTP, which is a highly lipophilic molecule that readily crosses the blood-brain barrier, this leads to a selective and irreversible loss of dopaminergic neurons in the SN in both non-human primates (Schober, 2004; Meredith and Rademacher, 2011) and rodents (Betarbet, et al., 2002; Blesa, et al., 2012).

Despite the popularity of these models, the fruit fly (Drosophila *melanogaster*), remains one of the most used model organisms for biomedical research for several reasons. The low cost, rapid generation time, and excellent genetic tools have made the Drosophila *melanogaster* (DM) an indispensable model for basic research. The fly genome has been completely sequenced and annotated, encoding about 14,000 genes on four chromosomes, making it an attractive research model (Pandey and Nichols, 2011). Besides, 75% of disease-related genes in humans have

functional orthologs in the fly (Reiter, et al., 2001; Lloyd and Taylor, 2010). *D. melanogaster* has many fundamental cellular processes, genes and signalling pathways in common with humans, and most genes linked to familial PD have at least one fly homolog (Muñez-Soriano, 2011). Furthermore, fruit flies can perform complex motor behaviours, such as walking and climbing (Muñez-Soriano, 2011).

1.17 The Life Cycle of Drosophila melanogaster

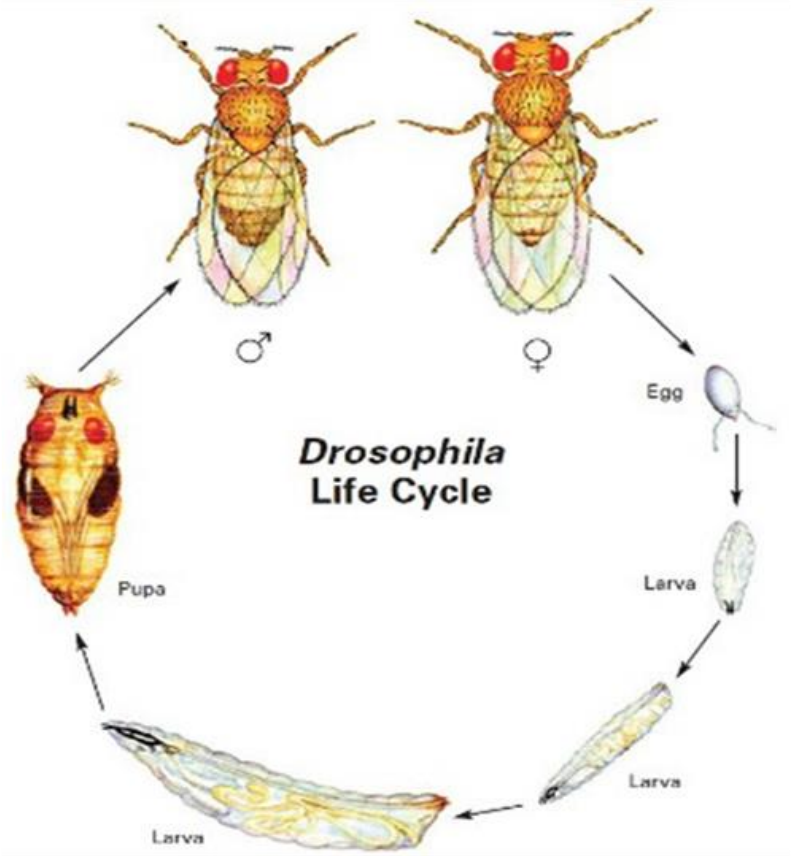


Figure 1.5: Drosophila melanogaster reproductive life cycle

D. *melanogaster* has a reproductive life cycle of 10 to 12 days at 25°C. Newly laid eggs hatch into a larva after 1 day. The larva stage lasts about 4 days and in the last 2 days of this stage, it crawls out of the food substrate and develops into a pupa. After 5 to 7 days of pupation, the adult fly emerges from its pupal case (eclosion) (Nichols, 2006). The average lifespan of wild-type (WT) *D. melanogaster* ranges from about 39 to 86 days, depending amongst other

factors on strain and gender (Sanz, 2010). For the males from the strain used in this study as WT, Dahomey, the average lifespan was reported to be 39 days (Sanz, 2010).

In the Drosophila *melanogaster*, the *PINK1* gene is located on the X chromosome, which means that all males are hemizygous, whereas females could theoretically be heterozygous or homozygous. However, the w^+ flies carried a balancer gene: FM7.GFP. This gene functions as a marker, as a suppressor of genetic cross-over and carries a recessive female sterile mutation (Greenspan, 2004). Thus, females homozygous for the balancer gene are not fertile. This trait is introduced to make sure that the w^- *PINK1* gene remains expressed in future generations and is not taken over by the balancer gene. The marker makes it possible to identify flies carrying the balancer gene, as it results in a bar phenotype in males and females (Greenspan, 2004). Male flies carrying the *PINK1* gene have a lack of eye colour, resulting in white eyes. These males are sterile and only produce progeny to a limited extent.

1.18 Why Drosophila Fly model

A compelling interest in DM as a research model may be attributed to the very rapid life cycle. A single mating pair can produce hundreds of genetically identical offspring within 10 to 12 days at 25^{o}C, in contrast to the rodent models which produce a handful offspring every 3 to 4 months (Pandey and Nichols, 2011). The fruit fly is a model organism, defined by four developmental stages (the egg, the larva, the pupa, and the adult stages) that are unique with specific advantages (Pandey and Nichols, 2011). The embryo, for instance, finds novel use in fundamental developmental studies that examine pattern formation, cell fate determination, organogenesis and neuronal development, and axon pathfinder.

The larva, especially the third instar larva, is commonly used to study developmental and physiological processes, as well as simple behaviour (e.g. foraging). At the larva stage, the future adult is fully represented as the imaginal disc (made up of undifferentiated epithelium). It undergoes extensive morphological changes from the late third instar (larva) stage through the pupal phase to the final adult structure (Pandey and Nichols, 2011). Thus, the pupa stage provides a good model to investigate certain developmental processes. In its complexity, DM has mirror structures that perform the equivalent functions of the mammalian heart, lung,

kidney, gut, and reproductive tract. Take the brain of the adult DM, for instance, over 100,000 neurons form discrete circuits and neuropil that mediate complex behaviours (such as circadian rhythms, sleep, learning and memory, courtship, feeding and aggression, grooming and flight navigation).

Interestingly, the observed response of flies to CNS acting drugs is akin to that observed in mammalian systems (McClung and Hirsh, 1998; Moore, et al., 1998; Bainton, et al., 2000; Nichols, et al., 2002; Rothenfluh and Heberlein, 2002; Satta, et al., 2003; Wolf and Heberlein, 2003; Andretic, et al. ,2008). Although there are some differences between flies and humans, the extent of conserved biology and physiology positions DM as an invaluable tool in the drug discovery process.

Genetic PD models have been established in *D. melanogaster* based on mutations found in α-synuclein, Parkin, PINK1, DJ-1 and LRRK2 genes (Muñez-Soriano, 2011). In our current investigation, a *D. melanogaster* model based on mutations in *PINK1* is used. The *D. melanogaster PINK1* gene encodes a protein containing the same domains as its human equivalent. *D. melanogaster PINK1* models have been generated by transposon-mediated mutagenesis and RNAi. *PINK1* mutants show male sterility, muscle degeneration, hypersensitivity to oxidative stress, mitochondrial defects, reduced lifespan, and dopamine neuronal degeneration accompanied by locomotor defects (Muñez-Soriano, 2011).

1.19 The genes associated with PD and the approximate frequencies

Monogenic mutation in specific genes can cause either autosomal recessive juvenile Parkinsonism (AR-JP) or autosomal dominant Parkinsonism (Nuytemans, et al., 2010). Mutations in six genes have been linked to either of these types of Parkinsonism. Four mutations for the AR-JP; PARKIN, DJ-1, phosphatase, and Tensin homolog (PTEN)-induced kinase 1 (PINK1) and P-type ATPase (ATP13A2) have been delineated. For the autosomal dominant Parkinsonism, two mutations have been identified, i.e. α-SYNUCLEIN -a non-A4 component of amyloid precursor (SNAC) and leucine repeat-rich kinase 2 (LRRK2) (Nuytemans, et al., 2010). It is interesting to note that PARKIN accounts for well over 50% of patients with AR-JP and some of these reported pathogenic mutations include missense and nonsense mutations, deletions, rearrangements, and duplications (Bonilla-Ramirez, et al., 2013). Bonilla-Ramirez, et al (2013) reported that the PARKIN gene which is located on chromosomes 6 (6q25.2-q27), contains 12 exons and encodes a 465 amino acid protein known as Parkin. Parkin is affiliated with the E3 ubiquitin ligase subset of the RBR protein family involved in protease degradation (Rankin, et al., 2011).

In the past two decades, concerted efforts have been made to unravel the genes associated with PD (Hatano et al., 2009). About 80% of cases of PD are sporadic or idiopathic type. Studies has unveiled the loci of genes responsible in patients with familial PD. Several PARK genes have been directly attributed to familial PD or as risk actors. Table 1.6 provides an overview of the genes/genetic factors as well as the chromosomal locations of the affected genes.

Table 1.6: The genes and genetic factors associated with PD and the approximate frequencies

Symbols/ Inheritance	Gene	Locus	Frequencies /onset	Functions	References
*PARK1/ PARK4 Dominant	α-Synuclein (SNCA).	4q21.3-q22	Around 40	Membrane trafficking	Romo-Gutierrez et al., (2015). Singleton et al., (2003)
*PARK2 Recessive	Parkin	6q25.2-6q27	<40	UPS, E3-ligase	Lucking et al., (2000) Poole et al., (2008) Romo-Gutierrez et al., (2015)
PARK3 Dominant	Unknown	2p13	35-89		Hatano et al., 2009
PARK5	UCHL1	4p13	~50	UPS, Ubiquitin, Hydrolase	Hatano et al., 2009
*PARK6	PINK1	1p36.12	1-8% /32 ±7	Mitochondria, kinase	Kompoliti & Verhagen, 2010
*PARK7 Recessive	DJ-1	1p36	In 1-2%/ 27-40	Oxidative stress	Romo-Gutierrez et al., (2015). Hatano et al., 2009
*PARK8 Dominant	LRRK-2	12q12	~65	Membrane trafficking, kinase	Yescas et al., (2010) Thaler et al., (2012) Romo-Gutierrez et al., (2015)
*PARK9 Recessive	ATP13A2	1p36	11-16	Lysosome? Autophagy?	Hatano et al., 2009 Park et al., (2011)
PARK10 Dominant	Unknown	1p32	65.8		Hatano et al., 2009 Grünewald et al., (2012)
PARK11 Dominant	GIGYF2	2q36-27	Late	IGF-1 signaling	Ramirez et al., (2006) Hatano et al., 2009
PARK12 X-linked	Unknown	Xq21–q25	Late		Hatano et al., 2009
PARK13 Dominant	HTRA2/ OMI	2p13.1	Late	Mitochondria, Protease	Hampshire et al., (2001).
*PARK14 Recessive	PLA2G6	22q13.1	>1% / 20-25	Phospholipase enzyme	Paisan-Ruiz et al., (2009). Sina et al., (2009). Lu et al., (2012). Tomiyama et al., 2011. Gui, et al.,(2012)
PARK15 Recessive	FBXO7	22q11.2-qter	10-19	UPS, E3-ligase	Hatano et al., 2009
PARK16 Risk factor	Unknown	1q32	-	-	Klein and Westenberger (2012)
PARK17 Dominant	VPS35	16q11.2	-	-	Klein and Westenberger (2012)
PARK18	EIF4G1	3q27.1			Klein and Westenberger (2012)

Dominant					
*APOE4 Risk	ε2 and 4	19q13.2	11.2 % of *APOE* ε2 carriers	-	Pankratz et al., 2006. Huan et al., (2004); Federoff et al., (2012)
*GBA Dominant /risk	GBA gene	1q22		Storage of lysosomal glycolipid	Klein and Westenberger (2012)
COMT	Catechol-O-methyltransferase	-	-	Monoamine degradation enzyme	Klein and Westenberger (2012)
*MAPT Risk	Tau	17q21.1	-	-	Lill et al (2012)

***Genetic factors associated with dementia in PD**

1.20 Research Focus and Experimental Model

The effects of pharmacological doses of heavy metals on neuronal cells have barely been studied. All organ systems are affected by heavy metal exposure. The most affected include the CNS, PNS, GI, renal, haematopoietic, and cardiovascular organ systems (Adal, 2015). The organ system affected, and the severity of the toxicity varies with the heavy metal involved, chronicity, and extent of exposure, as well as the age of the individual.

For this research, I will be considering three redox-active heavy metals: iron, Cu and Vd. Although the toxicological effects of these heavy metals have been well described in some detail, possible sub-toxic pharmacological, cell biological or neuroprotective effects following preconditioning, have not been investigated.

The PD pathology is characterised by distinct types of cellular defects: abnormal protein aggregation, oxidative damage that relates to mitochondrial dysfunction and a selective loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc). This research was designed to specifically investigate the possible effects of these heavy metals on these cellular defects. For *in vitro* studies, CAD (Cath.-a-differentiated) cell lines will be used. The CAD cells are variant of a CNS catecholaminergic cell line. It exhibits biochemical and morphological characteristics of primary monoamine neurons and provides a useful tool for studying PD. Importantly, it is a good model to study undifferentiated (immature) and undifferentiated (mature) neuronal neurons.

Additionally, CAD cells express enzymatically active tyrosine hydroxylase and accumulate L-DOPA, a precursor of dopamine. Ultra-structurally, processes from differentiated CAD cells have abundant parallel microtubules and intermediate filaments, and bear varicosities that contain both large dense-core vesicles/granules (120–160 nm) and smaller clear vesicles (60–80 nm). Thus, makes it suitable to use differentiation and the effects of environmental heavy metals on the different stages of development, in terms of oxidative and ER stress, and functional properties, pertinent to PD pathology. We further explored the interplay between Vd and iron in an *in vivo* model (WT and PINK1 mutant *Drosophila melanogaster*).

This model is universally used for relevant scientific investigations. Its low cost has a rapid generation time and is a powerful genetic tool for research work into ageing-related diseases. The mutant, PINK1 is based on loss of function of PINK 1 gene. This mutant is related to early-onset familial PD; it was reported that this gene is involved in mitochondrial fusion or fission dynamics, electron transport chain function and quality control, although the exact mechanism and interconnection between them are not fully understood (Pickrell and Youle, 2015). Dopamine degeneration seen in this PINK-1 model is accompanied with locomotive deficits (Park, et al.,2006) and the drosophila PINK1 is predicted to have over 60% similarity with human PINK1 and shares 43% amino acid identity with human (Muñoz Soriano and Paricio, 2011). In the brains of PD patients, the SNpc has also been found to have higher levels of iron than age-matched controls and elevated iron has been associated with mitochondrial dysfunction. Iron elevation in the SNpc might contribute to the oxidative and ER-induced damage observed in PD brains. We explored the relationship/interplay between Cu, iron and Vd toxicity to elucidate the links between environmental heavy metal stressors and PD disease.

1.21 Outline and Aims of the thesis

Heavy metals can be divided into two classes: first, those required by living organisms as essential micronutrients, such as calcium, manganese, molybdenum, zinc, Cu, and iron. The second, those devoid of any clear biological function, thus, potentially toxic even at a very low concentration. These include cadmium, chromium, mercury, lead and Vd (Marchetti, et al., 2005). Most of these latter heavy metals are released into the environment as a consequence of historical handcraft and industrial processes, and specialised cells, such as neurons, lack the intrinsic ability to distinguish between physiological and toxicological elements. Whilst Cu and iron can be placed in the first of these categories, as essential trace metal, Vd, on the other hand, falls into the class of metals with no known physiological function in humans. Clarkson (1993), in his review of molecular and ionic reviews of toxic metals, reported that these toxic elements can interact with specific sites on membrane transport protein and enzymes; he called this mechanism “ionic mimicry”. However, some micronutrient heavy metals ions that are involved in key steps of neurotransmission, can interfere in brain function, and cause different neurological and neurodegenerative disease.

1.22 Central Hypothesis

Sub - toxic doses of heavy metals differentially modulate downstream signalling, cytoarchitecture, and neuroplastic events, through mitochondrial oxidative stress pathways in healthy and PD neurons.

1.23 Aims

The overall aims of this thesis were to establish the effects of selected endogenous micronutrients (Cu, iron) and exogenous environmental (Vd) heavy metals on viability, neuronal oxidative status, function, development/cell architecture, in an *in vitro* and *in vivo* model of PD. Furthermore, we will explore the mechanism by which these pharmacological (sub-toxic) and cellular defects are accomplished, and finally how iron manipulation can protect against these deleterious effects.

Chapter 2: General Methods and Materials

2.1.1 Methods

2.1.2 CAD cell

For the cell culture experiments, Cath. A-differentiated (CAD) cells were used. CAD cell is a variant of a central nervous system (CNS) catecholaminergic cell line. Originally, this was derived from a brain tumour in a transgenic mouse carrying the wild-type SV40T antigen under transcriptional control of rat tyrosine hydroxylase promoter (Qi et al., 1997).

CAD cells do not have the immortalising oncogene but express neurone specific proteins as well as synaptic vesicles proteins. They are good models to investigate the effects of heavy metals on neuronal cell architecture for several reasons. First, they exhibit biochemical and morphological characteristics of primary neurons. They express enzymatically active tyrosine hydroxylase and accumulate DOPA- a precursor of dopamine, and, reversible morphological differentiation can be initiated. Neuronal cell differentiation can be initiated by the removal of serum from the media (DMEM). In serum-free media, cell proliferation ceases and cell differentiation commences which becomes very visible from Day 3 and extended long processes become established by Day 6.

2.1.3 CAD cell culture

Cath.-a-differentiated (CAD) cultures and experiments were performed in CL1 facilities under sterile conditions. CAD cells were grown at 37°C and in 5% CO2 on 75 cm^2 tissue culture flasks (Sarstedt, Newton, NC) in Dulbecco's modified Eagles' medium DMEM/F-12 Media - GlutaMAX™-I (GIBCO, Grand Island, NY), supplemented with 10% foetal bovine serum. (FBS; Sigma, St. Louis, MO) Cells were passaged every 3-4 days at a 1:4 dilution.

2.1.4 CAD cell line passaging

Original media from the T75 flask were decanted, then 10 ml complete media were then added to the flasks to dislodge the cells. These were then transferred into a sterile a 15ml sterile falcon tube and centrifuged at 1000 rpm for 5 mins. The supernatant was then decanted and 5 ml complete medium added to resuspend the pellet. The cells were divided 1:3-4 into 75 cm^2 flasks, with 20 ml total medium in each flask.

2.2 Cryopreservation and Storage of CAD cell lines

Sub-confluent cultures (70-80%) were dislodged by gentle pipetting, and then transferred to a 15 ml falcon tube and centrifuged at 200xg for 5 mins. at 4 ºC. The pellet was re-suspended in DMEM⁄F-12 Media - GlutaMAX™-I supplemented with 10% FBS and 10% DMSO. The cell suspension was immediately divided into aliquots of 2ml in cryogenic vials which were stored at –80 ºC for 24 hours and then transferred to liquid nitrogen the next day for storage.

2.2.1.1 Resuscitation of Frozen CAD cell lines

A cryogenic vial of cells from liquid nitrogen storage is collected wearing appropriate personal protective equipment. The vial was left to stand for about one minute (at room temperature) for it to warm up and then quickly transferred to a 37°C water bath, making sure the vial is not completely submerged. It was ensured that the water level did not exceed the thread and lid of the vial to prevent contamination. The cryovial was gently swirled in the water bath to enhance homogenous thawing of the frozen cell culture. With the content of the cryovial nearly completely thawed, the cryovial was sterilised with 70% (v/v) EtOH.

The ampoule was then wiped with a tissue soaked in 70% alcohol before opening. The whole content of the ampoule is then pipetted into a 15 ml sterile falcon tube. The pre-warmed medium was added to attain a total volume of 10 ml and the tube then centrifuged at 1000 rpm for 5 min. The supernatant was removed, and the pellet was re-suspended in DMEM⁄F-12 media - GlutaMAX™-I supplemented with 10% FBS. Using a 2 ml serological pipette, in the tissue culture flow hood, the content of the cryovial was pipetted into the pre-warmed 75 cm2 culture flask. The flask was placed in the 37°C CO2 incubator.

2.2.2 MTT cell proliferation assay

50 µL Phosphate Buffered Saline (PBS) (136.9 mM 2.68 mM KCl, 4.3 mM Na_2HP0_4, 1.4 mM KH_2P0_4, pH 7.4) containing 5 mg/ ml MTT was added to the cultures and incubated at 37°C and in 5% CO2 for 2.5 hours. Then the MTT- containing medium was removed, the surface of the wells was rinsed with 300 µl PBS before the application of 250 µL isopropanol. After the purple crystals had dissolved, the optical density of 100 µL sample was spectrophotometrically read at 595 nm. (Thermo Lab systems Multiskan Ascent, V1.3).

2.2.3 Lactic dehydrogenase (LDH) release assay

2.2.3.1 CytoTox 96 non-radioactive assay (Promega, UK)

CytoTox 96 is a non-radioactive colourimetric cytotoxicity assay. It is used to quantify lactate dehydrogenase (LDH); which is a stable cytosolic enzyme that is released during cell lysis (necrosis). The supernatant, which contained the LDH was retained and used for LDH assays. Before the assay, the samples were diluted with PBS (1:5). 50uL of each sample were transferred into each well of the assay plate (in triplicates) and 50uL of substrate mix was added to each sample and incubated for 30mins on the bench at room temperature- and protected from light. After 30 mins., 30uL of Stop Solution was added to each well and the absorbance was measured at 490nm on a Multiscan Ascent Plate Reader, Version 2.6.

2.2.4 Calcium imaging

Calcium ions play an integral role in neuronal function. They are intracellular signals that can elicit such responses as altered gene expression and neurotransmitter release from synaptic vesicles. Within the cell, calcium concentration is highly dynamic due to the presence of pumps that selectively transport these ions in response to a variety of signals. Calcium imaging takes advantage of intracellular calcium flux to directly visualize calcium signalling in living neurons.

In this investigation, cultured CAD cells were probed with the molecular dye; Calcium-Green-1 AM (Invitrogen) as specified in the manufacturer manual (ref). Calcium Green is used to investigate how intracellular calcium fluctuates in relation to neuronal activity. A 2mM stock of the Calcium-Green probe was prepared in DMSO D-8779 500Ml, lot 100K3729 by Sigma). This was stored at -20ºC. On the day of the imaging experiments, the probe stock was brought to room temperature and then diluted in HEPES physiological buffer (150mM NaCl, 1mM $MgCl_2$, 10mM HEPES, 2mM $CaCl_2$, 5mM KCl) to a 1µM solution.

The cells were plated in flat bottom µ-dishes, (brand: 60 µ-Dish, 35mm, high glass flat bottom-81158 Ibidi GmbH). The cells were washed with (once) 1mL of physiological buffer and incubated for 40 mins. in 1mL of the probe solution. After the incubation period, the cells were washed again with the HEPES physiological buffer and left in the buffer until the start

of the imaging experiments. All the drugs applied to the clonal cells during the experiments were dissolved in the HEPES physiological buffer as stock solutions 10 times more concentrated than the final desired concentration for the experiments.

To evaluate the effects of Cu (at a final concentration of 40μM) on cellular calcium fluxes, baselines were taken for around 5 seconds and then Cu was injected into the dish. Careful pipetting was ensured by injecting against the dish wall to avoid causing fluid turbulence, thereby compromising the images. The cells were imaged in the presence of Cu (10 & 40μM) and potassium chloride (KCl). The incubation period for each treatment was for the periods of 250 cycles (0.63s each) before the application of the same volume of the depolarising buffer (500mM of KCl in HEPES physiological buffer, giving a final concentration of 49.6mM) and then imaged for another period of 250 cycles. KCl, which caused depolarization of the neurons due to a rise in the intracellular calcium concentration, serve as good positive control. All the solutions were stored at 37ºC to avoid any stress on the cells during the sample preparation and the imaging experiment.

For the imaging, the Zeiss LSM 850 with Airyscan microscope was used. The laser used for imaging was set at 514nM:1.1% (Ch2GaAsp:524-620). The spectrum window was set between 24620 and a pinhole of 49um was used. A size 63 oil lens with an LA (lens aperture) of 1.4 was used to capture each image and the time between each frame (time series) was set at zero. The duration for one frame was 633.02ms. To extract the figures and fluorescence intensities, the Zeiss software was used. Cells bodies were isolated by the selection of their corresponding areas and graphs of fluorescence intensity divided by the corresponding area versus time (in imaging cycles) were plotted in the software. The reported figures correspond to isolated cells with the strongest basal signals, gathered from replicates.

2.2.5 Cell differentiation

To differentiate CAD cells, the growth media (with 10% FBS) in which the cells had been plated was removed and replaced with serum-free DMEM Nutrient Mixture F-12. After

replacement by serum-free media, the cells were returned to the incubator and left for six days to differentiate.

2.2.6 Cell Lysate Preparation

For different experiments, protein lysates were required. To prepare the lysates, cells were plated on plates or flasks. Before detaching the cells, they were rinsed with cold phosphate-buffered saline (PBS) -to get rid of the media. The flask, with new fresh PBS, was placed on an iced bucket for some mins. Afterwards, the cells were detached from the plate or the flask and transferred into a 15ml falcon tube which was centrifuged for 5 mins. at 1000 rpm at RT. After centrifuging, the supernatant was discarded, and the cell pellet was resuspended in cold PBS, transferred to Eppendorf tubes, and immediately stored on ice. In the next step, samples (in Eppendorf) were centrifuged for 5 mins. at 1000 rpm and 4°C and the supernatant was discarded afterwards. Cell pellets were transferred into a tissue grinder and grounded with 1ml of PBS. Subsequently, the samples were transferred into an Eppendorf tube and stored at -20°C.

2.2.7 Western Blot

2.2.8 Gel Casting

To cast the gels for a sodium-dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), the Hoefer® Dual Gel Caster (see Table X) was used. This consists of a glass plate, ceramic tile, spacers, and caster. The glass plate was placed on a ceramic tile with the T-shaped spacers placed between the glass and the tile on each side to ensure a gap of 1 mm between the glass and ceramic tile. Glass and tile were fixed unto the Hoefer® Dual Gel Caster and checked for leakages by filling the gap with deionized water (dH_2O). When no dH_2O exited the system, the dH_2O was discarded and replaced by the prepared running gel (Table 2). The percentage of acrylamide used was responsible for the pore size of the gel and determined the time proteins take to run through the gel.

For all the experiments, a 10% SDS gels were made. The gap between the glass and the ceramic tile was filled up to approximately ¾ with running gel. 1 – 2 ml saturated butanol to enhance

an even gel surface and to dispose of possible air bubbles. After 20 – 30 mins. the gel was polymerised, and the stacking gel was prepared. The stacking gel contains a smaller percentage of acrylamide. This part of the gel was not used to separate the proteins but to ensure that all proteins loaded enter the running gel at the same time.

Before the stacking gel could be placed on top of the running gel, the butyric acid was discarded. Once the stacking gel was poured on top of the running gel, the T-shaped comb was positioned in the stacking gel creating 10 wells for the sample loading carried out later. The stacking gel polymerised within 20 mins. Prepared gels may be used afterwards or stored o/n in TBS soaked paper towels- this enhances polymerisation. Gels that were stored at +4°C o/n were unwrapped and placed in the Hoefer® Mini Vertical Protein Electrophoresis Unit and loaded with the samples.

2.2.9 Sample Preparation

To investigate specific protein abundance, cell samples generated as described (in 2.2.9) were thawed and 10 µl of each sample were transferred to a new sample tube. 10 µl sample was mixed 1:1 with Laemmle sample buffer Samples were prepared either in reducing (R) or non-reducing (NR) conditions. As for reducing agent 0.1 M DTT (dithiothreitol, Table 1) was diluted 1:5 in the sample. Samples were then mixed and heated in a 95°C hot water bath for 5 mins. before being spun down on a table-top centrifuge.

2.2.9.1 SDS-PAGE

The SDS-PAGE was used to separate proteins by their molecular mass. Therefore, a total volume of 10 µl of each sample prepared as described (in 2.210.2) was loaded into one well in the stacking gel (see table 2.2). Due to the Laemmle sample buffer, all proteins are negatively charged and run from the cathode to the anode through the gel when connected to power. The running gel containing 10 % acrylamide (See Table 2.2) forms a grid forming pores that small proteins can easily pass through but make it difficult for bigger proteins to run through. The gel was run at 10mA for 15 mins. to help the samples to settle in the stacking gel before the current was increased to 15mA (but the double current for 2 gels) and the gel was run for another 1-1.5 hours until the sample buffer (See Table2.2) got to the bottom end of the gel. As

a scale for protein size, a standardised marker (HyperPAGE,) was run on every gel next to the samples.

2.2.9.2 Blotting

After the SDS-PAGE, proteins were blotted on a nitrocellulose membrane using electric current. For the protein transport from the gel unto the membrane, the membrane was pre-incubated in transfer buffer. Transfers were done on a lockable cassette. The cages were filled in the following order: sponge, two filter papers, nitrocellulose membrane, gel, two filter papers, and sponge. All materials used were soaked in transfer buffer (See Table 2.2) for at least 30 mins. before transfer - to ensure that neither the gel nor the membrane was running dry. After the cages were locked, they were placed in a Hoefer® Mighty Small Transfer Tank filled with transfer buffer with the cathode facing the side of the gel and the anode facing the nitrocellulose membrane. The transfer was performed at 50 V and >300 mA enabling the negatively charged proteins to travel towards the anode and therefore entering the membrane when exiting the gel. Two cooling units were placed on the sides of the tank to prevent temperature increases due to the electricity. The transfer was due 2.5 hours later.

2.2.10 Analysis of metal content in CAD cells

CAD cells were passaged and seeded as previously described (Sections 2.1.3 and 2.1.4) into two 75cm3 flasks. After 24 hours the process of differentiation was initiated as previously described (See Section 2.2.5) and the undifferentiated cells were left to proliferate. After 100% confluency was attained, the undifferentiated cells were rinsed with cold PBS and detached. After detaching, the falcon tubes were centrifuged for 5 mins. (at 1500 rpm) and transferred into 1.5ml Eppendorf tubes, centrifuged again and stored at -20^{o}C for ICP-MS analysis for metal contents. Similarly, the differentiated (6 days DIV) cells were detached and stored at -20^{o}C for ICP-MS.

2.2.10.1 Sample Preparation

1.5ml of Nitric acid (65% HNO3) was added to each thawed cell pellets and vortexed. The cells were left for 24 hours to be digested in the fume hood, and vortexed again. 1.2ml of the digested sample was removed and dispensed into fresh Eppendorf tubes and centrifuged (5’,

@14,000rpm). 500uL of the digest was then diluted with 4.5ml 2.5% HNO3. 1ml of the clarified sample was decanted into a 15ml falcon tube. The clarified sample was diluted 1/10 (2% nitric acid). The analysis was carried out with Ag as an internal standard (Ag 100 ppb) which was added to the samples and standard curve. ICP-MS analysis was done in duplicates.

Standard solutions of known metal concentration were analysed before and after the samples ("front standard curve" and "back standard curve") and these were used to define limits for quantitation. The final working range was noted and used to quantify the metal content.

2.2.11 Immunofluorescence

Immunofluorescence (IF) assay was performed to establish the effect of Vd on ER stress, PDI expression and neuronal differentiation. CAD cells were plated on coverslips (18mm), which were placed in 6-well plates. 2ml of CAD cells suspended in FBS-supplemented in DMEM/F-12 media was added to a 6cm cell culture dish (TPP© Tissue Culture Dishes, Sigma, Z707678), with additional 2ml of fresh media; to give a 1:1 dilution of cells. These were left for 24 hours to attach to the coverslips and differentiated as described in 2.2.5. For chronic studies, cells were spiked with Vd (10µM or 100µM), one-hour post differentiation and left on SFM for 6 days.

For acute studies, cells could differentiate (6 days DIV) and spike with Vd (10µM or 100µM) and left for 24 hours. The media was removed in preparation for antibody application and the cells were washed twice in cold PBS supplemented with 1mM calcium and 0.5mM magnesium (PBS^{++}). Each wash was left for 5 mins. After washing, the cells were fixed with 4% paraformaldehyde (PFA) to preserve the cell's structure and to preserve cell structure and to prevent decay- this is made possible by PFA enhancing the formation of covalent bonds between proteins. The cells were incubated in PFA for 1mins. at RT. Subsequently, the PFA was removed and the cells were washed as previously described.

To eliminate unspecific antibody binding to free aldehyde groups in the samples- "quenching" was necessary. Cells were incubated in 50mM ammonium chloride (NH_4Cl) in PBS^{++} for 15

mins. at RT. NH_4Cl quenches free aldehyde groups and allowing a better signal contrast. Following this, NH_4Cl was discarded and the cells were washed again in PBS^{++} three times for 5 mins. in cold PBS^{++}. 0.2% Bovine serum (BSA) in PBS++ was used to block unspecific binding sites. Cells were incubated in BSA two times for 5 mins. at RT. The coverslip was taken out of the blocking solution and placed face-down on the drop of antibody solution. Coverslips with control cells were placed on one drop of 0.2% BSA in PBS^{++}. Cells were incubated in antibody solution for 2 hours (for MAP2) and 1 hour (for PDI) at RT and then washed trice in cold PBS^{++} for 5 mins. between each wash. Secondary antibodies (See Table 2.5) were applied in the same way as described for the primary antibodies and incubated for 25 mins.

Due to the light-sensitive nature of the secondary antibody, Samples were covered with foil to prevent fluorophore-exhaustion. Excessive antibodies were washed off with cold PBS two times for 5 minutes. For nucleus-localisation, DNA was stained with DAPI diluted 1:1000 in PBS^{++} and applied as previously described for antibodies. DAPI intercalates into the DNA and emits fluorescent light when excited with light in the ultraviolet range. After 5 minutes. of incubation at RT, coverslips were mounted on microscopy slides using mounting medium by *Vectashield*® (See Table 2.2) as mounting medium; the cell-coated side of the coverslip was placed face down. The edges of the coverslips were sealed with nail polish.

Immunofluorescent pictures were taken by the confocal *ZEISS ApoTome Microscope*. The secondary antibodies tagged with Alexa-fluorophore-594 gave a signal with a wavelength of 594 nm when excited with a wavelength of approximately 570 nm. Immunofluorescent images of DAPI and Alexafluorophore-594 were taken separately and merged using the *ZEISS ApoTome* software

2.2.12 Drosophila melanogaster stocks and culture conditions.

Drosophila *melanogaster* PINK1 ($^{w-}$ $PINK1^{B9}$/FM7.GFP^{w+}) mutant and WT (WT) were used for this experiment. Flies were kept in an incubator with a 12-hour day-night cycle at 25^{o}C. Fly food was prepared by mixing 15.025g of instant medium (Applied Scientific Jazz-Mix Drosophila Food) with 46ml of deionized water (formula with this food: water ratio was

found to have the best consistency) per bottle; 250mL. Following this, each bottle was slightly shaken to ensure even distribution of water. Each bottle was plugged with a foam plug and left to set for at least an hour at room temperature before transferring the flies (progenies) into them. Fresh food was prepared every two weeks and flies were flipped into new bottles.

2.2.13 Dosage and treatment

A low dose (oxidative stress dose) of Vd (1µM) was used. This concentration of Vd was calculated using the equation by Hong, et al (2011) to convert dosages used in human studies to effective and non-toxic doses that could be used in the Drosophila *melanogaster* experiments. To investigate the effect of chronic exposure of Vd on motor activities, lifespan, iron chelation and oxidative stress markers, 3 or 4 groups (depending on the experimental design) were used. These consisted of: a control group (with no treatment), positive control (treated with L-dopa; 1.6mg) a treatment group (Vd-1µM). Each group comprised of five replicates and each bottle had 10 flies.

To prevent oxidation of L-Dopa, 20.8mg of ascorbic acid was added to each bottle containing L-Dopa (like the amount used in a study by Pendleton *et al.,* 2002). At the start of each experiment, flies were allowed to lay eggs on food once the larvae appeared, the adult flies were released from the bottles, after 6 days the progenies were transferred to fresh food and redistributed onto treatments the next day (which is counted as day zero). To establish the effect of synthetic iron chelation on motor activity and lifespan, a dose-dependent effect of DFO (0 µM, 5 µM, 10 µM, and 20 µM) was tested –in both WT and mutant flies. From the result of this experiment, established optimal concentration of synthetic iron chelator Deferoxamine (DFO) Mesylate salt were used added in the subsequent experiment-: 5 µM; WT and 20 µM; PINK1.

2.2.14 Climbing assay and life span

The motor functions were tested against different treatment regimen for the symptoms in *PINK1* mutant vs WT flies. This was measured by testing their climbing ability. Fourteen days after the flies were originally placed on food with treatment, their progeny was tested

using a climbing test adapted from Pendleton, et al., 2002; Nichols, et al., 2012 and Cha, et al., 2005 - conducted with moderate modifications. All groups were tested at random, groups of 10-15 flies (depending on the experiments) were transferred into empty 100-ml Pyrex graduated cylinder with a foam plug, and a height of 8 cm horizontal line above the bottom of the cylinder was drawn in a paper as a criterion. The flies were allowed 10 mins. to acclimatise. The flies were then gently tapped down and allowed to climb up past the 8 cm mark (in 8 seconds) on the chart, and afterwards tapped down again.

A digital camera was placed in front of the cylinders at 30 cm from the paper, and a timer was used to record the time. The total number of flies that crossed the 8 cm mark was recorded (as the "Escaped flies"- in this escaped ability test). This was repeated two more times. The climbing assay was performed at 10 am and at the same time every two to three days. An average of the total number of flies that escaped was noted and the percentages of flies that escaped were then calculated; against the total number of flies that survived at each time-point- this was defined as climbing scores. Each treatment group contain five replicates. The mean of each group was calculated using the data of the three replicates tests. Adjusted two-way analysis of variance (ANOVA) with post-test Bonferroni correction was performed using GraphPad Prism, version 7 (GraphPad Software, San Diego California USA).

2.2.15 Sample preparation for biochemical assays

At the end of exposure (14 days), the flies from each group of control and treated (Vd only; Vd + DFO) groups were anaesthetized in ice. The flies were then snapped frozen in liquid nitrogen and vortexed at high speed to separate the fly head from the body (since we are only interested in measuring the RONS in the head). The detached fly heads were transferred into pre-weighed Eppendorf tubes and weighed. It was then homogenised in 0.1M phosphate buffer, pH 7.0 (1 mg: 10µL), centrifuged for 10 min at 4000g (temperature, 4^{o}C). The supernatants obtained were stored at -20^{o} C and used to determine the RON's level, protein, and total thiol content. The assays were performed in duplicates for each of the three replicates of the treatment groups (Vd only; Vd + DFO) treatment groups.

2.2.16 Measurement of DCFH oxidation for RONS – Kinetic assay

To determine the RONS level following chronic exposure to Vd and influence of Fe chelation, 2', and 7'-Dichlorofluorescein (DCFH) oxidation was measured as an index of oxidative stress according to the method of Perez-Severiano, et al (2004). From the frozen supernatant, five microliters (5µL, 1:10 dilution) of each supernatant from Vd only- and Vd + DFO- treated and control flies (for both WT and mutant) were transferred into a 96-well plate. Subsequently, 5µL of 200 µM DCFH-DA (final concentration of 5 µM) was added to the samples and the fluorescence product of DFH oxidation (i.e. DCF), was measured for 10 mins. (at 30-sec intervals), using Synergy H4 hybrid multi-mode microplate reader (See Table 4.1) with excitation 488 and 525 nm emission. All the experiments were conducted in duplicates for each of the three replicates of Vd ±DFO – treated and control flies (for both WT and mutant). The rate of DCF formation was expressed in percentage of the control group.

2.2.17 Total Thiol (T-SH) assay – WT & Mutant.

The total thiol content for both WT and mutant flies was determined using the method of Ellman (1959). The reacting mixture contained 170 µL of 0.1 M potassium phosphate buffer (pH 7.4), 20 µL of the sample as well as 10 µL of 10 mM DTNB. It was incubated for 30 mins. at room temperature, the absorbance was measured at 412 nm and used to calculate the sample total thiol levels (in µmol/mg protein) using GSH as standard.

2.3 Materials

2.3.1 List of Materials for Western Blot

Table 2.1: Materials and suppliers

Product	**Manufacturer**
Acrylamide (30%)	Bio-Rad
Anti-rabbit IgG, HRP-linked	Cell Signaling Technologies
Bromophenol	Sigma Aldrich
Butanol	Sigma Aldrich
Chemiluminescence	film GE Healthcare DAPI
DTT	Sigma Life Science
Foetal bovine serum	Sigma Life Science
Fura-2 Calcium Dye	Thermo Fisher Scientific
Gibco™ DMEM/F12	Thermo Fisher Scientific
Gibco™ PBS	Thermo Fisher Scientific
Goat-anti-rabbit IgG HRP-linked	Cell Signaling Technologies
Hoefer® Dual Gel Caster	Hoefer Inc., USA
Hoefer® Mini Vertical Protein Electrophoresis	Hoefer Inc., USA
HyperPAGE	Bioline
Mighty Small Transfer Tank	Hoefer Inc., USA
MTT	Invitrogen
Nitrocellulose membrane	GE Healthcare
Parafilm®	Pechinery Plastic Packaging
Rabbit-anti-GAPDH	Proteintech
Rabbit-α-PDI polyclonal	Benham Group, Durham University, UK
SDS	Sigma Aldrich
TEMED	Sigma Aldrich
Tween 20	Thermo Fisher Scientific
Xograph Imaging System	Xograph Healthcare
ZEISS LSM 880 with Airyscan	Carl Zeiss AG

2.3.2 List of Materials

Table 2.2: Materials for Western blot, Calcium Imaging and corresponding suppliers

Product	Manufacturer
Acrylamide (30%)	Bio-Rad
Ammonium Chloride	Sigma Life Science
Ammonium persulfide	Sigma Life Science
Anti-rabbit IgG, HRP-linked	Cell Signaling Technologies
Bromophenol	Sigma Aldrich
BSA	Sigma Scientific
Butanol	Sigma Aldrich
Calcium Chloride	Sigma Aldrich
Chemiluminescence film	GE Healthcare
DAPI	Thermo Fisher Scientific
DMSO	Sigma Aldrich
DTT	Sigma Life Science
Foetal bovine serum	Sigma Life Science
Fura-2 Calcium Dye	Thermo Fisher Scientific
Gibco™ DMEM/F12	Thermo Fisher Scientific
Gibco™ PBS	Thermo Fisher Scientific
Glycine	Acros Organics
Goat-anti-rabbit IgG HRP-linked	Cell Signaling Technologies
Hoefer® Dual Gel Caster	Hoefer Inc., USA
Hoefer® Mini Vertical Protein Electrophoresis unit	Hoefer Inc., USA
HyperPAGE Bioline	Bioline
Magnesium Chloride	Sigma Aldrich
Methanol	Fisher Scientific
Mighty Small Transfer Tank	Hoefer Inc., USA
MTT	Invitrogen
NaCl	Sigma Aldrich
Nitrocellulose membrane	GE Healthcare
Parafilm®	Pechinery Plastic Packaging
PFA	Fisher Scientific
Rabbit-anti-GAPDH	Proteintech
Rabbit-a-PDI polyclonal	Benham Group, Biosciences, Durham University, UK
SDS	Sigma Aldrich
ß-mercaptoethanol	Sigma Life Science
TEMED	Sigma Aldrich
Triton X-100	Merck
Trizma® Base	Sigma Life Science
Tween 20	Thermo Fisher Scientific
Vectashield mounting media	VECTASHIELD®
Xograph Imaging System	Xograph Healthcare
ZEISS ApoTome	Carl Zeiss AG
ZEISS LSM 880 with Airyscan	Carl Zeiss AG

2.3.3 List of Materials & Chemicals

Table 2.3: Materials for *in vivo* experiments and corresponding suppliers

Product	**Supplier**
WT (WT) flies - Dahomey; Drosophila *melanogaster*	Dr Ed Okello's lab- Institute of Cellular Medicine, Medical School, Newcastle University
PINK1 mutant flies – ($^{w-}$ PINK1 ^{B9}/FM7. GFP $^{w+}$) -Drosophila *melanogaster* PINK1	Dr Ed Okello's lab- Institute of Cellular Medicine, Medical School, Newcastle University
250ml flies' bottles	Scientific Laboratory Supplies
Applied Scientific Jazz-Mix Drosophila Food	Fisher Scientific (Loughborough, UK)
Carbon dioxide	Sigma Aldrich (Dorset, UK)
Fly pad	Blades Biological (Kent, UK)
Controlled room (12 hourly day-night cycle @25^{o}C)	Lab
Brush	Blades Biological (Kent, UK)
Filter using Whatman #1 paper	Fisher Scientific (Loughborough, UK)
Microplate reader - Thermo scientific multiskan FC.	Thermo Fisher Scientific Oy P.O Box 100, FI-01621 Vantaa, Finland
Refrigerated centrifuge (4^{o}C)- Eppendorf Centrifuge 5415R. Spec:CF3CH2F.	Eppendorf AG 22331 Hamburg, Germany
Liquid nitrogen	Biosciences Nitrogen Tank
Synergy H4 hybrid multi-mode microplate reader	BioTek Instruments, Inc., P.O. Box 998, Highland Park, Winooski, Vermont 05404-0998 USA.
Vortex (machine) Genie-2; Model:G-560E	Scientific Industries Inc. Bohemia, NY, 11716, USA
Plugs	Blades Biological (Kent, UK)

2.3.4 List of Reagents and Buffer Composition for Western blot

Table 2.4: Composition of Buffers used

Buffer	Composition
Blocking buffer, western blot	5 % SMP in PBS
Electrode buffer	190 mM glycine 25 mM Tris-Cl 0.1 % SDS pH 8.6
HEPES physiological buffer Calcium imaging	150 mM NaCl 5 mM KCl 2 mM CaCl2 1 mM MgCl2 10 mM HEPES pH 7.4
Laemmli sample buffer	60 mM Tris-Cl 10 % glycine 5 % ß-mercaptoethanol 2 % SDS 0.01 % bromphenol blue pH 6.8
Running gel	0.375 M Tris Base 10 % acrylamide 0.1 % SDS 0.1 % APS 0.04 % TEMED pH 8.8
Stacking gel	0.125 M Tris Base 5 % acrylamide 0.075 % APS 0.075 % TEMED pH 6.8
Transfer buffer	190 mM glycine 25 mM Tris Base 20 % methanol In dH2O
Washing buffer, western blot	2.5 % SMP in PBS 0.02 % Tween 20

2.3.5 List of Antibodies for Western blot

Table 2.5: List of primary and secondary antibodies used

Primary AB	**Dilution**	**Application**
Rabbit-α-PDI polyclonal	1:3000	Western blot Rabbit
Rabbit-α-PDI polyclonal	1:2000	Immunofluorescence
Rabbit-α-PDI polyclonal	1:2000	Western blot
Rabbit- α-GAPDH	1:2000	Western blot
MAP2		Western blot
Secondary AB	**Dilution**	**Application**
Goat-α-Rabbit-HRP	1:2000	Western blot
α-Rabbit AlexaFluorophore594	1:500	Immunofluorescence

2.3.6 List of Antibodies for immunofluorescence and Biochemical assays

Table 2.6: List of primary and secondary antibodies used immunofluorescence

Primary AB	**Dilution**	**Application**
Rabbit-α-PDI polyclonal	1:3000	Western blot Rabbit
Rabbit-α-PDI polyclonal	1:200	Immunofluorescence
Rabbit-α-PDI polyclonal	1:2000	Western Blot
Rabbit- α-GAPDH	1:2000	Western Blot
MAP2	1:1000	Western Blot
MAP2	1:50	Immunofluorescence
Secondary AB	**Dilution**	**Application**
Goat-α-Rabbit-HRP	1:2000	Western Blot
α-Rabbit	1:500	Immunofluorescence
Alexa Fluorophore 596	1:500	Immunofluorescence

Table 2.7: Chemicals for biochemical assays and corresponding suppliers

Product	Supplier
Absolute Ethanol	Fisher Scientific, UK
Ascorbic acid (vitamin C powder)	Sigma Aldrich (Dorset, UK)
Bovine Serum Albumin (BSA)	Sigma Aldrich (Dorset, UK)
Bradford Reagent	Sigma Aldrich (Dorset, UK)
Coomassie Brilliant Blue G-250	Sigma Aldrich (Dorset, UK)
DCFH-DA (2,7-Dichloroflouresceine diacetate)	Sigma Aldrich (Dorset, UK)
Deferoxamine (DFO) Mesylate salt	Sigma Aldrich (Dorset, UK)
Dibasic Phosphate buffer (K_2HPO_4)	Sigma Aldrich (Dorset, UK
DTNB (Ellman's Reagent) (5,5-dithiol-bis-(2-nitrobenzoic acid)	Thermofischer Scientific
Ethanol	Sigma Aldrich (Dorset, UK)
L-DOPA (3,4-Dihydroxy-L-phenylalanine)	Sigma Aldrich (Dorset, UK)
Methanol	Fisher Scientific, UK
Methanol	Fisher Scientific, UK
Monobasic Phosphate buffer (KH_2PO_4)	Sigma Aldrich (Dorset, UK)
Phosphoric acid (H_3PO4)	Sigma Aldrich (Dorset, UK)
Potassium Phosphate Buffer	Fisher Scientific, UK
Reduced Glutathione- GSH	Abcam

Chapter 3: *In-vitro* effect of subtoxic concentration of Cu relevance to PD

3.1 Introduction

This chapter is devoted to elucidating the effects of Cu, an endogenous heavy metal, on neuronal metabolic function, cytotoxicity, functional responses, and neuronal maturation, with possible implications on synaptic plasticity and neurodegeneration. The increased concentrations of Cu in the cerebrospinal fluid of patients with PD, its cellular toxicity in dopaminergic neurons, as well as the formation of reactive oxygen species (ROS) informed the need to explore the potential effect(s) of low and sub-toxic concentrations of Cu as a possible contributor to the aetiology of PD. However, whilst most studies have focused on mechanisms underlying Cu-induced neurotoxicity, the sub-toxic pharmacological effects of such heavy metals have been somewhat undervalued. We seek, in this study, to explore this important issue.

Some micronutrient heavy metal ions that are involved in key steps of neurotransmission, can interfere with brain function and have been implicated in different neurodegenerative diseases. This chapter is aimed at elucidating the effect of sub-toxic pharmacological effects of Cu on the cellular functionality of neuronal cells at different stages of development; undifferentiated, differentiating, and differentiated Cath. A-differentiated (CAD) cells.

3.1.1 Copper: an essential element

Cu is an essential trace element involved in many important physiological processes in biological systems. It represents the prosthetic group of enzymes that takes part in the transfer of electrons in several key reactions in metabolism (Luza and Speisky, 1996; Chen, et al., 2009). In the brain, Cu-dependent enzymes, such as dopamine β hydroxylase, Cu-monoamine oxidase, and peptidyl glycine α-amidating monooxygenase, catalyses the formation and the metabolism of neuronally active substances. Due to the high Cu level in the brain, the role of this metal in CNS functions is invaluable. Unbound Cu is deleterious and potentially carcinogenic; under this condition, its redox activity may lead to cellular damage, due to release of reactive oxygen species (ROS) (Arciello, et al., 2006); through modification of proteins, lipids and nucleic acids, with clear structural and functional implications.

ROS (superoxide anion, hydrogen peroxide and hydroxyl radical) are physiological by-products of oxidative metabolism of cells, the electron transport chain of mitochondria –being also a major source of ROS and a target of this action (Halliwell, et al., 1990). In the biological system Cu, as well as Iron, can promote ROS formation, bind directly to free thiols of cysteines, leading to oxidation and cross-links between proteins, This inevitably leads to enzyme inactivation and the impairment of structural proteins (Cecconi, et al. ,2002).

3.1.2 Cu and Oxidative Stress

Cu plays a critical role in cellular functions. It is central in neural cells where it is utilised for neurochemical activities as well as general metabolism. Its capacity to cycle between the two oxidation states (Cu^{+} and Cu^{2+} respectively) is employed by various enzymes involved in various biochemical reactions essential for brain development and function (Hatori, et al., 2016). Two vital Cu-dependent enzymes include cytochrome c oxidase (involved in electron transfer and ATP production in the mitochondria) and superoxide dismutase (SOD1 and SOD3). These are responsible for detoxification of reactive oxygen species (ROS) in the cytosol and cell surface. These two subserve the housekeeping functions in cells. The other group contributes to the functional identity of specific neurons including dopamine-β-hydroxylase (central in the biosynthesis of norepinephrine), and peptidyl-glycine-a-monooxygenase (PAM) – involved in the production of all amidated neuropeptides (Hasan and Lutsenko, 2012).

3.1.3 Cu homeostasis and PD

Due to the compartmentalisation of proteins requiring Cu, there is the need for timely delivery of Cu to these compartments for functional maturation of the resident enzymes (Hatori, et al., 2016). It is noted that Cu delivery from the cytosol to its target protein is facilitated by chaperones (Field, et al., 2002). The chaperone for SOD is CCS (Cu Chaperone for SOD) which delivers Cu to SOD1 (Williams, et al.,2016). A plethora of chaperones mediate the delivery of Cu transfer into mitochondria and subsequent incorporation of Cu into cytochrome c oxidase. These are Cox11, Cox17, SCO1 and SCO2.

In the cytosol, Atox1 (a cytosolic chaperone) shuttles Cu to the Cu transporters ATP7A and ATP7B. These are both located in the secretory pathway (Field, et al., 2002; Lutsenko, et al.,

2012). These transporters (ATP7A and ATP7B) transports cytosolic Cu into the lumen of the trans-Golgi network (TGN) and various vesicles; energy from this process is derived from the hydrolysis of ATP. This subsequently activates PAM, DBH, SOD and other Cu-dependent enzymes (Hatori, et al., 2016). Identities of metallochaperones have been well defined. However, the mechanism underpinning the regulation of Cu flow to the different cellular compartment is yet to be elucidated. It has been suggested that the distribution of Cu between proteins is driven/mediated by the relative abundance of Cu-binding molecules and the differences in their affinity for Cu (Banaci, et al., 2010).

This principle is true for the cytosolic distribution of Cu in steady-state. Prompt regulation of intracellular Cu fluxes is critical for the development of distinctive neurochemical characteristics of neurons. During the transition from the proliferative phase to a differentiated state, neuronal cells undergo remarkable metabolic restructuring which is defined by changes in the metabolome (Lutsenko, et al., 2008) and the protein expression profiles (Banci et al., 2010). There is an active synthesis of neurotransmitters and neuropeptides in differentiated neurons within the secretory pathway. These processes require Cu (Bousquet-Moore, et al., 2010).

The role of Cu as a neurotransmitter, its location in the secretory pathway and its neurochemical activities makes the dysregulation of this metal pivotal in the pathogenesis of numerous neurological diseases such as Parkinson Disease, Alzheimer Disease, Amyotrophic Lateral Sclerosis and Huntington Disease (Desai and Kaler, 2008).

Dysregulation of Cu homeostasis may result in the serious disorders such as hepatocerebral dystrophy (Wilson's Disease), renal tubular dysfunction, liver cirrhosis, the emergence of Kayser-Fleischer rings and damage of different brain structures, in particular the thalamus, subthalamic nuclei, brainstem, and frontal cortex (Das and Ray, 2006; Manto, 2014). Besides, this disorder may manifest as Parkinsonism and/or cognitive impairment (Lorincz, 2010). This cascade of events is a consequence of oxidative stress induced by free Cu^{2+} (Ranjan, et al., 2015).

Furthermore, impairment of Cu may cause a decrease in neuronal cell viability or induce synaptic alterations (Sadiq, et al., 2012; Marchetti, et al., 2014), upsets neural synaptic activity *in vivo* and *in vitro*. It is interesting to note that major sources of Cu toxicity are associated with exposures to environmental contamination and occupational hazards among others (Gaetke, et al., 2014).

3.2 Methods

3.2.1 MTT cell proliferation assay

50 µL Phosphate Buffered Saline (PBS) (136.9 mM 2.68 mM KCl, 4.3 mM Na_2HP0_4, 1.4 mM KH_2P0_4, pH 7.4) containing 5 mg/ ml MTT was added to the cultures and incubated at 37°C and in 5% CO2 for 2.5 hours. Then the MTT- containing medium was removed, the surface of the wells was rinsed with 300 µl PBS before the application of 250 µL isopropanol. After the purple crystals had dissolved, the optical density of 100 µL sample was spectrophotometrically read at 595 nm (Thermo Lab systems Multiskan Ascent, V1.3).

3.2.2 Lactic dehydrogenase (LDH) release assay

3.2.2.1 CytoTox 96 non-radioactive assay (Promega, UK)

CytoTox 96 is a non-radioactive colourimetric cytotoxicity assay. This was used to quantify lactate dehydrogenase (LDH). This is a stable cytosolic enzyme that is released during cell lysis (necrosis). The supernatant, which contained the LDH was retained and used for LDH assays. Before the assay, the samples were diluted with PBS (1:5). 50uL of each sample were transferred into each well of the assay plate (in triplicates) and 50uL of substrate mix was added to each sample and incubated for 30 mins. on the bench at room temperature and protected from light. After 30 mins., 30uL of Stop Solution was added to each well and the absorbance was measured at 490nm on a Multiscan Ascent Plate Reader, Version 2.6.

3.2.3 Calcium imaging

In this investigation, cultured CAD cells were probed with the molecular dye; Calcium-Green-1 AM (Invitrogen) as specified in the manufacturer manual (ref). Calcium Green is used to investigate how intracellular calcium fluctuates with neuronal activity. A 2mM stock of the Calcium-Green probe was prepared in DMSO D-8779 500Ml, lot 100K3729 by Sigma). This was stored at -20ºC. On the day of the imaging experiments, the probe stock was brought to room temperature and then diluted in HEPES physiological buffer (150mM NaCl, 1mM $MgCl_2$, 10mM HEPES, 2mM $CaCl_2$, 5mM KCl) to a 1µM solution.

The cells were plated in flat bottom µ-dishes (brand: 60 µ-Dish, 35mm, high glass flat bottom-81158 Ibidi GmbH). The cells were washed with (once) 1mL of physiological buffer and incubated for 40 mins. in 1mL of the probe solution and once more after incubation

period- with the HEPES physiological buffer and left in the buffer till the start of the imaging experiments- ready for imaging. To evaluate the effects of Cu (at a final concentration of 40μM) on cellular calcium fluxes, baselines were taken for around 5 seconds and then Cu was injected into the dish. Careful pipetting was ensured by injecting against the dish wall to avoid causing fluid turbulence, thereby compromising the images.

The cells were imaged in the presence of Cu (10 & 40μM) and potassium chloride (KCl). The incubation period for each treatment was for the periods of 250 cycles (0.63s each) before and after the application of the same volume of the depolarising buffer (500mM of KCl in HEPES physiological buffer, giving a final concentration of 49.6mM).

3.2.4 Western Blot

3.2.4.1 SDS-PAGE

The SDS-PAGE was used to separate proteins by their molecular mass. Therefore, a total volume of 10 µl of each sample prepared as described (in Section 2.2.9.1) was loaded into one well in the stacking gel (See Table 2.4). Due to the Laemmle sample buffer, all proteins are negatively charged and run from the cathode to the anode through the gel when connected to power. The running gel containing 10 % acrylamide (See Table 2.1) forms a grid forming pores that small proteins can easily pass through but make it difficult for bigger proteins to run through.

The gel was run at 10mA for 15 mins. to help the samples to settle in the stacking gel before the current was increased to 15mA (but the double current for 2 gels) and the gel was run for another 1-1.5 hours until the sample buffer (See Table 2.4) got to the bottom end of the gel. As a scale for protein size, a standardised marker (HyperPAGE,) was run on every gel next to the samples.

3.2.4.2 Blotting

After the SDS-PAGE, proteins were blotted on a nitrocellulose membrane using electric current. For the protein transport from the gel unto the membrane, the membrane was pre-incubated in transfer buffer. Transfers were done on a lockable cassette. The cages were filled

in the following order: sponge, two filter papers, nitrocellulose membrane, gel, two filter papers, and sponge. All materials used were soaked in transfer buffer (See Table 2.4) for at least 30 minutes. before transfer to ensure that neither the gel nor the membrane was running dry.

After the cages were locked, they were placed in a Hoefer® Mighty Small Transfer Tank filled with transfer buffer with the cathode facing the side of the gel and the anode facing the nitrocellulose membrane. The transfer was performed at 50 V and >300 mA enabling the negatively charged proteins to travel towards the anode and therefore entering the membrane when exiting the gel. Two cooling units were placed on the sides of the tank to prevent temperature increases due to the electricity. The transfer was due 2.5 hours later.

3.3 Results

3.3.1 Dose-dependent effect of Cu on undifferentiated, differentiating, and differentiated CAD cells

As a first step, a dose-response effect of Cu on the viability of undifferentiated, differentiating, and differentiated CAD cells was investigated. This was aimed at determining the subtoxic concentration of Cu for further experiments to help choose a suitable range of doses as a subtoxic dose to use for the series of experiments planned. Dose-response effect of Cu at different stages of development (undifferentiated, differentiating and differentiated) of CAD cells- MTT

Concerning metabolic function, Cu did not affect mitochondrial functionality at lower doses for both acute and chronic exposures- at different stages of maturation. However, it reduced mitochondrial viability for chronic differentiating and acutely treated cells- at higher concentrations.

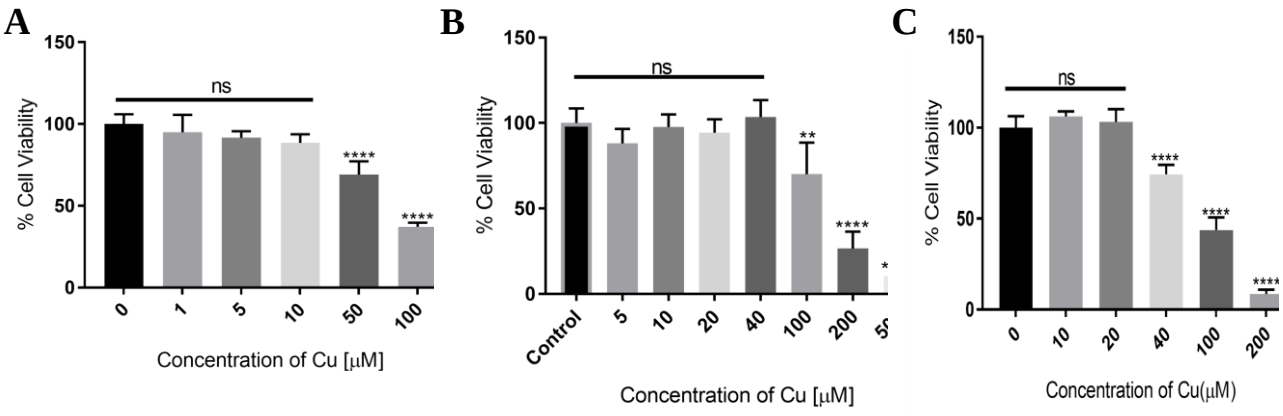


Figure 3.1: : Dose-response effect of Cu at Different level of development of CAD cells.

Results of dose-response treatment of CAD cells at different stages of development. Acute (24hours) treatment of undifferentiated cells (Panel A), chronic (6 days) treatment of differentiating cells (Panel B) and acute treatment of differentiated cells (Panel C). The mean IC_{50} values were determined using GraphPad Prism© 7.05 fitting the data to a sigmoidal fit of a variable slope. The mean IC_{50} values for undifferentiated CAD cells = 8 x 10^{-5} M; differentiating cells = 12 x 10^{-5} M; and differentiated cells = 14 x 10^{-5} M were similar. All values are means ± SD, from at least 6 separate experiments and n=4 for each experiment. ** $p < 0.01$; *** $p < 0.001$.

3.3.2 Effect of Cu exposure on undifferentiated, differentiating, and differentiated CAD – MTT

The subtoxic concentration of Cu (10µM) did not affect mitochondrial viability in both acute and chronic exposed CAD cells. 40µM reduced mitochondrial viability for chronic differentiating and acutely treated cells- but with no effect on undifferentiated cells.

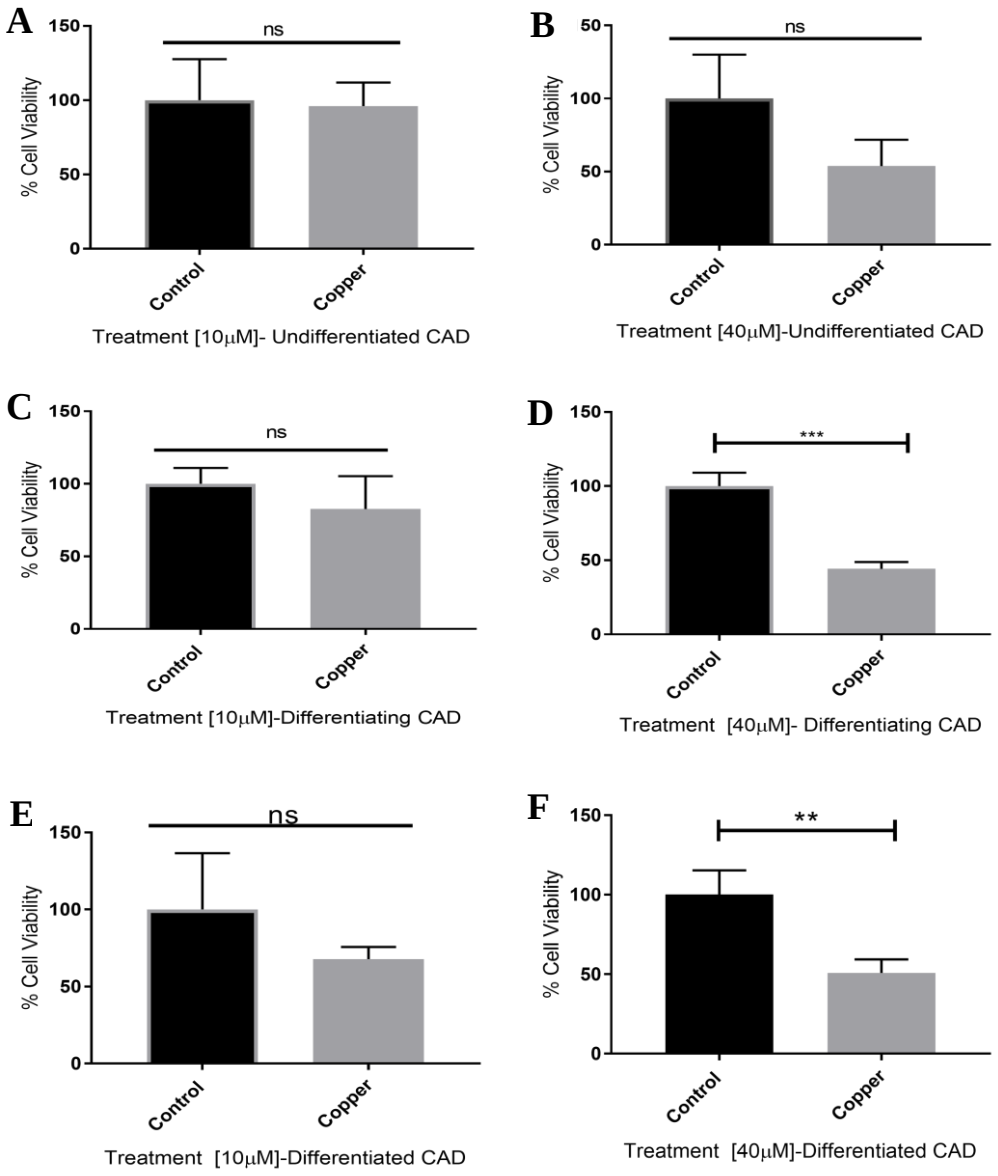


Figure 3.2: Exposure of CAD cells to sub-toxic (10 µM) and low-dose Cu (40 µM)- MTT

An MTT assay was carried out to confirm the effect of Cu (24 hours) exposure on undifferentiated (A, B) and differentiated cells (E and F) and chronic (6 days) exposure on differentiating CAD cells (C and D). Cu concentrations tested were 10 µM and 40 µM. Values are mean for n= 4 samples. (* $p<0.05$; **$P<0.01$; *** $p<0.001$). ns = non-significant. Overall, Cu (10 µM) did not affect MTT values (mitochondrial viability) (Fig. 2.2A, C and E) for both acute and chronic exposures. In contrast, Cu (40 µM) had no significant effect upon

undifferentiated cells, but reduced MTT values for chronic differentiating and acutely treated differentiated cells (Fig. 2.2B, D and F).

3.3.3 Lactate dehydrogenase assay

Since an MTT assay is not a clear indicator of cytotoxicity, and LDH assay was used to find out whether the culture conditions and treatments elicited a cytotoxic effect on neurons. LDH assays are another type of colourimetric assay used to quantify the levels of LDH released into the cell culture medium due to cell lysis. The resulting red colour from the enzymatic reaction can be measured spectrophotometrically (absorbance), and a stronger colour indicates a higher degree of cell lysis. An increase in the amount of LDH release is indicative of toxicity.

3.3.4 Effect of 10 µM and 40 µM Cu exposure on undifferentiated, differentiating, and differentiated CAD - lactate dehydrogenase (LDH)

Subtoxic dose (10 µM) treatment of CAD cells at different stages of development showed a decreased released of LDH in undifferentiated, differentiating and differentiated cells. Also, treatment with 40 µM showed a decrease in LDH release in both differentiating and differentiated CAD cells, with reference to the control. However, treatment with 40 µM in undifferentiated showed an increase (non-significant) in LDH release.

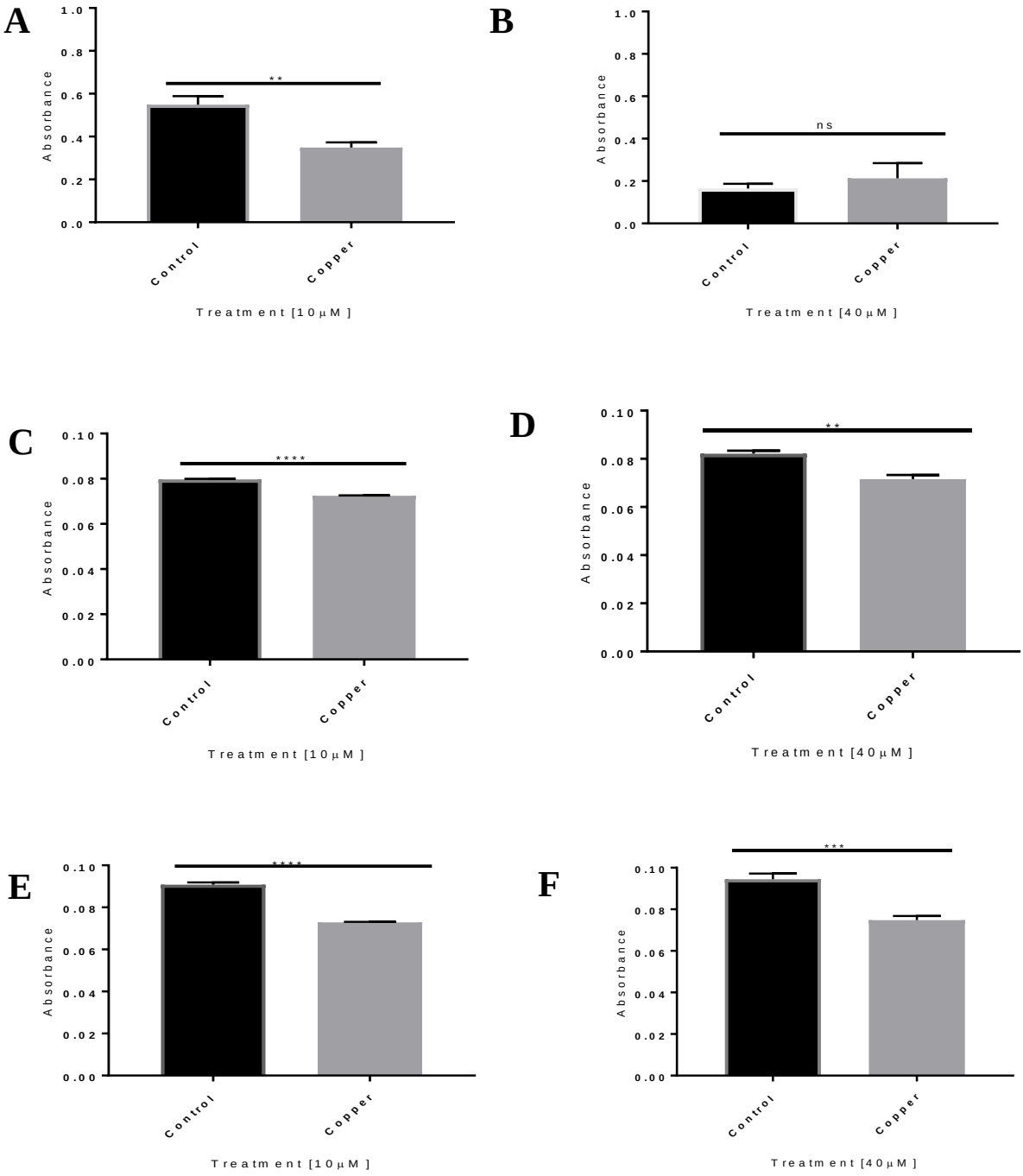


Figure 3.3: Exposure of CAD cells to sub-toxic (10 µM) and low-dose Cu (40 µM)- LDH

An LDH assay was carried out to probe the effect of Cu (24 hours) exposure on undifferentiated (A, B) and differentiating cells (E and F) and chronic (6 days) exposure on differentiating CAD cells (C and D). Cu concentrations tested were 10 µM and 40 µM. Values are mean for n= 4 samples. (* $p<0.05$; **$P<0.01$; *** $p<0.001$). ns = non-significant. Acute and chronic exposure with 10 µM Cu is not cytotoxic there is a suggestion of modest but significant neuroprotection in culture. Acute exposure of differentiated cells and chronic exposure of differentiating cells to 10 µM and 40 µM Cu again shows lack of toxicity at these concentrations, again with a suggestion of neuroprotection, with lower LDH release in the presence of Cu.

3.3.5 Deferoxamine (DFO)-mediated iron-chelation on undifferentiated, differentiating, and differentiated CAD cells.

The influence of iron chelation on cell viability (MTT) in acute exposure of differentiated and undifferentiated CAD cells to Cu was explored. There is an accumulation of iron in PD patient, which may potentiate oxidative stress, which is implicated as an underlying cause of neuronal cell death. The next set of experiments were designed to investigate the effect of Fe chelation on acute and chronic exposure of Cu at different concentrations of Cu. This was performed to elucidate the potential effect of iron chelation on cell viability, hence, ameliorate possible mitochondria stress response due to Cu insult.

Table 3.1: Effects of Iron chelation upon Cu-induced toxicity (Acute and chronic treatments) in CAD cells

CAD cell stage & treatment regime	Control	+ 25 µM DFO	+ 50 µM DFO
Acute undifferentiated	8×10^{-5} M	$\geq 10 \times X10^{-5}$ M	$2 \times X10^{-5}$ M *
Chronic differentiating	12×10^{-5} M	14×10^{-5} M	$13 \times X10^{-5}$ M
Acute differentiated	14×10^{-5} M	n.d.	$3 \times X10^{-5}$ M*

nd= not determined.

3.3.6 Effect of Deferoxamine (DFO)-mediated iron-chelation on undifferentiated, differentiating, and differentiated CAD cells.

MTT assay on mitochondrial functionality showed that acute exposure CAD cells (at different stages of maturation) to 50µM DFO increase the sensitivity of undifferentiated and differentiated cells to Cu (approximately 4-fold) with reference to controls. For the chronically treated differentiating CAD cells, there was no apparent difference in Cu IC_{50} in the presence of DFO (Table 3.1).

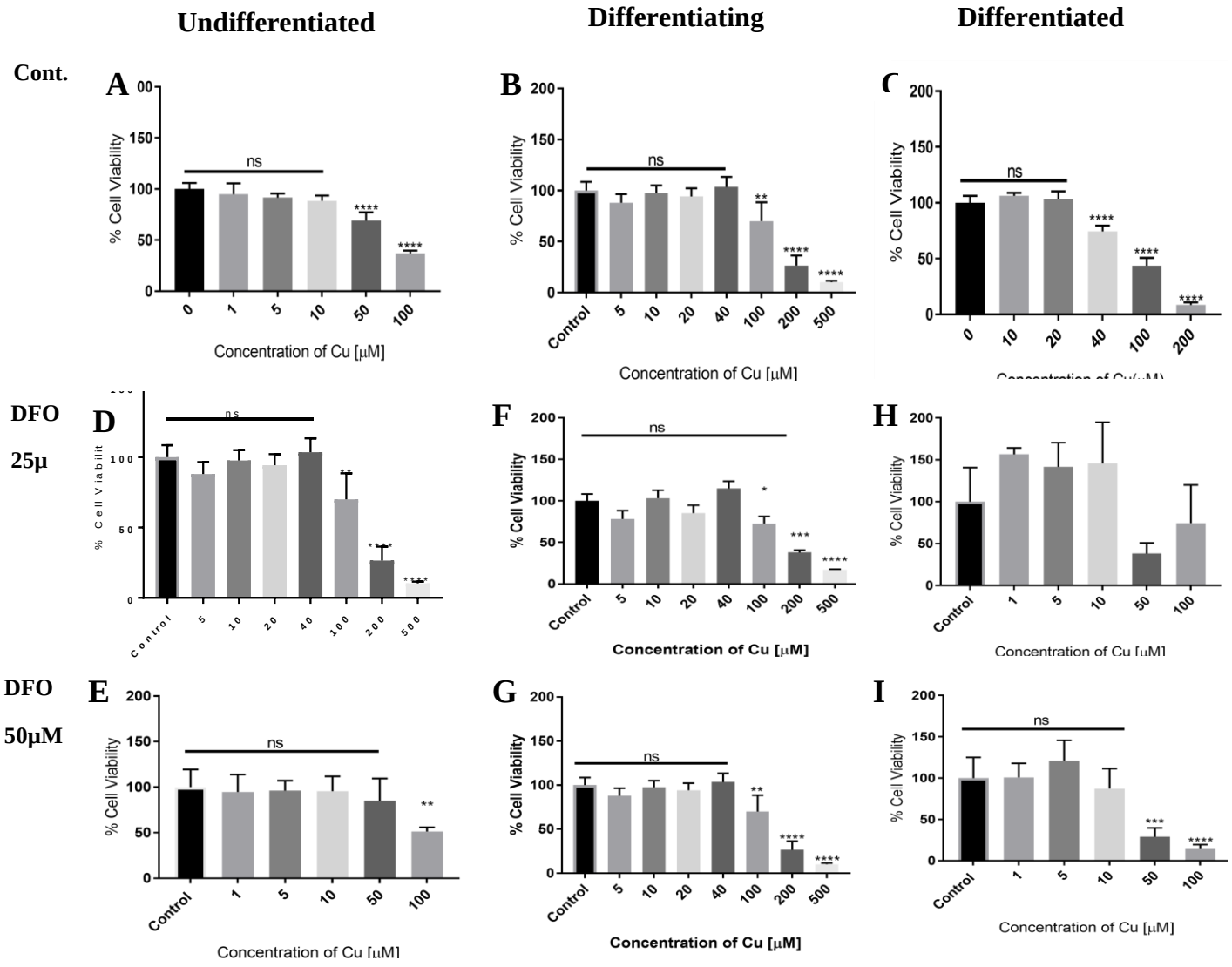


Figure 3.4: Fe chelation in undifferentiated, differentiating and differentiated CAD Cells.

Effects of iron chelation (DFO) upon Cu-induced cell toxicity following acute 24h for undifferentiated and differentiated and chronic 6-day exposure for differentiating CAD cells.

Values are mean for n= 4 samples. (* $p<0.05$; **$P<0.01$; *** $p<0.001$; ****$p<0.0001$) ns = non-significant.

3.3.6.1 Effect of Cu upon functional CAD monoaminergic Neurons – calcium imaging

Following identification of two subtoxic Cu concentrations (10 µM and 40 µM), the latter displaying mitochondrial oxidative stress effects, the effects of Cu on functional responses were explored using a calcium imaging approach in undifferentiated and differentiated CAD cells. Baseline control recording was measured initially in undifferentiated CAD cells (Fig. 2.5).

3.3.6.1.1 Control calcium signal

Basal reading was obtained before investigating the functional response of cells to Cu. KCl, used as a positive control, showed a surge in calcium flux in the cell body of CAD cells with reference to the same cell before injection of KCl.

Cell body- before depolarisation **Cell body after depolarisation**

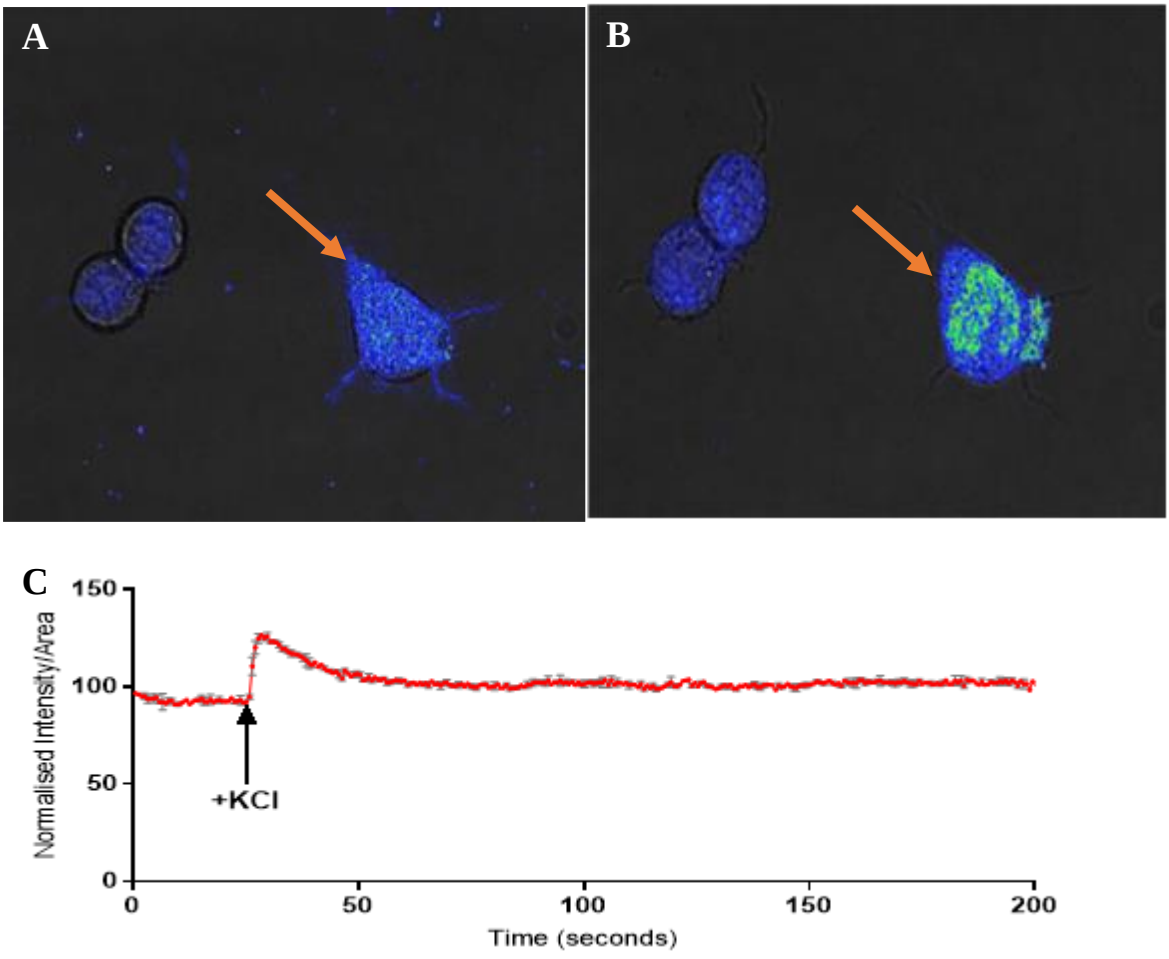


Figure 3.5: Basal reading showing the functional response of undifferentiated CAD cells upon KCl induced depolarisation.

Panel A/B and the adjunct Fig. (C) shows representative baseline recordings of calcium signal in CAD neuronal cell body. Panel B shows the effect of depolarisation induced calcium signalling following injection of KCl (50 mM). A surge in calcium signal is seen which is represented by a spike and an extended signal (approx. 50 secs).

3.3.6.1.2 Effect of Cu upon calcium imaging in undifferentiated CAD cells

Cu (10 µM) elicited no detectable effect on calcium signalling (Fig. 3.6A) but completely suppress the depolarisation response in the cell bodies of undifferentiated cells (Fig. 2.6B) compared to control (Fig. 2C).

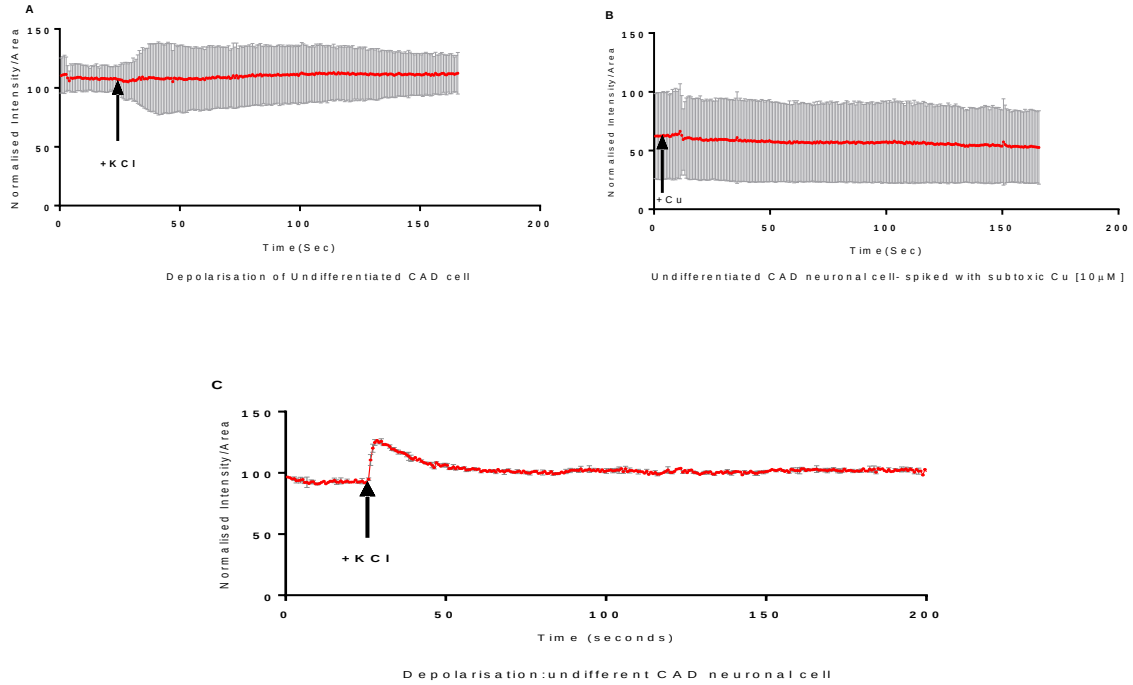


Figure 3.6: Calcium modulation in undifferentiated CAD cells in the presence of [10 µM] Cu.

Spiking with Cu (10 µM) did not elicit any calcium signal (A). In the presence of Cu, KCl-induced-depolarisation was very modest (B) compared to the control response. Representative images and data for n=6 separate experiments with at least 10 individual cells per experiment

3.3.6.1.3 Effect of Cu upon Calcium Imaging in differentiated CAD Cells

The next sets of experiments were designed to elucidate the effect subtoxic and low doses of Cu (10 µM and 40 µM) on both the cell body and neuronal process of differentiated CAD cells. There was no marked difference in the effect of Cu on both the cell body and process - with subtoxic dose (10 µM) as against treatment with low dose (40 µM), where a prominent surge in Ca influx was noted in the cell body and a concurrent inhibition in the processes, following KCl-induced mobilisation.

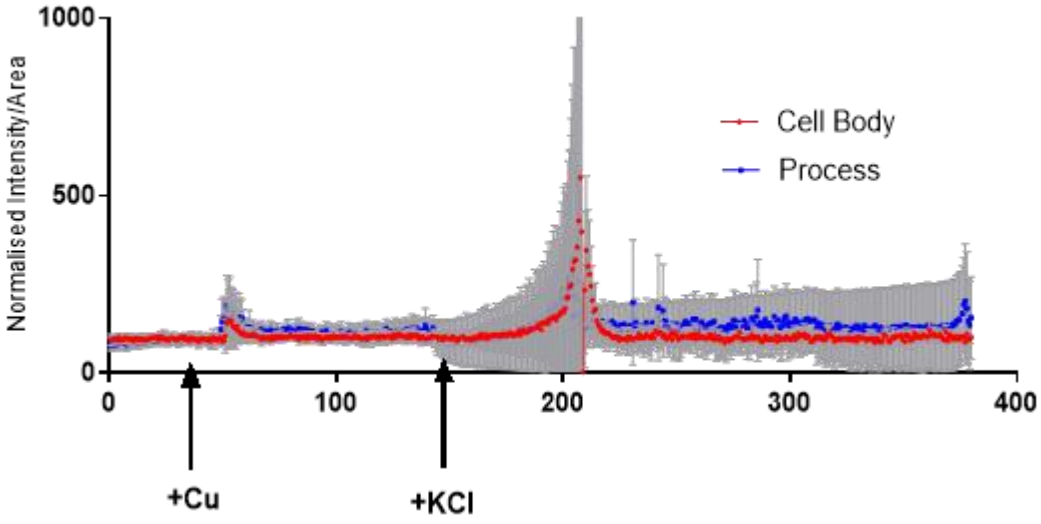


Figure 3.7: Effect of Cu (10µM) on differentiated CAD cells.

Sub-toxic dose Cu (10 µM) caused an increase in signal intensity in the cell soma and process-with a slightly higher increase in signal strength in the processes. There was a delay in signal intensity following KCl-induced depolarisation. Selective suppression of depolarisation (KCl-induced) calcium intensity is apparent in the processes (with normalised intensity/area <400) but this was absent in the cell body (normalised intensity/area >600) of differentiated neurons. The signal intensity of the cell body was returned to baseline (post depolarisation) but that of the processes failed to return to baseline.

3.3.6.1.4 Effect of Cu (40µM) upon Calcium Imaging in differentiated CAD Cells

Low dose (40µM) treatment of differentiated CAD cells indicated an opposite effect on both the cell body and the processes. There was a surge in Ca signal following KCl-induced depolarisation, in the cell body. In contrast, there was an inhibition of Ca signal in the process.

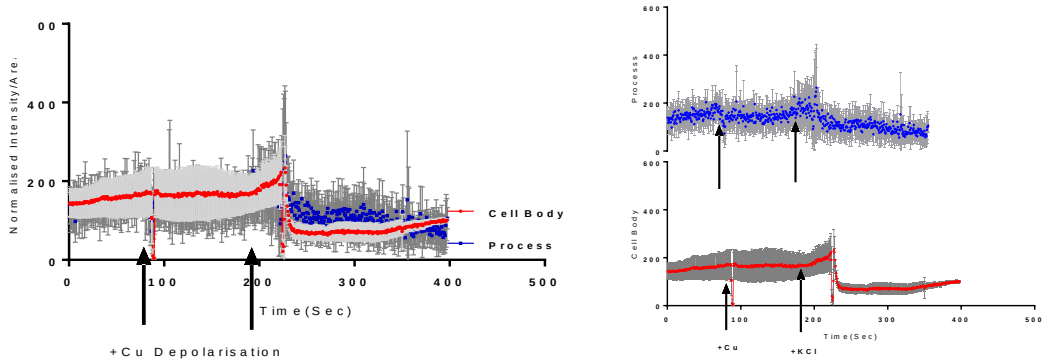


Figure 3.8: Effect of Cu (40µM) on differentiated CAD cells.

Low-dose Cu (40µM) bi-directionally modulates calcium levels in the neuronal processes (inhibits-blue) and cell body (elevates-red) of DIV 6 differentiated CAD neurons in cultures. Low-dose Cu (40µM) selectively suppresses depolarisation-induced calcium in the processes, but not the cell body of differentiated neurons in cultures (Fig. 2.9), which has implications for neurotoxicity and synaptic plasticity. Representative images and data for n=6 separate experiments with at least 10 individual cells per experiment.

3.3.6.1.5 Bidirectional modulation of calcium homeostasis +/- Cu (40µM) in differentiated neuronal CAD cells

The inhibitory effect of Ca (40µM) on the process of differentiated CAD cells was noted in the process (indicative a blockage of neurotransmitter release) as against the cell body where an increase in Ca signal was observed.

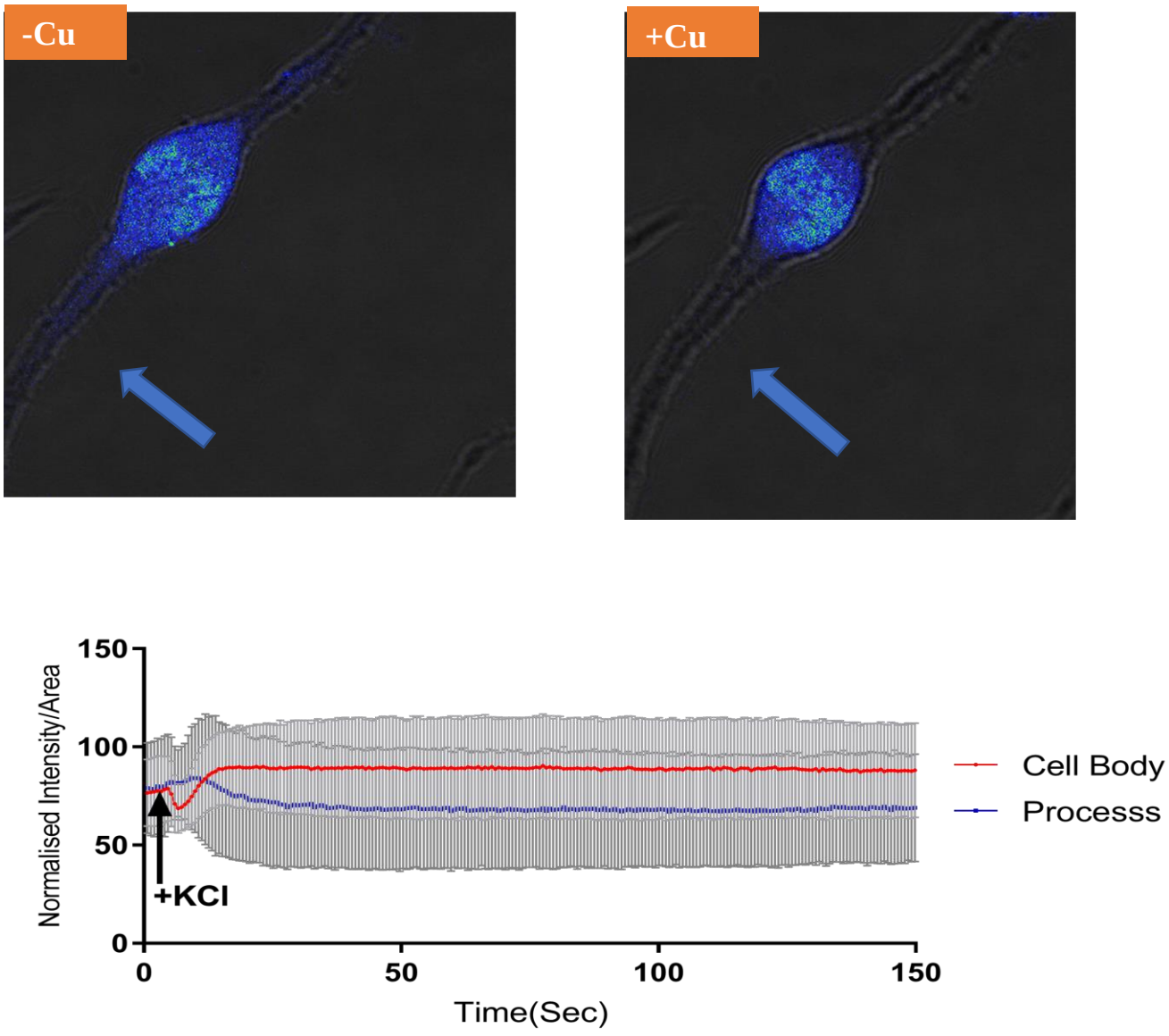


Figure 3.9: Effect of Cu (40µM) on Calcium-induced depolarisation in differentiated CAD cells

Calcium modulation in neuronal cells. Live cell imaging with CAD cells with low dose Cu (40µM) bi-directionally modulates calcium levels-inhibiting calcium signal in the process (blue line) and concurrently elevates the calcium level in the cell body (red line). Representative images and data for n=6 separate experiments with at least 10 individual cells per experiment.

3.3.7 Effect of Cu (24 hours) on differentiated CAD cells

Microtubule-associated protein 2 (MAP-2) is a structural protein, necessary along with other cytoskeletal proteins, to maintain neuroarchitecture. It is sensitive to many physiological inputs, and plays a vital role in the growth, differentiation, and plasticity of neurons, responding to growth factors, neurotransmitters, synaptic activity, and neurotoxins. Modification and rearrangement of MAP-2 is an early obligatory step in many processes which affects neuronal function. The brain microtubule-associated protein MAP-2 family is composed of high-molecular-weight (MAP2a and MAP2b) and low-molecular-weight (MAP2c and MAP2d) isoforms. This investigation was designed to elucidate the possible effect of Cu (sub-toxic doses) on differentiation using MAP-2 as a marker.

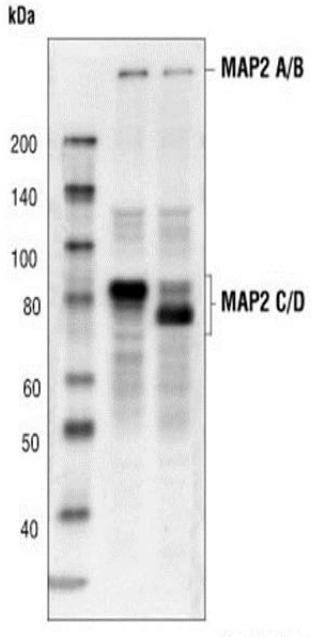


Figure 3.10: MAP-2 Isoforms: Multiple isoforms are expressed in neurons: MAP2A-280kDa, MAP2B - 270kDa, MAP2C, MAP2D - 70 & 75kDa - (Sanchez, et al., 2000); G.W. Huntley, 1998).

3.3.7.1 Effect of Cu (10µM) on differentiated CAD cells - 24 hours

The selective effects of Cu upon cell bodies and processes reported in the previous section were explored further by investigating the effects of Cu (10µM and 40µM) on MAP-2 expression in differentiated CAD cells. Sub-toxic Cu negatively modulated differentiation by selectively reducing MAP-2a isoform, with no significant effect upon MAP-2b isoform, for both 10 and 40 µM concentrations of Cu (Figures 3.11 and 3.12).

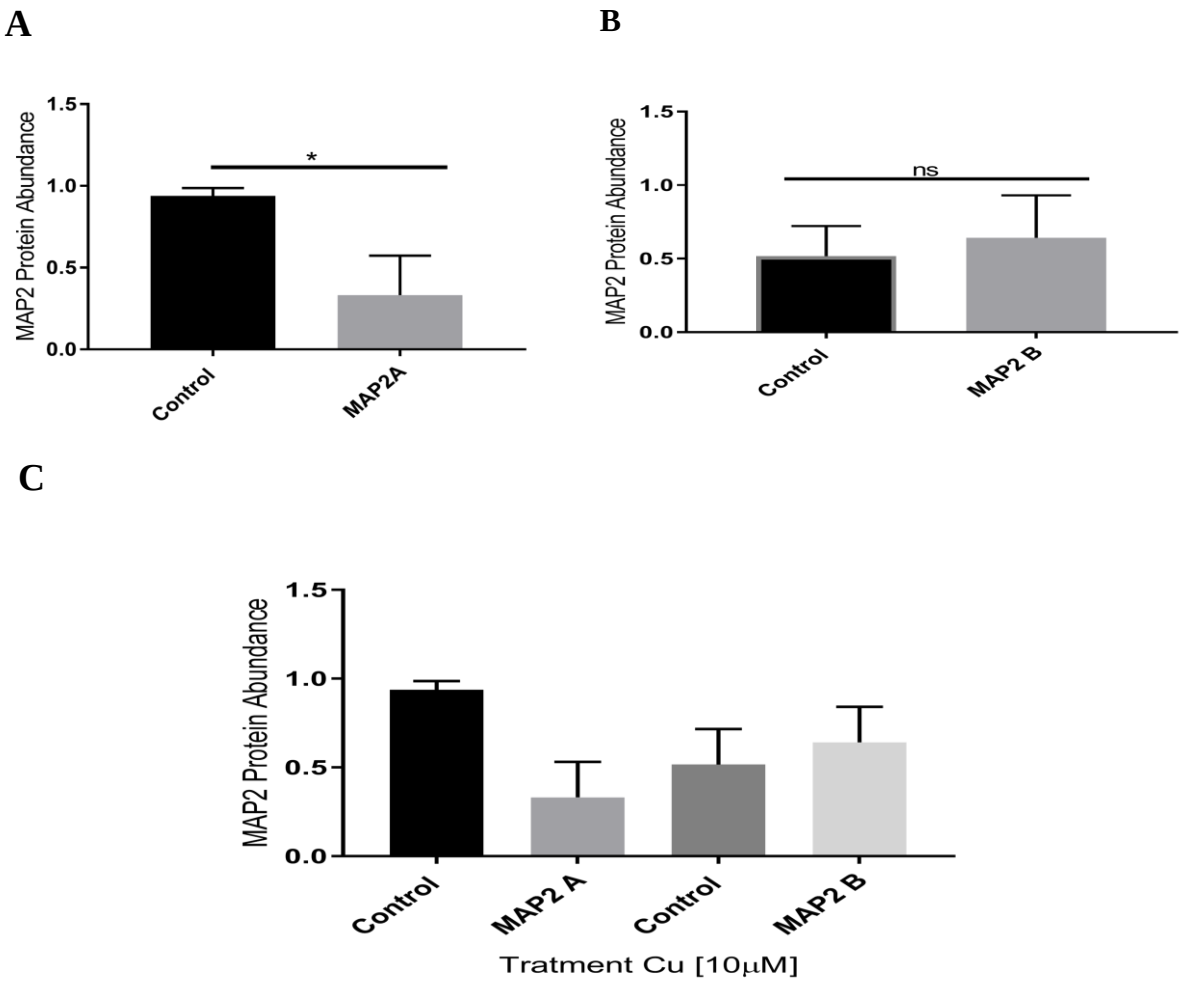


Figure 3.11: Quantitative analysis of immunoblot data on MAP-2 expression following acute exposure (24 hours) of Cu (10µM) compared to control on differentiated CAD cells.

Results show there was a significant reduction in the expression of MAP-2A (Panel A, $p<0.05$) in comparison to untreated control. There was a moderate increase in MAP 2B in comparison to control, but not statistically significant (Panel B, $p>0.05$). Panel C shows a comparison of MAP2A vs MAP2B. Figures are mean values ±SD, n =6

3.3.7.2 Effect of Cu (40µM) on differentiated CAD cells - 24 hours

Acute exposure (24 hours) of differentiated CAD cells to low dose Cu (40µM) resulted in a reduction in expression of MAP 2a and MAP 2b isoforms.

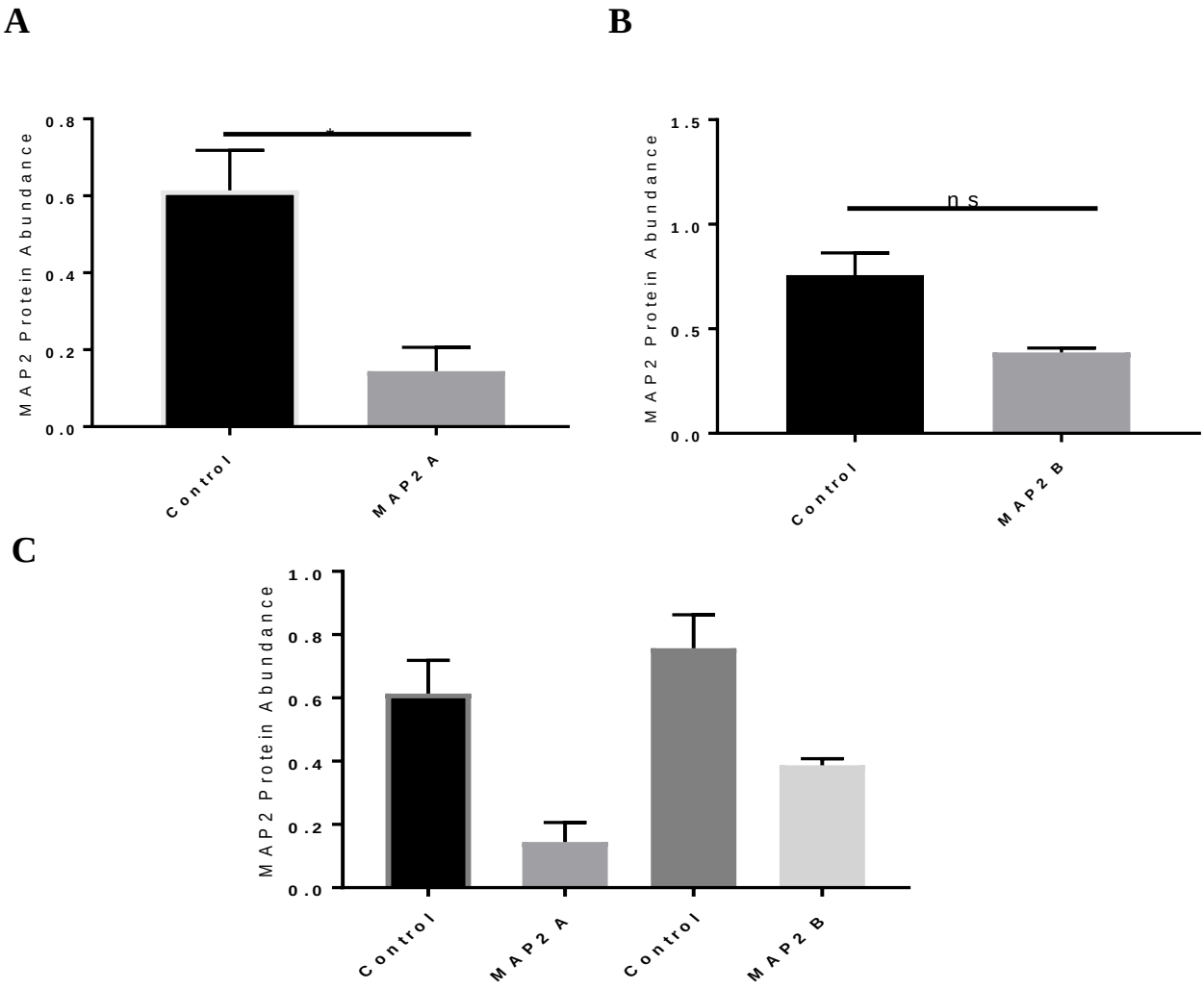


Figure 3.12: Quantitative analysis of immunoblot data on MAP-2 expression following acute exposure (24 hours) of Cu (40µM) compared to control on differentiated CAD cells. A significant reduction in the expression of MAP-2A (Panel A, $p<0.05$) in comparison to control, but not statistically significant ((Panel B, $p>0.05$). Panel C shows a comparison of MAP-2A vs MAP-2B. Figures are mean values ±SD, n=6.

3.4 Discussion

Cu homeostasis is tightly regulated by the activity of Cu pumps at the cellular levels. Failure of these pump leads to the accumulation of Cu and cell toxicity. Impaired functions of mutated Cu-ATPase (ATP7B), which exports Cu through the trans-Golgi network causes Wilsons' Disease (an autosomal recessive disorders) which is associated with Cu overload and resultant cell degeneration (Brewer, 200; Brewer, 2009). Sokol, et al (1994) and GU, et al (2000) investigated the mechanisms underlying liver Cu toxicity in Wilson's Disease. In their findings, they suggested that the mitochondrion is the prime target.

Arciello, et al (2004) in their investigation of Cu-dependent toxicity, using SH-SY5Y neuroblastoma cells noted that Cu (in complete medium for 24 hours) had a toxic effect which they adduced to the impairment of the capability of the mitochondria dehydrogenase to reduce tetrazolium salt, and to a lesser extent the loss of the integrity of the plasma membrane. They concluded that the mitochondria are an early and susceptible target of Cu-mediated oxidative stress in neuronal cells and posited that mitochondrial damage triggers the neurodegenerative processes associated with Cu overload in Wilson's Disease.

This chapter is devoted to elucidating the effect of sub-toxic concentrations of the endogenous heavy metal Cu on the cell biology and functionality of monoamine neuronal cells at different stages of maturation. As a first step, the dose-response effect of Cu was performed using acute (24 hours) and chronic (6 days) exposure times on metabolic activity (MTT) and cytotoxicity (LDH). This was to determine suitable concentrations for subsequent experiments on neuronal functionality and differentiation. Neuron metabolic rates were relatively resistant to applied Cu with similar IC_{50} values (approx. 10mM) (Fig. 2.1).

Based on the results, 10µM Cu was shown to have no significant effect upon metabolic rate or cytotoxicity, while 40µM of Cu reduces metabolic rate for differentiating (6 days exposure) and differentiated (24 hours exposure) cells but remained non-cytotoxic for all conditions. This will probably reflect the increased excitability of differentiated cells. Cu is critical in neuronal transmission (Zischka and Einer, 2018), memory and learning (Kapkaeva, et al., 2017). A

disturbance to Cu homeostasis could be a consequence of either genetic or environmental factors or a combination of both factors (Gaetke, et al., 2014).

Accumulation of Cu in specific brain regions such as the thalamus, subthalamic nuclei, brainstem, and cortex has resulted in Cu-induced neuronal cell loss; as seen in Wilson's Disease (Kapkaeva, et al., 2017). Accumulated Cu level in this disease pathology has been noted to be in the range of 128-,000µg/g dt. (Faa, et al.,1995). Significant neurological disorder such as tremor, ataxia, bradykinesia, rigidity, chorea, and dystonia (Cordato, et al., 2004) and biochemical interruptions (disturbance in glucose consumptions) have been associated with Wilson's Disease (Cordato, et al., 2004). Increased Cu-induced oxidative stress has been noted in these changes (Ranjan, et al., 2015). Functional brain concentration of Cu^{2+} is about 100 µM depending on certain conditions and specific sites (Kapkaeva, et al., 2017). Shao (2013) and his colleagues estimated the basal level of Cu (in the brain dialysate) to be 1.7µM but noted that this value increased to 10µM following global cerebral ischaemia. However, Kapkaeva, et al (2017) in their investigations proposed an oxidative mechanism of Cu^{2+} toxicity, when they observed that a 50µM of Cu^{2+} in culture, induced a strong dose-dependent death of neuronal hippocampal cells. They also observed a further reduction in cell viability of cultured hippocampal neurons on the application of 200µM of $CuCl_2$ (24 hours exposure).

In contrast, 10µM $CuCl_2$ did not have any significant influence on cell viability. Previous studies show that application of 10µM of Cu^{2+} is not cytotoxic for cultured neurons (Stelmashook, et al., 2016; Stelmashook, et al., 2019). This is in concert with our findings where we demonstrated that 10µM of Cu had no effect on mitochondrial viability for both acute and chronic exposures (with LDH assay confirming its non-toxicity on both cells undifferentiated and differentiated cells).

The perturbation of Cu^{2+} homeostasis not only results in neuronal cell death but also causes synaptic alterations (Sadiq, et al., 2013; Opazo, et al., 2014). Results of calcium modulation were ambivalent; 40µM Cu bi-directionally modulates calcium levels in the neuronal processes and cell body. Whilst an inhibitory effect occurred in the processes, there was an elevation of calcium signal in the cell of (DIV 6) differentiated CAD neurons in cultures (Fig. 2.8). It also

selectively suppresses depolarisation-induced calcium in the processes (Fig 2.9), but not the cell body of differentiated neurons in cultures. This has implications for neurotoxicity and synaptic plasticity. These findings are in concert with the findings of Colvin, et al (2015) where they noted that Cu levels are distributed diffusely in neurons, but higher in the processes relative to other metal ions, such as zinc.

Kapkaeva, et al (2017) noted that treatment of cultured hippocampal neurons with 10µM Cu^{2+} significantly reduced the spike activity of cultured neuronal network (which was attenuated by the antioxidant - Trolox). Besides, they posited that when neurons were exposed/treated with sub-toxic Cu^{2+} it interrupts synaptic function and breaks the distribution of the signal in the neuronal network. This probably explains the selective suppression of KCl-induced depolarisation when differentiated CAD cells were spiked with 10µM Cu. The decrease in calcium intensity was apparent in the processes with normalised intensity <400 normalised intensity/area but absent in the cell body (normalised intensity >600 normalised intensity/area). Whilst the signal intensity of the cell body was returned to baseline (post-depolarisation) the signal intensity in the processes failed to return to baseline). Kapkaeva, et al (2017) further asserts that bioelectric activity of neurons is a probable key target of Cu-induced neurotoxicity in which free radicals can be involved.

In terms of sensitivity of differentiated cells to Cu, Ogra and his colleagues (2016) suggested that the intracellular Cu concentration is increased by differentiation. In their research, they demonstrated that the process of differentiation induces Cu accumulation in PC12. This was followed by a concomitant increase in Cu transporter 1 (Ctr1) as a compensatory mechanism to ameliorate Cu accumulation. The increased Cu in differentiated cells are bound to MT-3 that they suggested as the possible factors underpinning the progression of the pathological changes observed in the nerve cells.

Similarly, Hatori, et al (2016) observed an increase in cellular demand for Cu upon neuronal differentiation, notably in the secretory pathway. The flow of Cu to this compartment is facilitated through transcriptional and metabolic regulation (Hatori, et al., 2016). During neuronal differentiation, there is a change in the oxidation state of glutathione in the cytosol

and the redox state in the cytosol facilitates the reduction of the Cu chaperone – Atox1. The reduction of this chaperone results in the liberation of metal from its binding sites. Related to this (the expression of Atox1) is the upregulation the Cu transporter, ATP7A.

These events result in a higher flux of Cu across the secretory pathway, thereby, increasing the supply of the co-factor to Cu-dependent enzymes which are elevated in differentiated neurons (Hatori, et al., 2016). This probably explains why differentiating and differentiated cells were more sensitive to Cu toxicity in both chronic and acute exposure. As previously reported (Fig. 2.2 B, D and F) 40µM of Cu had no significant effect upon undifferentiated cells, but reduced MTT values for chronic differentiating and acutely treated differentiated cells- implying a reduced metabolic rate in both differentiating and differentiated cells.

Altered metal homeostasis has been reported in PD (Rubio-Osornio, et al., 2013; Montes, et al., 2014). For example, augmentation of Fe and a decrease in Cu levels in the substantial nigra was reported (Kozlowski, et al., 2012). This elevated (and unbound) iron may contribute to increased oxidative stress, alpha-synuclein oligomerization, and Lewy body formation (Montes, et al., 2013). Besides, decreased Cu along with increased iron has been found in the caudate nucleus of PD patients (Montes, et al., 2013). Cu influences iron content in the brain through ferroxidase ceruloplasmin activity. Therefore, decreased protein-bound Cu in the brain may enhance the iron accumulation and the associated oxidative stress. Herein, we explored the interaction between iron and Cu-mediated metabolic rate reduction.

Acute exposure of 50µM of DFO (a synthetic chelator) increased the sensitivity of undifferentiated and differentiated cells to Cu, by approximately four-fold compared to the control. This was not apparent in chronically treated differentiating cells. An adaptive response may probably explain the lack of sensitivity to Cu in chronically treated cells. The increase in sensitivity of undifferentiated and differentiated cells following iron chelation (with DFO) (acute) may not necessarily be related to the chelation of iron within the cells.

Gangania, et al (2017) in their investigation while elucidating the role of iron and Cu in the pathogenesis of PD, noted that serum Cu and Fe levels were significantly decreased in PD patients compared to the controls, but ceruloplasmin levels were slightly raised. Using the Spearman's Correlation Coefficient to investigate the correlation, between iron-Cu, Cu-ceruloplasmin, iron-ceruloplasmin in PD patients and controls, they reported no obvious correlation among parametric pairs- implying that the concentrations of Fe, Cu and ceruloplasmin may be relatively independent of each other (Gangania, et al., 2017). It is unclear if Cu modulates Fe flux or Fe modulates Cu flux. However, it is established that tight regulation of both Cu and Fe transport is critical to avoid neurological perturbation associated with metal imbalance (in cases such as PD and AD) and disease specifically associated with Cu (Menkes and Wilson's Diseases) or iron (Friedreich's Ataxia and ceruloplasminemia).

Cu deficiency has been noted to result in elevated iron levels in the liver, which is partly due to decreased levels of ceruloplasmin activity. In contrast, Prohaska and Gybina (2005) observed that iron concentration is decreased following dietary Cu deficiency in rat brains. This may be associated with lower serum Fe because the injection of Cu-deficient rats with Fe reversed the serum and brain iron deficit (Prohaska and Gybina, 2005; Pyatskowit and Prohaska, 2008).

As a redox-active metal, iron (Fe) is essential for cell respiration and metabolism. Pourcelot, et al (2015) demonstrated that cells deprived of Fe (by the addition of a chelating agent) resulted in growth arrest. However, this was reversed after replacing the media with an iron source (after 24 hours). Brain regions responsible for motor functions have been shown to have more Fe than non-motor related regions. Hence movement disorders are often associated with Fe loading (Myhre, et al., 2013; Ashraf, et al., 2018). While metal chelation may be beneficial, the ubiquity of their action in the body and their non-selectivity calls for care and caution in the application.

Cytoskeletal homeostasis is very vital for the growth, survival, and maintenance of a functional nervous system (Ramocki and Zoghbi, 2008; Wilson and Gonzalez-Billault, 2015). Microtubules are important for neuronal growth, morphology, migration, and polarity. In this

study, we investigated MAP-2 - a differentiation marker to investigate the effects of subtoxic and metabolic stress concentrations of Cu upon neuronal maturation. Three isoforms of MAP-2 have been delineated; high molecular weight MAP2a and MAP2b and the low molecular weight MAP2c have been identified in the neurons (Dehmelt, et al., 2005).

In this present study, MAP2a and MAP2b were the focus, as the only two detected. Following acute exposure (24 h) of differentiated CAD cells (peak differentiation - 6 days in culture) to a sub-toxic dose of Cu [10µM], expression of MAP2a, but not MAP2b, was significantly reduced. This finding agrees with extant literature which suggests that MAP2c is expressed in the developing brain and it is down-regulated during brain maturation, whereas the high molecular weight MAP2b is expressed in both developing and adult brain.

It has also been established that MAP2a appears only after brain maturation (Dehmelt, et al., 2005). Since MAP-2 is associated with microtubule assembly, cargo trafficking and plays a vital role in determining as well as stabilising the shapes of dendrites during neuronal development. The observed differential decreased expression of MAP2a in differentiated cells in response to Cu (40 µM) may relate to the observed differential modulation of overall metabolic activity and calcium signalling in the neuronal processes (Fig. 2.9), as well as the apparent suppression of depolarisation-induced calcium in the processes (but not in the cell body). This may have implications for synaptic plasticity and neurotoxicity. Recent evidence suggests that PD pathogenesis might be underscored by early cytoskeleton dysfunction (Brunden, et al., 2017; Pellegrini, et al., 2017).

Overall, sub-toxic Cu (endogenous heavy metal) has a range of pharmacological effects upon monoaminergic neurons concerning metabolic function, signalling and maturation, with evidence for deleterious pharmacological effects exacerbated by reduced iron content. The next chapter will compare these finding to an exogenous environmental heavy metal, which has been proposed as a potential candidate for causing PD, namely Vd.

Chapter 4: In-vitro effect of low and oxidative stress doses of vanadium and the influence of iron with relevance to PD

4.1 Introduction

Age is a major risk factor in the development of PD, With the global demographic shift to an ageing population, the prevalence of PD and its socio-economic impact is estimated to increase significantly by the year 2050. Environmental exposure to heavy metals is another putative risk factor in the development of PD. One of such metals is Vd - a contaminant in fossil fuels.

Environmental pollution from oil spills and gas flaring as seen in the Nigerian Niger Delta is a source of Vd exposure to humans (Fatola, et al., 2019; Oghenetega, et al., 2019). Despite our current understanding of the mechanistic basis of the relationship between Vd toxicity and PD (oxidative stress, inflammation, ER stress, autophagy, to mention but a few), the search for effective therapies is still on.

Vd, a heavy metal, is found in many carbon-containing deposits such as crude oil, coal, oil shale and tar. It is found mostly in the soil as vanadate which is generally very soluble and taken up by plants at levels reflecting its availability. As a heavy metal nutraceutical, Vd has been used at low doses in different health supplement formulations as antidiabetic (Domingo and Gomez, 2016), anticancer (Ray, et al., 2006; Chakraborty, et al., 2007; Korbecki, et al., 20120 and many more Fatola, et al., 2019).

Elementary Vd (V) is a soft, corrosion-resistant, steel grey solid in group Vb of the periodic table and belongs to the first transition series. Vd occurs in two isotopic forms: ^{50}V and ^{51}V- the most abundant being ^{51}V (99.75% natural abundance) with atomic number 23 and atomic weight of 50.94. Some physicochemical properties are presented in Table 4.1. It forms compounds, mainly, in three oxidation states: +3, +4 or +5. The most stable of these oxidation states is +4, in which Vd forms the oxoVd (+4) ion (vanadyl; VO^{2+}) (Crans, et al., 2004). Free elemental Vd is rare in nature, but it can be found in about 65 different minerals, including magnetite, carnotite and patronite. It can be found in phosphate rock and crude oils.

Its presence in crude oil means it constitutes an environmental pollutant as a bi-product of combustion as fumes in car exhaust and industrial effluents. Exposure to this metal, therefore, constitutes a health risk to humans and animals (Shrivasta, et al., 2007; 2011).

Vd is valuable in the manufacturing industry and has gained ubiquitous relevance in welding and construction industries (Wenning and Kirsch, 1988). It is used as alloys to strengthen steels, jet engines (Plunkett, 1987; Olopade and Connor, 2011; Fatola, et al., 2019). The main route of entry into humans is via the nasal mucosa (Olopade and Connor, 2011). Vd has no known biological activity. It can be found in trace amounts certain types of food (such as mushrooms, parsley, dill weed, black pepper, shellfish, and some grains).

A balanced diet provides about 0.01mg/day of Vd. In high doses, Vd is toxic, however, in minute doses it has increasingly been employed as a useful health supplement. Thus, presenting a "pharmacological paradox". As a heavy metal nutraceutical, Vd has been used in different health supplement formulations and employed in treating health conditions, such as anaemia, diabetes, heart, disease, high blood pressure, high cholesterol, obesity. Inhaling large amounts of Vd can result in lung problems, such as bronchitis or pneumonia. It has been shown that workers exposed to Vd peroxide are more susceptible to eye, nose, and throat irritation (Hall, et al., 1997; Cooper, 2007; Gorguner and Akgun, 2010).

As a heavy metal nutraceutical, Vd has been used at low doses in different health supplement formulations, as antidiabetic (Domingo and Gomez, 2016), anticancer (Ray, et al., 2006; Chakraborty, et al., 2007; Korbecki, et al., 20120 and many more Fatola, et al.,2019).

4.1.1 Properties of Vanadium

Table 4.1: Physical and Chemical Properties of Vanadium

Properties	Description	Source
Physical description	Sodium vanadate appears as colourless to yellow crystal or cream coloured solid	Barceloux and Barceloux (1999)
Molecular weight	121.929 g/mol	Barceloux and Barceloux (1999)
Atomic weight	50.94u	Assem, et al (2015)
Atomic number	23	Assem, et al (2015)
Density	6.11g/cm^3	Assem, et al (2015)
Melting point	1910 ±10^oC	Assem, et al (2015)
Boiling point	3420^oC	Assem, et al (2015)
Halides	Pentafluoride, tetrachloride, oxovanadium chloride, dichloride and trichloride	Crans, et al (2004)
Oxidation states	−3, −1, +1, +2, +3, +4, +5 (an amphoteric oxide)	Crans, et al (2004)
Solubility:	< 1mg/ml @ 22.22 ^OC	Barceloux and Barceloux (1999)
Acid	Dissolves in acid giving dioxovanadium (+5) ion (VO_2^+) –which is chemically and structurally similar to phosphate (PO_4^{3-})	Crans, et al (2004)
Base	Dissolves to form vanadate (VO_4^{3-})	
Compound	Sodium metavanadate ($NaVO_3$)	Assem, et al (2015)
Oxides	Vanadium (+3) dissolves in acid giving a hexaaqua ion Vanadium (+3) salts are strongly reducing	Crans, et al (2004)

This chapter is aimed at investigating the effects of Vd, an exogenous heavy metal, on neuronal metabolic function, signalling responses, oxidative and ER stress, and neuronal maturation, all with possible implications for synaptic plasticity and neurodegeneration.

4.2 METHOD

4.3 MTT ASSAY

Cell viability assay – was carried out as previously described in Section 2.2.6.

4.3.1 Analysis of metal content in CAD cells

CAD cells were passaged and seeded as previously described (Section 2.2.2 and 2.2.3) into two 75cm3 flasks. After 24 hours the process of differentiation was initiated as previously described (See 2.2.9) and the undifferentiated cells were left to proliferate. After 100% confluency was attained, the undifferentiated cells were rinsed with cold PBS and detached. After detaching, the falcon tubes were centrifuged for 5 minutes (at 1500 rpm) and transferred into 1.5ml Eppendorf tubes, centrifuged again and stored at -20^{o}C for ICP-MS analysis for metal contents. Similarly, the differentiated (6 days DIV) cells were detached and stored at -20^{o}C for ICP-MS.

4.3.1.1 Sample Preparation

1.5ml of Nitric acid (65% HNO_3) was added to each thawed cell pellets and vortexed. The cells were left for 24 hours to be digested in the fume hood, and vortexed again. 1.2ml of the digested sample was removed and dispensed into fresh Eppendorf tubes and centrifuged (5', @14,000rpm). 500uL of the digest was then diluted with 4.5ml 2.5% HNO3. 1ml of the clarified sample was decanted into a 15ml falcon tube. The clarified sample was diluted 1/10 (2% nitric acid). The analysis was carried out with Ag as an internal standard (Ag 100 ppb) which was added to the samples and standard curve. ICP-MS analysis was done in duplicates. Standard solutions of known metal concentration were analysed before and after the samples ("front standard curve" and "back standard curve"). These were used to define limits for quantitation. The final working range was noted and used to quantify the metal content.

4.3.2 LDH release assay

Lactic acid dehydrogenase (LDH) release assay- was done as previously described in Section 2.2.3

4.3.3 Live Single-Cell Calcium Imaging

Calcium mobilisation in undifferentiated and differentiated CAD cells was investigated by live single-cell calcium (Ca2+) imaging as described in Section 2.2.4

4.3.4 Western blot

Blotting by the SDS-PAGE was done as previously described in Sections 2.2.7 and 2.2.9.1

4.3.5 Immunofluorescence

Immunofluorescence (IF) assay was performed to establish the effect of Vd on ER stress, PDI expression and neuronal differentiation. CAD cells were plated on coverslips (18mm), which were placed in 6-well plates. 2ml of CAD cells suspended in FBS-supplemented in DMEM/F-12 media was added to a 6cm cell culture dish (TPP© Tissue Culture Dishes, Sigma, Z707678), with additional 2ml of fresh media; to give a 1:1 dilution of cells. These were left for 24 hours to attach to the coverslips and differentiated as described in Section 2.2.5.

For chronic studies, cells were spiked with Vd (10µM or 100µM), one-hour post-differentiation and left on SFM for 6 days. For acute studies, cells were allowed to differentiate (6 days DIV) and spike with Vd (10µM or 100µM) and left for 24 hours. The media was removed in preparation for antibody application and the cells were washed twice in cold PBS supplemented with 1mM calcium and 0.5mM magnesium (PBS^{++}). Each wash was left for 5 minutes. After washing, the cells were fixed with 4% paraformaldehyde (PFA) to preserve the cell's structure and to preserve cell structure and to prevent decay. This is made possible by PFA enhancing the formation of covalent bonds between proteins. The cells were incubated in PFA for 1min. at RT. Subsequently, the PFA was removed and the cells were washed as previously described.

To eliminate unspecific antibody binding to free aldehyde groups in the samples - "quenching" was necessary. Cells were incubated in 50mM ammonium chloride (NH_4Cl) in PBS^{++} for 15 minutes at RT. NH_4Cl quenches free aldehyde groups and allowing a better signal contrast. Following this, NH_4Cl was discarded and the cells were washed again in PBS^{++} three times for

5 minutes in cold PBS^{++}. 0.2% Bovine serum (BSA) in PBS++ was used to block unspecific binding sites. Cells were incubated in BSA two times for 5 minutes at RT. The coverslip was taken out of the blocking solution and placed face-down on the drop of antibody solution. Coverslips with control cells were placed on one drop of 0.2% BSA in PBS^{++}.

Cells were incubated in antibody solution for 2 hours (for MAP2) and 1 hour (for PDI) at RT and then washed thrice in cold PBS^{++} for 5 minutes between each wash. Secondary antibodies (See Table 2.5) were applied in the same way as described for the primary antibodies and incubated for 25 minutes. Due to the light-sensitive nature of the secondary antibody, samples were covered with foil to prevent fluorophore-exhaustion. Excessive antibodies were washed off with cold PBS two times for 5 minutes.

For nucleus-localisation, DNA was stained with DAPI diluted 1:1000 in PBS^{++} and applied as previously described for antibodies. DAPI intercalates into the DNA and emits fluorescent light when excited with light in the ultraviolet range. After 5 mins. of incubation at RT, coverslips were mounted on microscopy slides using mounting medium by *Vectashield*® (See Table 2.2) as mounting medium; the cell-coated side of the coverslip was placed face down. The edges of the coverslips were sealed with nail polish. Immunofluorescent pictures were taken by the confocal *ZEISS ApoTome Microscope*. The secondary antibodies tagged with Alexa-fluorophore-594 gave a signal with a wavelength of 594 nm when excited with a wavelength of approximately 570 nm. Immunofluorescent images of DAPI and Alexafluorophore -594 were taken separately and merged using the *ZEISS ApoTome* software.

4.4 List of Materials

Table 4.2: Materials and corresponding suppliers

Product	Manufacturer
Acrylamide (30%)	Bio-Rad
Ammonium Chloride	Sigma Life Science
Ammonium persulfide	Sigma Life Science
Anti-rabbit IgG, HRP-linked	Cell Signaling Technologies
Bromophenol	Sigma Aldrich
BSA	Sigma Scientific
Butanol	Sigma Aldrich
Calcium Chloride	Sigma Aldrich
Chemiluminescence film	GE Healthcare
DAPI	Thermo Fisher Scientific
DMSO	Sigma Aldrich
DTT	Sigma Life Science
Foetal bovine serum	Sigma Life Science
Fura-2 Calcium Dye	Thermo Fisher Scientific
Gibco™ DMEM/F12	Thermo Fisher Scientific
Gibco™ PBS	Thermo Fisher Scientific
Glycine	Acros Organics
Goat-anti-rabbit IgG HRP-linked	Cell Signaling Technologies
Hoefer® Dual Gel Caster	Hoefer Inc., USA
Hoefer® Mini Vertical Protein Electrophoresis unit	Hoefer Inc., USA
HyperPAGE Bioline	Bioline
Magnesium Chloride	Sigma Aldrich
Methanol	Fisher Scientific
Mighty Small Transfer Tank	Hoefer Inc., USA
MTT	Invitrogen
NaCl	Sigma Aldrich
Nitrocellulose membrane	GE Healthcare
Parafilm®	Pechinery Plastic Packaging
PFA	Fisher Scientific
Rabbit-anti-GAPDH	Proteintech
Rabbit-a-PDI polyclonal	Benham Group, Biosciences, Durham University, UK
SDS	Sigma Aldrich
ß-mercaptoethanol	Sigma Life Science
TEMED	Sigma Aldrich
Triton X-100	Merck
Trizma® Base	Sigma Life Science
Tween 20	Thermo Fisher Scientific
Vectashield mounting media	VECTASHIELD®
Xograph Imaging System	Xograph Healthcare
ZEISS ApoTome	Carl Zeiss AG
ZEISS LSM 880 with Airyscan	Carl Zeiss AG

4.4.1 List of Antibodies

Table 4.3: List of primary and secondary antibodies used

Primary AB	**Dilution**	**Application**
Rabbit-α-PDI polyclonal	1:3000	Western blot Rabbit
Rabbit-α-PDI polyclonal	1:200	Immunofluorescence
Rabbit-α-PDI polyclonal	1:2000	Western Blot
Rabbit- α-GAPDH	1:2000	Western Blot
MAP2	1:	Western Blot
MAP2	1:50	Immunofluorescence
Secondary AB	**Dilution**	**Application**
Goat-α-Rabbit-HRP	1:2000	Western Blot
α-Rabbit	1:500	Immunofluorescence
Alexa Fluorophore 596	1:500	Immunofluorescence

4.5 Results

4.5.1 Dose-dependent effect of vanadium on undifferentiated, differentiating, and differentiated CAD monoamine neuronal cells.

The first set of experiments were designed to explore the dose-dependent effects of Vd on a range of immature, maturing, and matured monoamine neurons in culture. Immature CAD cells (undifferentiated) were exposed to Vd for 24 hours, whilst maturing cells (differentiating) were exposed for 6 days- spanning the period it takes for the process of differentiation to be complete (chronic exposure); after which (at the end of 6 days) mitochondrial viability assay was done. However, the matured (differentiated) cells, they were exposed to Vd by day 6 and left for 24 hours (acute exposure).

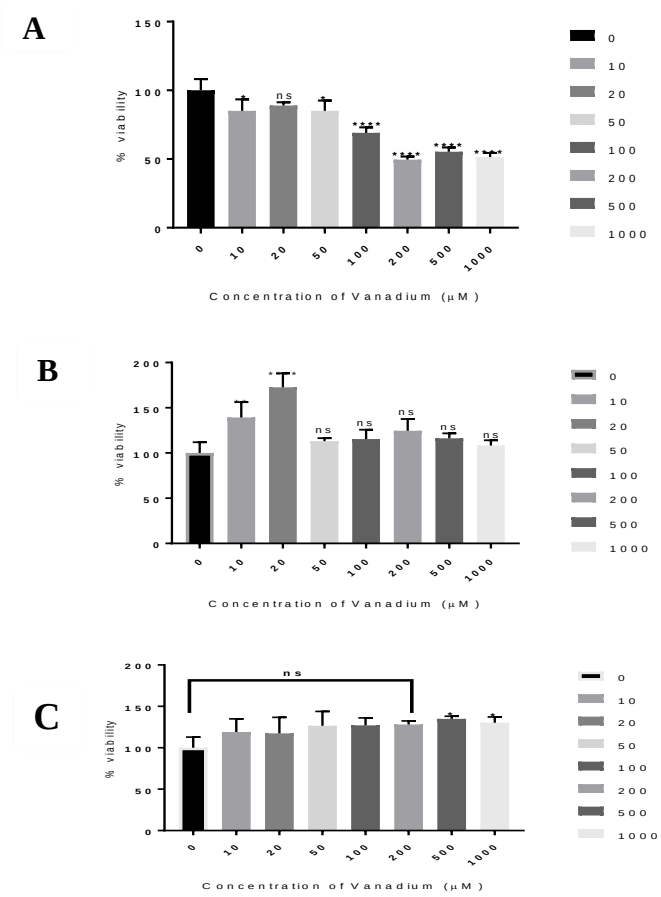


Figure 4.1: Dose-dependent effect of vanadium on undifferentiated, differentiating, and differentiated CAD cells

Acute (24 hours) treatment with (sodium vanadate (vanadium) was performed for undifferentiated cells (Panel A), chronic (6 days) treatment for differentiating cells (Panel B) and acute treatment for differentiated cells (Panel C). Both differentiating and differentiated cells were insensitive to Vd up to 1000µM. In contrast, undifferentiated cells were sensitive to Vd, above 100 µM. Approx. 50% of the cells were insensitive to 1000µM. All values are means ± SD, from at least 6 separate experiments and n=4 for each experiment. ns $p>0.05$; *$p<0.05$; **$p<0.01$; ***$p<0.001$ versus zero Vd control.

4.5.2 The Temporal effects of vanadium on different stages of CAD cell development in culture - undifferentiated, differentiating and differentiated

Dose-dependent effect of Vd on CAD cells. This was aimed at investigating the effect of chronic long-term Vd exposure on mitochondrial functionality (MTT assay) at different stages of development. Undifferentiated cells were spiked with Vd 24 hours after plating and exposed for 9 days; differentiating cells for 9 days while differentiated cells were exposed for 5 days (after 6 days post-differentiation).

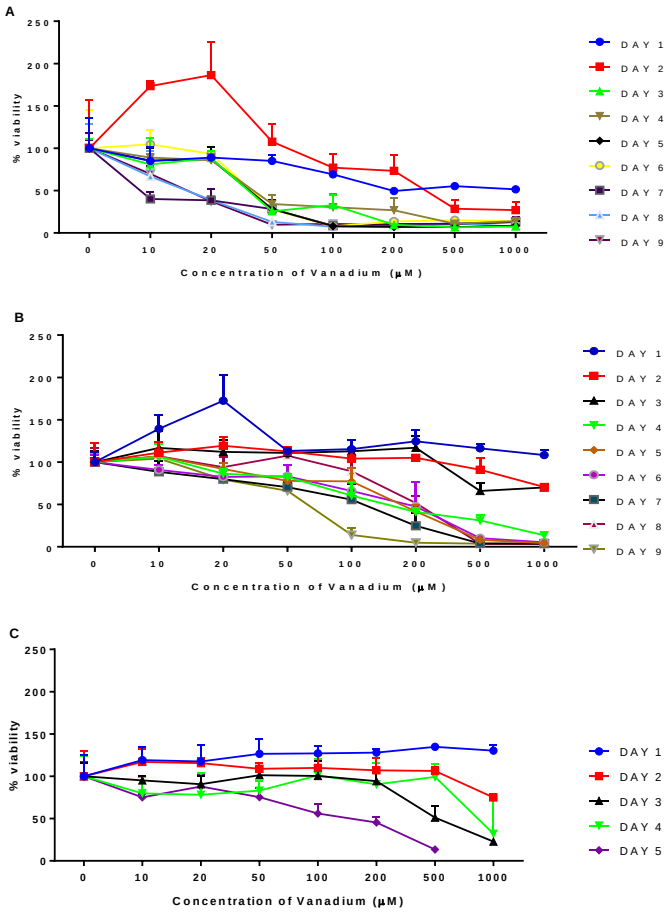


Figure 4.2: A time-course chronic exposure of vanadium (0 – 1000µM) to CAD neurons at different stages of maturating cells.

An MTT assay was carried out to investigate the effect of chronic exposure of Vd (0-1000 µM) on undifferentiated (A), differentiating (B) and differentiated (C). Vd concentrations tested were 10, 20, 50, 100, 200,500 and 1000µM. The mean IC_{50} values (See Table 3.1) were estimated using GraphPad Prism© 7.05 fitting the data to a sigmoidal fit of the variable slope when possible. These data indicated that undifferentiated cells were more sensitive to Vd exposure compared to differentiating and differentiated cells; a decline in the viability of viability with an increasing number of days indicative of an ageing-associated decline in cell viability. All values are means ± SD, from at least 3 separate experiments and n=4 for each experiment.

4.5.3 Dose-dependent effect of vanadium on undifferentiated and differentiated CAD monoamine neuronal cells, evaluation of their iron content - for days 1 & 5

The levels of metals in undifferentiated and differentiated cells were then determined with a view to establishing if Fe levels at different stages of development may account for the variations in sensitivity of CAD cells to Vd exposure. We thus, established that the increased sensitivity of the undifferentiated cells was associated with significantly higher ($p<0.005$) levels of intracellular iron (about 3-fold increase) in undifferentiated versus differentiated CAD cells (Fig.4.4).

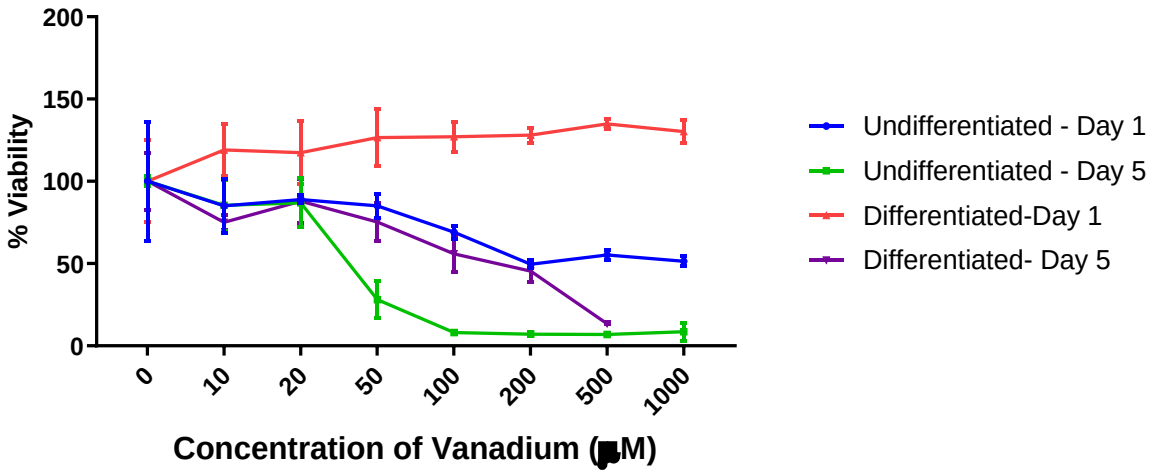


Figure 4.3: Dose-response to vanadium on oxidative stress (mitochondrial viability) in undifferentiated and differentiated CAD cells- for days 1 & 5.

Results from mitochondrial viability investigation showed that undifferentiated neuronal cells are more sensitive (ca. 10-fold) to Vd than differentiated cells (panel B). This correlates with the significantly higher ($p<0.005$) levels of intracellular iron in undifferentiated versus differentiated CAD cells (See Fig.4.4). Values are mean ± SD for n = 4 replicates for mitochondrial viability assay.

4.5.4 IC50 cell viability values for CAD cell sensitivity to vanadium

Values showing temporal sensitivity to Vd following chronic exposures of CAD neurons to Vd in undifferentiated, differentiating, and differentiated cells.

Table 4.4: Estimated IC50 (µM) cell viability values for CAD cell sensitivity to vanadium

Day post-treatment Vd	Undifferentiated cells approx. IC_{50} (µM)	Differentiating cells approx. IC_{50} (µM)	Differentiated cells approx. IC_{50} (µM)
1	>1000	>1000	>1000
2	300	>1000	>1000
3	30	>1000	500
4	30	150	750
5	8	150	200
6	30	150	*
7	15	100	*
8	15	50	*
9	15	50	*

*.= not determined

4.5.5 Metal content- in Undifferentiated and Differentiated CAD cells

An inevitable consequence of ageing is an elevation of brain iron (Fe) levels in specific brain regions. There is significant variation in the concentration of Fe between different brain regions. It has been observed that the differential distribution of iron in the brain has a functional association. For example, regions associated with motor functions tend to have more Fe than non-motor related regions (Crichton, et.al, 2006). It has also been reported that Vd toxicity is exacerbated in the presence of Fe (Todorich, et al., 2011). This experiment was aimed at elucidating the levels of metals in undifferentiated and differentiated cells to establish if Fe levels at different stages of development may account for the differences in sensitivity/response of CAD cells to Vd exposure.

4.5.6 Metal concentration in undifferentiated and differentiated cells Showing the values in parts per billion (ppb) of some metals analysed by ICP-MS.

Values for Mg, Ca, Mn, and Fe as determined by ICP-MS (A, B & C). Other metals: Co, Ni, Cu, and Zn were analysed as well, but the concentrations of these metals were below the confidence limits (CL) and detection limit of the assay. Values for Mg, Ca, Mn, and Fe are within upper and lower confidence limits and that can be considered quantitative were noted. Values for Co, Ni, Cu, and Zn were outside the confidence limits and were not considered, therefore, not used quantitatively to determine their levels in the cells.

4.5.7 Metal concentrations in CAD cells

The concentrations of selected analysed metals in part per billion (ppb) were recorded in Tables 4.5. The concentration of these metals in ppb/mg/ of protein content: Table 4.6.

Table 4.5: Metal content in CAD cells in part per billion (ppb)

CAD cells	Mg	Ca	Mn	Fe
Undifferentiated	226.85	20.27	1.11	14.79
Differentiated	63.31	70.82	0.49	4.8

Table 4.6: Metal content in CAD cells in part per billion (ppb) in mg/of protein content

CAD cells	Mg	Ca	Mn	Fe
Undifferentiated	400.15	35.76	1.95	26.08
Differentiated	230.02	257.29	1.77	17.51

4.5.8 Metal concentration in undifferentiated and differentiated CAD cells; values in parts per billion (ppb)/mg/protein and Atoms of Fe.

Comparing the metal content in undifferentiated and differentiated CAD cells. Results obtained shows that there is significantly higher ($p<0.005$) levels of intracellular iron in undifferentiated cells compared to differentiated CAD cells.

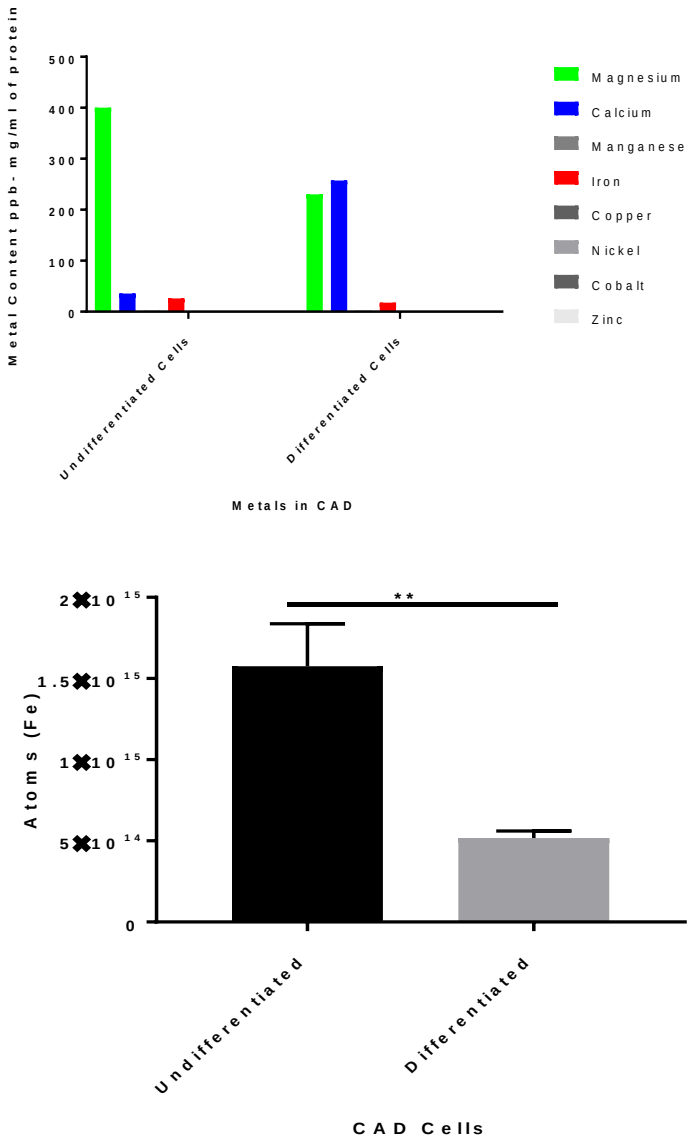


Figure 4.4: Metal content in undifferentiated and differentiated CAD cells.

Shows concentrations of a selection of analysed metals in part per billion/mg/of protein (ppb/mg/protein) (upper panel). There is a notable difference in metal levels in both the immature and maturation stages (lower panel). The levels of Mg and Fe is higher in undifferentiated cells compared to differentiated cells. In contrast, Ca levels are higher in differentiated cells compared to undifferentiated cells. All values are means ± SD, from 2 separate experiments and n=4 for each experiment. Student t-test - **$p<0.005$.

4.5.9 Effect of synthetic iron chelation upon CAD monoaminergic cells - dose-dependent effects; undifferentiated and differentiated cells

This experiment was designed to establish the effect of a synthetic chelator-Deferoxamine (DFO) Mesylate salt on CAD cells and to select a range of doses that are non-toxic for the subsequent series of experiments planned.

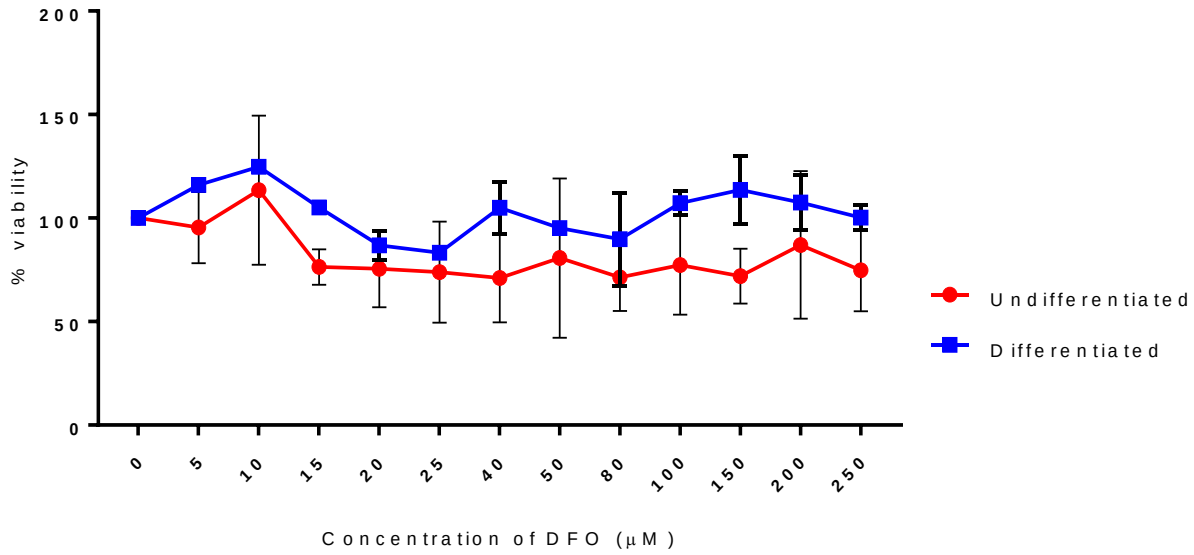


Figure 4.5: MTT assay; a dose-dependent effect of synthetic iron chelator DFO on the viability of CAD cells

No significant effects are seen on both undifferentiated and differentiated cells with DFO up to 250µM. A trend is evident for modestly higher sensitivity in differentiated cells Vs undifferentiated although this did not attain statistical significance ($p > 0.05$).

4.6 Vanadium and Fe chelation

Iron is an essential transition metal which is critical for many physiological functions in the cell. It is tightly regulated. As an enzyme co-factor, it protects the cell from oxidative damage. However, in the aberrant state, it is toxic and implicated in iron-mediated oxidative and carbonyl stress resulting in the alteration of internal homeostasis milieu. In the mitochondria, an iron-containing protein, cytochromes, are involved in the sequential transfer of electrons along the respiratory chain - which entails oxidation and reduction cycling of iron. Vd toxicity is exacerbated in the presence of Fe in oligodendrocytes progenitor cells.

This study was designed to investigate the possible benefits or otherwise of Fe chelation on cell viability. The synthetic Fe chelator (DFO) was compared with a natural product (Aloysia *citrodora Palau*) which has been identified by our group as an efficient Fe chelator (Abuhamdah, et al., 2015).

4.6.1 Effect of iron chelation on cell viability: In the presence of vanadium

The effect of both synthetic (DFO) and natural Fe chelators (*A. citrodora* essential oil) upon Vd (200µM)-induced oxidative stress (mitochondrial viability), following chronic (6 days) exposure in differentiating CAD cells was investigated. Both DFO and Aloysia oil, significantly reversed vanadium-induced toxicity, as compared with Vd-only treated cells.

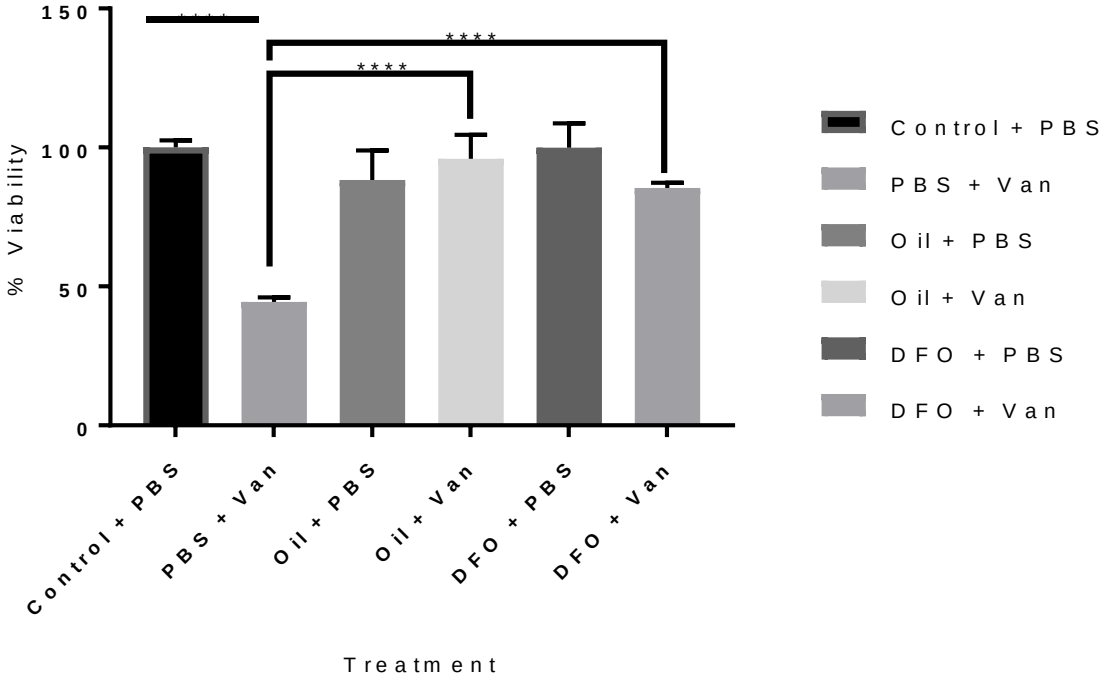


Figure 4.6: Effect of synthetic and natural Fe chelator on chronic exposure to vanadium of differentiating cells.

The effect of the synthetic (DFO) and natural Fe chelator (Aloysia *citrodora Palau L.* essential oil) upon Vd (200µM)-induced oxidative stress (mitochondrial viability), following chronic (6 days) exposure for differentiating CAD cells. Both synthetic and natural chelators, significantly reversed Vd-induced toxicity, as compared with Vd only treated cells. The concentration of DFO used was 10µM and Aloysia *citrodora Palau* was 0.01mg/ml. Values are mean ± SD for n= 4 samples. ****$p<0.0001$. A repeated measure one-way ANOVA (with Tukey's Multiple Comparisons Test) was performed.

4.7 Cytotoxicity of vanadium assay: The effect of 100µM vanadium exposure on differentiated CAD cells - LDH release

An LDH assay was used to establish if the culture conditions and treatments elicited a cytotoxic effect on neurons. LDH assays are a colourimetric assay used to quantify the levels of LDH released into the cell culture medium due to cell lysis. The resulting red colour from the enzymatic reaction can be measured spectrophotometrically, and a stronger colour indicates a higher number of lysed cells.

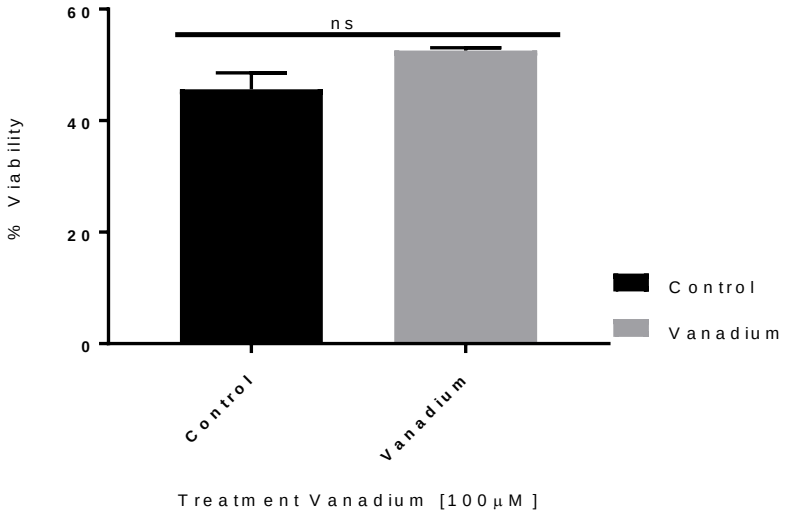


Figure 4.7: Exposure of differentiated CAD cells to oxidative stress dose (100µM) of vanadium cytotoxicity assay (LDH release).

Acute exposure of differentiated CAD cells to oxidative stress dose (100µM) of Vd for 24 hours showed no significant difference between the control group and the Vd treated group ($p > 0.05$).

4.8 Calcium and neuronal transmission

In the neurons, calcium plays a two-fold role. First, as a charge carrier and secondly, as an intracellular messenger - which is involved in neuronal migration, growth-cone dynamics, dendritic development, and synaptogenesis. Calcium ions generate multi-faceted signals that control crucial functions in all types of neurons (Berridge, et al., 2000). In neuronal cells, calcium signals deploy their highly specific functions in well-defined cellular compartments. There functions range from vital cell functions, complete cell cycle to cell proliferation and ultimately cell death (Neher and Sakaba, 2008).

In the nervous system, the functional role of calcium ion can be delineated across the entire neuronal cell circuitry (pre-synaptic, post-synaptic and the soma - specifically the nucleus - as well as the cytosol among others). For example, Ca^{2+} influx in the pre-synaptic neuron triggers exocytosis of neurotransmitters (within the storage vesicles). Postsynaptically, a brief rise in the calcium level in dendritic spines is crucial for the induction of synaptic plasticity (Zucker, 1999). In the nucleus, another cellular sub-compartment, calcium signal regulates gene transcription (Lyons and West, 2011).

Eminently, intracellular calcium signals regulate a wide range of processes over a range of time, from the microseconds scale (neurotransmitter release) to the minutes and hours range as seen in gene transcription (Berridge, et al., 2003). Therefore, the time-course, the amplitude and importantly the auction site in the specific sub-compartment, are key determinants of the function of intracellular calcium signals. Therefore, visualization and quantitative investigation of neuronal calcium functions are essential to understanding the role of xenobiotics in the pathogenesis of neurodegenerative diseases/PD.

4.9 Effect of vanadium on calcium mobilisation in undifferentiated CAD cells

Effect of sub-toxic and oxidative stress doses of Vd on cell signalling was elucidated. In these investigations Live imaging of undifferentiated cells (cell bodies) (Figs.4.9.1 and 4.9.2) and differentiated cells (cell bodies and processes) (Fig. 4.9.3 and 4.9.4) were exposed to Vd (10µM and 100µM).

4.9.1 Effect of vanadium (10µM) on calcium mobilisation in undifferentiated CAD cells

Sub-toxic dose (10µM) had no clear effect on the intracellular calcium levels in cell bodies.

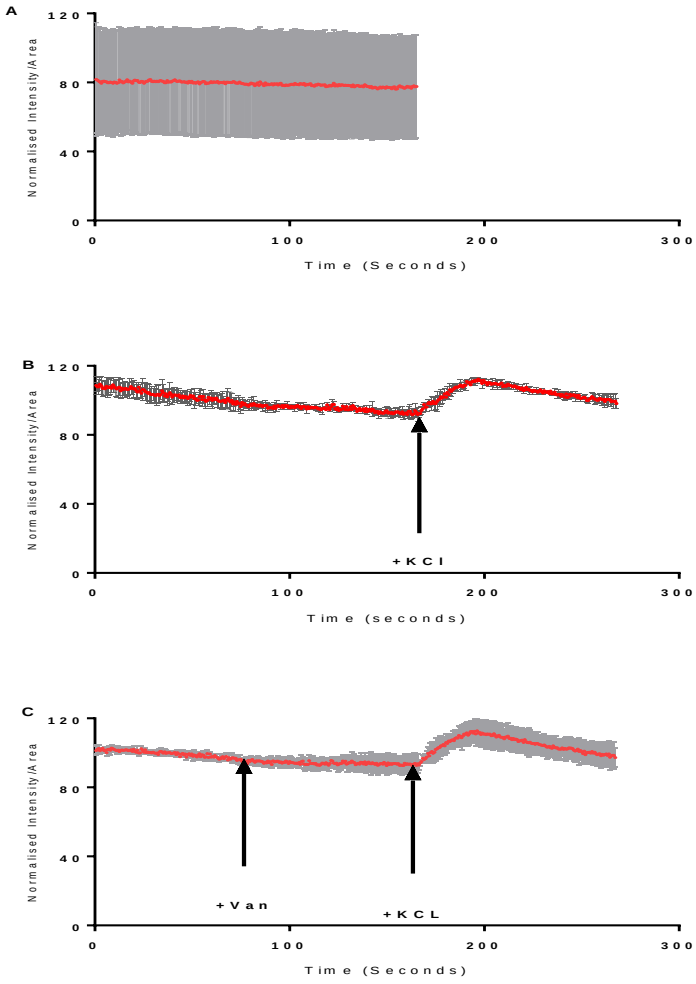


Figure 4.8: Effect of Vanadium (10 µM) on undifferentiated CAD cells.

Results show Baseline levels (A); Control depolarisation (B); and effect if Vd upon cell body before and upon depolarisation (panel C) (Fig.3.7). A sub-toxic dose (10µM) of Vd did not affect the calcium modulation and did not modify depolarization-induced calcium elevation.

Representative images and data for n=6 separate experiments with at least 10 individual cells per experiment.

4.9.2 Effect of vanadium (100µM) on undifferentiated CAD cells

Oxidative stress dose of Vd (100µM) had a short inhibitory effect and showed reduced/delay KCl-induced depolarisation.

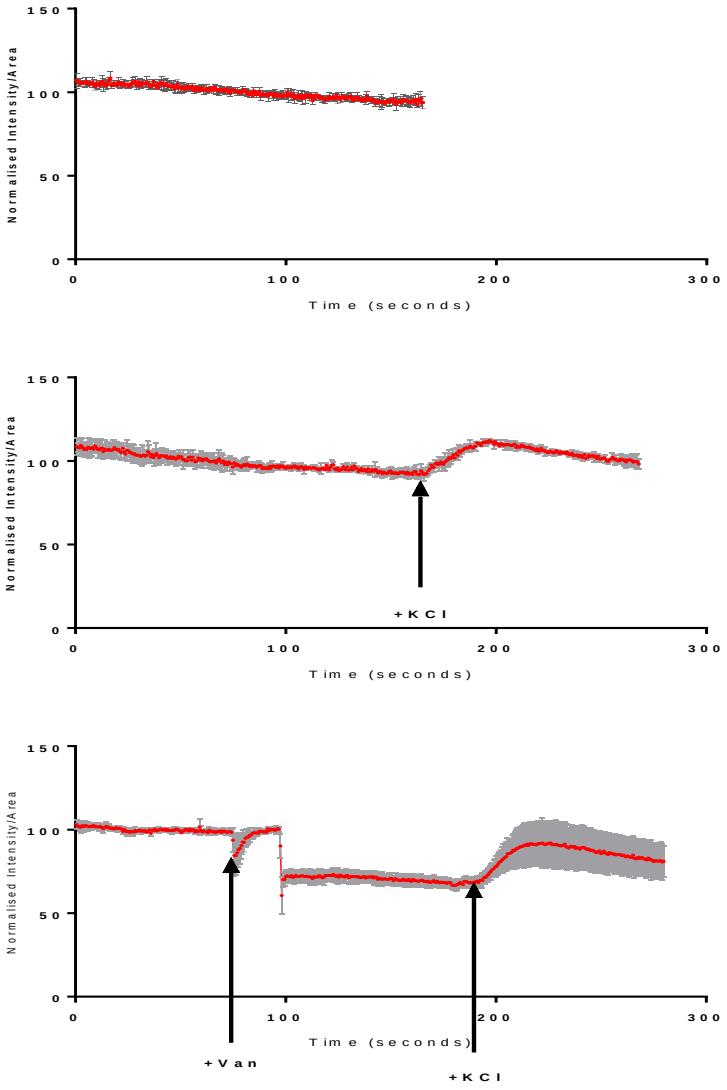


Figure 4.9: Effect of vanadium (100 µM) on undifferentiated CAD cells.

Results show Baseline levels (A); Control depolarisation (B); and effect of Vd upon cell body prior to and upon depolarisation (panel C). A dual effect can be observed in live-cell imaging with undifferentiated CAD cells with sub-toxic, oxidative stress dose of Vd (100µM). It shows rapid transient inhibitory spike followed by protracted inhibition and a delayed depolarisation effect. Representative images and data for n=6 separate experiments with at least 10 individual cells per experiment.

4.9.3 Effect of vanadium (10µM) upon differentiated CAD cells

A biphasic effect was seen following the spiking of differentiated cells with a subtoxic dose of Vd. Whilst there was a slight dip (inhibitory) in calcium signalling in the cell body, there was a slight increase in the processes - which had a modulatory effect on KCl-induced depolarization.

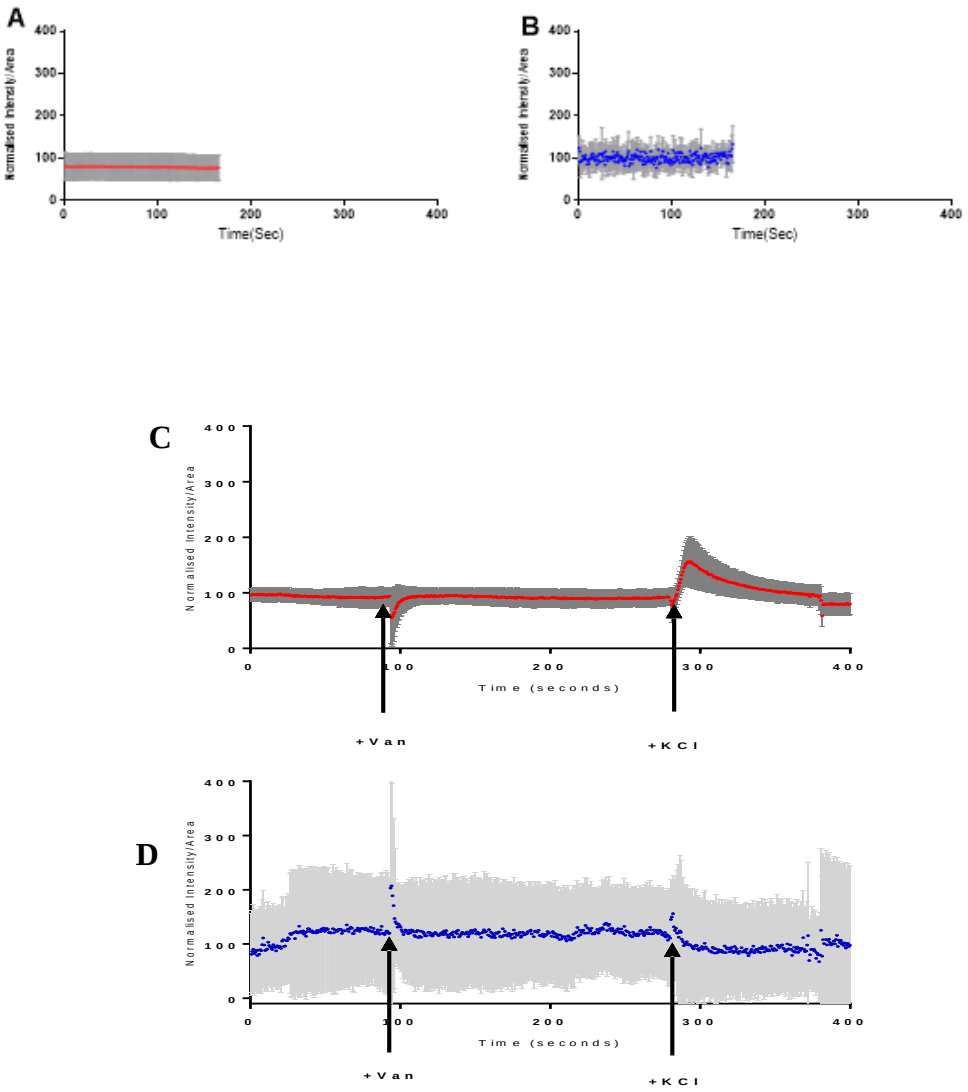


Figure 4.10: Effect of vanadium (10 µM) on differentiated CAD cells.

Live cell Ca^{2+} imaging of differentiated CAD cells. Panel A & B show control basal reading in cell body and processes. A modest double spike is evident; in the presence of Vd (Cell body (panel B) and cell processes (panel C). The effect of Vd (10µM, non-toxic/mild stress dose) upon cell body before and upon depolarisation (panel B). In the presence of Vd, the KCl-induced depolarisation was very pronounced compared to the control response; in contrast, a modest single spike in the processes can be observed. Representative images and data for n=6 separate experiments with at least 10 individual cells per experiment.

4.9.4 Effect of vanadium (100µM) upon calcium imaging in differentiated CAD cells

Oxidative stress dose of Vd (100µM) did not affect the cell body but suppressed Ca signalling in the processes. KCl-induced depolarisation was delayed in the cell body but was completely inhibited in the processes.

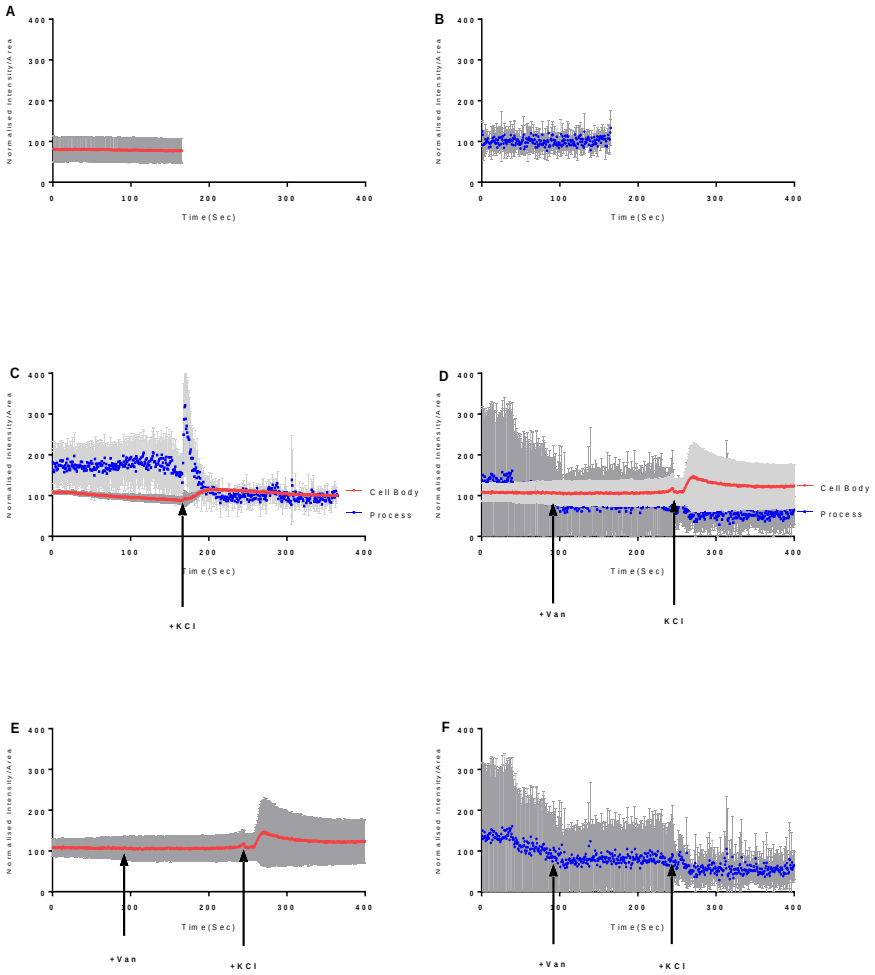


Figure 4.11: Effect of vanadium (100µM) differentiated CAD cells

Result of baseline imaging in the presence of physiological vehicle (HEPES) + Control Depolarisation in the cell body (panel A) and processes (panel B). KCl induced depolarisation in cell body and processes (panel C). Live imaging cell body +/- Van/KCl (panel D); cell body only (panel E); and process only (panel E). Low dose Vd (100µM) had no apparent effect on the cell body (panel E); in contrast, it inhibits calcium mobilisation in the processes (panel F) as compared to the control (panel B). interestingly, it selectively suppressed the depolarisation-induced calcium in the process but caused a delayed increase in cell body compared to the control. Representative images and data for n=6 separate experiments with at least 10 individual cells per experiment.

4.10 Western blot: Effect of vanadium on cell differentiation

As previously stated (See section 2.2.7), MAP-2 is sensitive to many physiological inputs and plays a vital role in the growth, differentiation, and plasticity of neurons, responding to growth factors, neurotransmitters, synaptic activity, and neurotoxins (Johnson and Jope, 1992; Gu, 1995; Niang, et al., 1998). The focus of this study was to investigate the possible effect of acute exposure to Vd (10µM and 100µM) of CAD cells on differentiation using MAP-2 as a marker.

4.10.1 Quantitative analysis of immunoblot data on MAP2 expression following acute exposure (24 hours) of vanadium (10µM) on differentiated CAD cells.

Low dose Vd (10µM) increase (slightly) the expression of MAP 2C & 2D proteins**.**

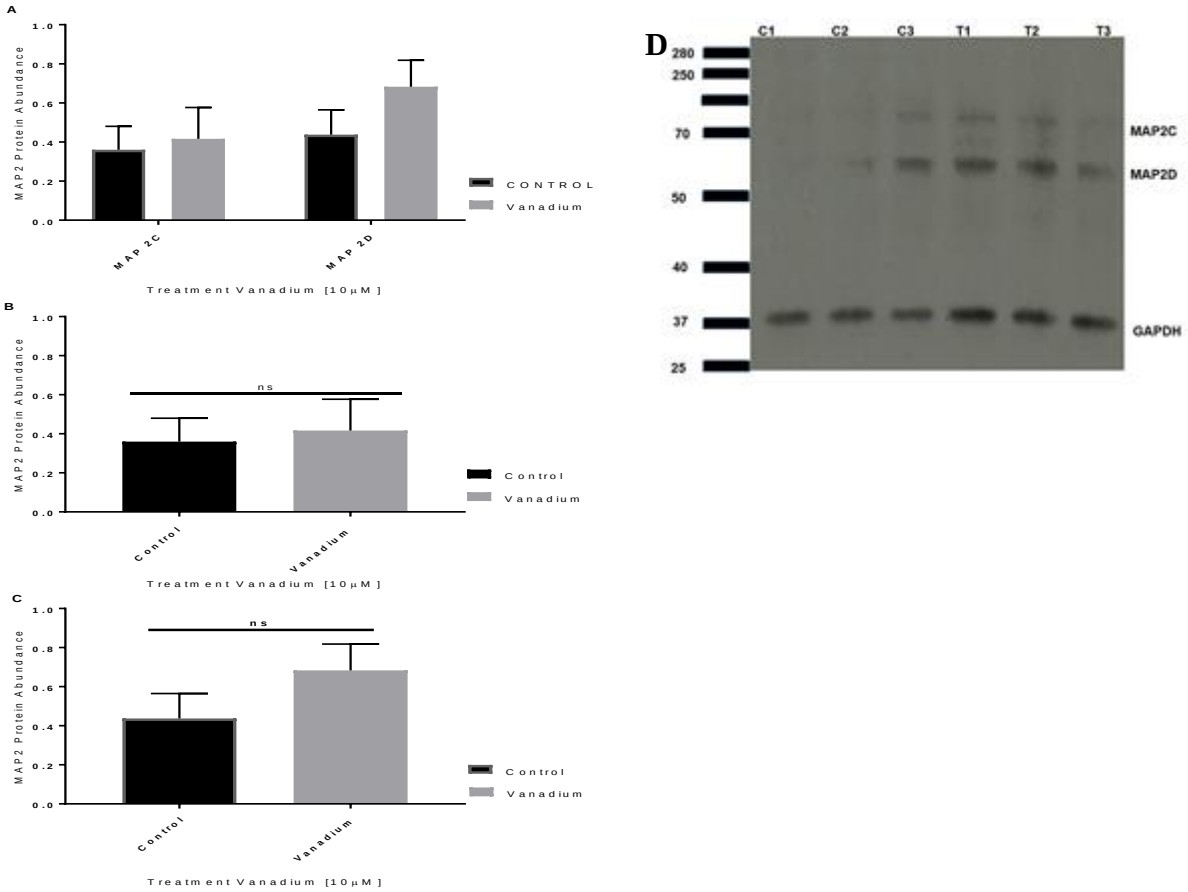


Figure 4.12: Protein abundance, by Western blot, of MAP2 in differentiated CAD cells treated with vanadium (10 µM)..4.13.

Two isoforms of MAP2 can be seen from the blot inserts (panel D). An upper band MAP2C and a lower band MAP2D (panel A). Results show a slight increase in the expression of both isoforms following treatment- 2C (panel B) and 2D (panel D). Student t-test of control/GAPDH and Vd/GAPDH of both isoforms shows no significant difference ($p>0.05$) following treatment with 10 µM Vd. Blots were quantified with image J. Inserts show representative of western blots for 10 µM (panel D). All values are means ± SD, from at least 5 separate experiments and n=3 for each experiment. ns = not significant ($p>0.05$).

4.10.2 Quantitative analysis of immunoblot data on MAP-2 expression following acute exposure (24 hours) of vanadium (100µM) to differentiated CAD cells.

Oxidative stress dose (100µM) of Vd significantly reduced the expression of MAP2C seen in treatment with 10µM. A clear absence of MAPs 2A, B, D is apparent**.**

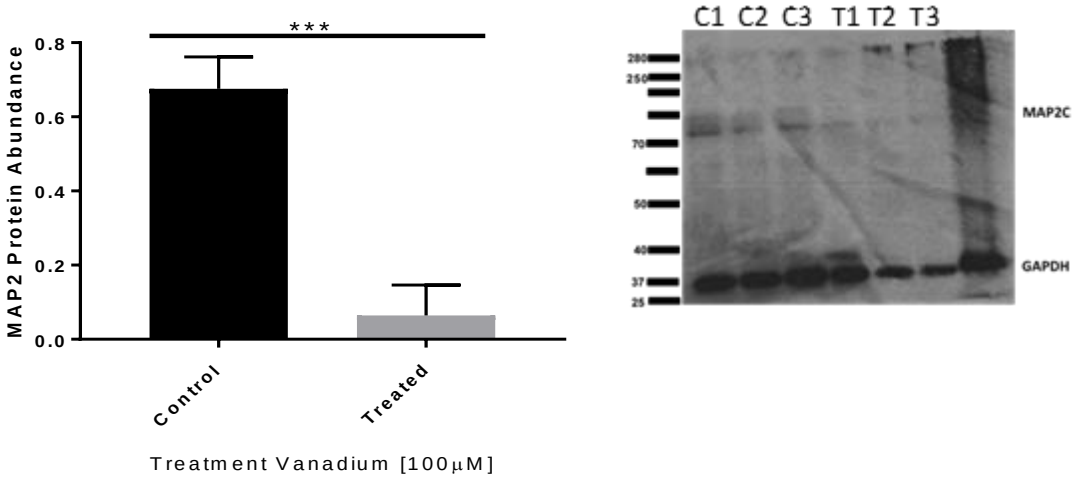


Figure 4.13: Protein abundance, by Western blot, of MAP2C in differentiated CAD cells treated with vanadium (100µM).

The result shows a significant decrease in the expression of MAP2C isoforms following treatment. Student t-test of control/GAPDH and Vd/GAPDH shows a significant difference ($p<0.001$) following treatment with 100µM Vd. Blots (insert) were quantified by image J. All values are means ± SD, from at least 5 separate experiments and n=3 for each experiment and unpaired T-test with Welch's correction was performed. ***= $p<0.001$.

4.11 **Effect of vanadium upon ER Stress (PDI)**

Protein disulphide isomerase (PDIs) are molecular chaperones that belong to the family of foldases which are located primarily in the endoplasmic reticulum and catalyse the formation and isomerization of disulphide bonds. It aids in protein folding as well as perform key physiological functions in protein quality control, cell death, and cell signalling. Protein misfolding is implicated in most neurodegenerative disease (such as PD, Alzheimer, and amyotrophic lateral sclerosis, among others). Altered expression of PDIs is a prominent and key feature of these neurodegenerative conditions. These series of experiments were designed to investigate the effects of acute and chronic exposures of sub-toxic (low stress) and sub-toxic high-stress dose Vd on differentiating and differentiated CAD cells.

4.11.1 PDI (Reducing condition): Quantitative analysis of immunoblot data on PDI following chronic exposure (6 days) to differentiating CAD cells; +/- vanadium (10µM; 100µM)

Under reducing conditions, chronic treatment with low dose Vd (10µM) resulted in two prominent bands of PDI which is indicative of post-translational modification. Low dose treatment caused a moderate decrease in PDI expression while oxidative stress dose (100µM) caused an up-regulation.

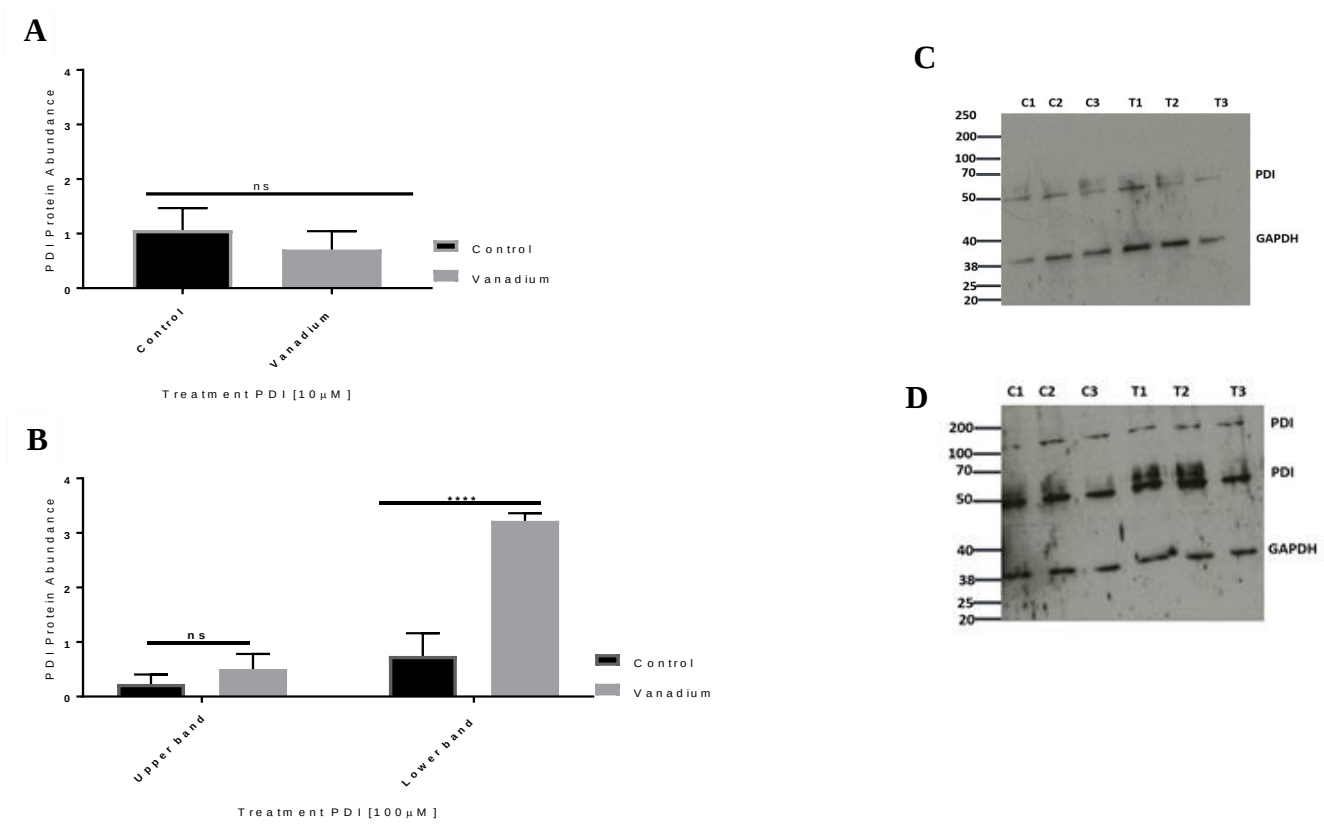


Figure 4.14: Quantitative immunoblotting of PDI expression following chronic exposure (6 days) of vanadium (10µM & 100µM) in differentiating CAD cells.

The PDI Mr 50,000 protein species is more prominently expressed than Mr 100,000 species ($p < 0.001$). There was no significant difference in PDI expression (lower band, Mr 50,000) following exposure to non-toxic (10µM) concentration of Vd (Panel A). In contrast, results indicated a significant increase in the expression of PDI (lower band, Mr 50,000), but the lower expression in the Mr 100,000 species, following exposure to 100µM Vd. Student unpaired T-test with Welch's correction was performed for control/GAPDH and Vd/GAPDH displayed a significant difference ($p< 0.0001$) in the of Mr 50,000 species (a 3.5- fold increase) compared to the control. Blots were quantified with image J. Inserts show representatives of western blots for 10µM &100µM (panel C & D). All values are means ± SD, from at least 5 separate experiments and n=3 for each experiment. ****$p<0.0001$; ns= not significant.

4.11.2 PDI (Reducing condition): Quantitative analysis of immunoblot data on PDI following acute exposure (24 hours) to differentiated CAD cells; +/- vanadium (10µM; 100µM)

Under reducing conditions, acute treatment with oxidative stress dose of vanadium (100µM) also resulted in two prominent bands of PDI: indicative of post-translational modification. There was no significant change in PDI expression in relation to the control group.

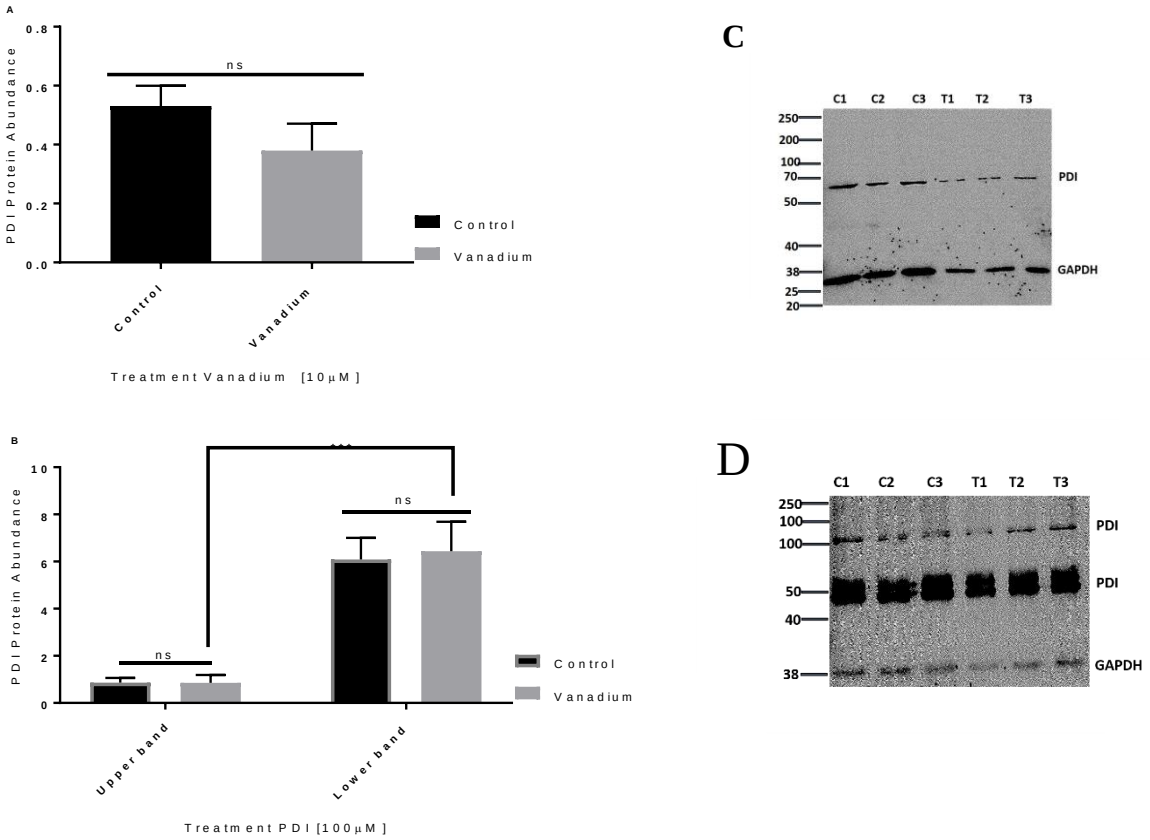


Figure 4.15: Quantitative analysis of immunoblot data on PDI expression following acute exposure (24 hours) of vanadium (10µM & 100µM) compared to control on differentiated CAD cells.

The PDI Mr 50,000 protein species is 13-fold more prominent than 100,000 species ($p < 0.001$). Results show a modest non-significant reduction in PDI expression following acute exposure to Vd mild stress concentration (10µM); $p>0.05$. Similarly, an oxidative stress concentration (100µM) shows no significant change in PDI expression for both species of PDI (Panels B and D). Student and unpaired T-test with Welch's correction was performed of control/GAPDH and Vd/GAPDH of both concentrations (10µM & 100µM) were not significant ($p>0.05$). Blots were quantified by image J. Inserts show representative of western blots for 10µM & 100µM (panel C & D) respectively. All values are means ± SD, from at least 5 separate experiments and n=3 for each experiment. ***$p<0.001$; ns= not significant.

4.12 Analysis of PDI under non-reducing conditions

The expression of PDI was probed by gel electrophoresis, under non-reducing conditions, following chronic and acute exposure of maturing and matured CAD cells to Vd.

4.12.1 PDI: (non-reducing condition): Quantitative analysis of immunoblot data on PDI following chronic exposure (6 days) to differentiating CAD cells; +/- vanadium (10µM; 100µM)

Under non-reducing conditions, chronic treatment with low dose Vd (10µM) resulted in a significant decrease in PDI expression but caused a slight increase in PDI expression with oxidative stress dose (100µM).

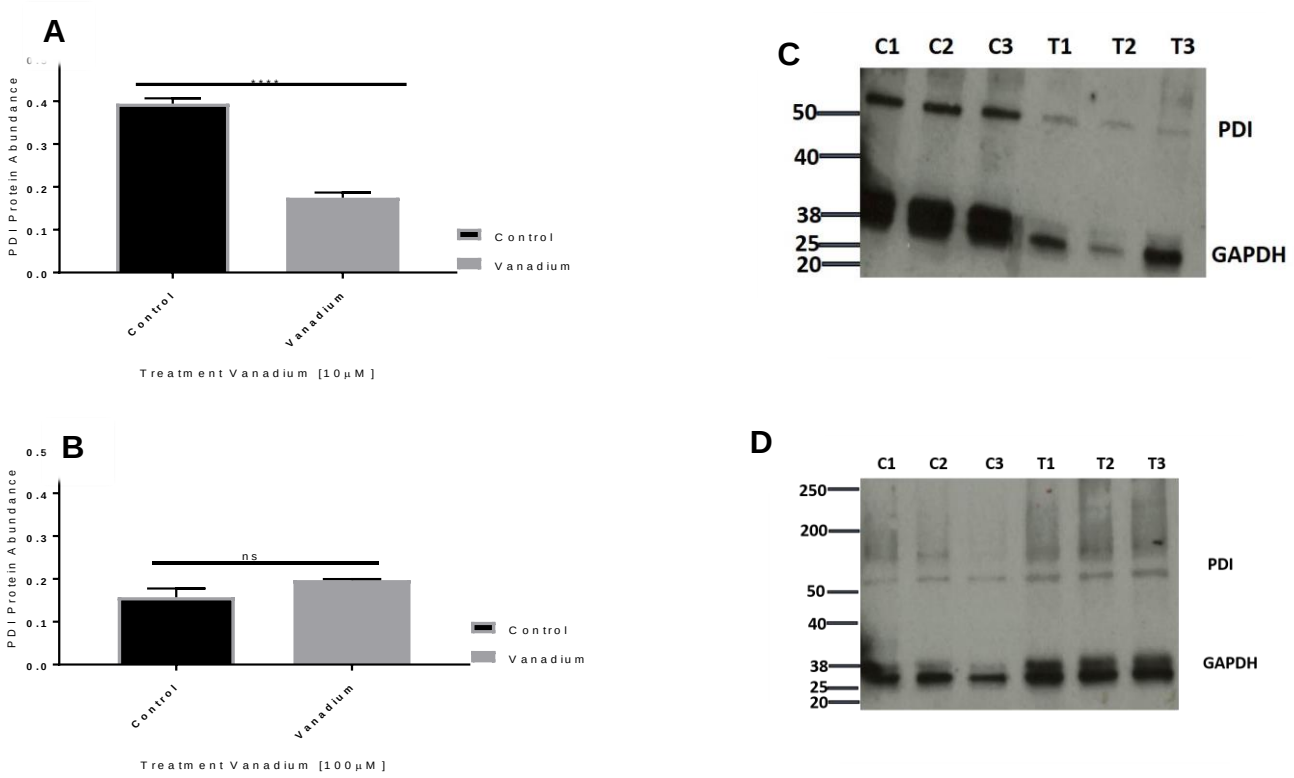


Figure 4.16: Results of quantitative analysis of immunoblot data on PDI expression following chronic exposure (6 days) of vanadium 10µM (Panel A) and 100µM (panel B).

There was a significant decrease in PDI expression ($p<0.0001$) following treatment with a sub-toxic concentration of Vd (10µM – panel A) and non-significant increase ($p>0.05$) with 100 µM of Vd (panel B). Blots were quantified by image J. Inserts show representative of western blots for 10µM & 100µM Vd (panel C & D respectively). All values are means ± SD, from at least 4 separate experiments and n=3 for each experiment. Student's unpaired T-test with Welch's correction was performed. ***$p<0.0001$; ns= not significant.

MAP2 Immunofluorescence of CAD cells following treatment with vanadium (10µM and 100µM) at different stages of development

Using immunofluorescence, we investigated the possible effect of Vd treatment on differentiation. Exploring the effect of treatment on maturing and matured cells using differentiation marker as the primary antibody (MAP2) and Alexa fluorophore as secondary antibody CAD cells were exposed to sub-toxic (10µM and 100µM doses) to elucidate the chronic effect following 6 days of chronic exposure of cells to Vd (differentiating cells) and acute 24 hours exposure (differentiated cells).

4.12.2 MAP2- Immunofluorescence of CAD cells, chronic exposure (6 days) - differentiating cells; Sham vs Treated (100µM).

Protein misfolding (an indication of ER stress) is associated with dendritic spine loss. Immunofluorescence (of MAP2) following chronic exposure of Vd to oxidative stress dose (100µM) shows significant loss of processes in differentiating cells.

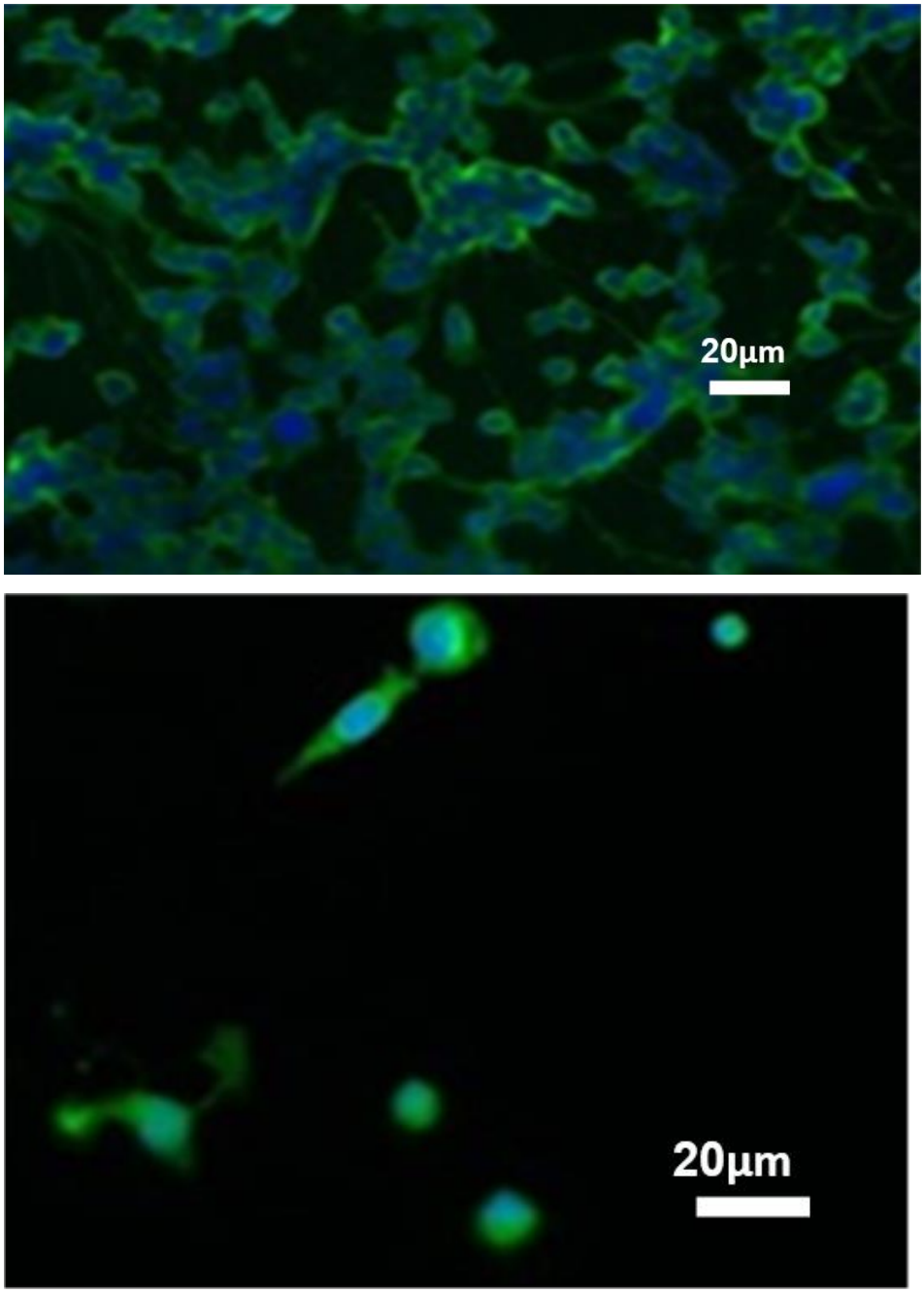


Figure 4.17: MAP2 localised to neuronal processes in Differentiating (chronic exposure- 6 days) CAD cells. Immunofluorescence shows that MAP2 expression was localised to neuronal processes of differentiating CAD cells (Sham treated cells- upper panel) and a significant reduction in the expression of MAP2 in treated cells following chronic exposure to Vd (100µM- bottom panel). DAPI was used as a nuclear stain (DAPI: 1:1000; incubated

for 10mins). While MAP2 was stained with Alexa-Fluor 594 (1:500; incubated for 25mins). Images were obtained on a widefield fluorescence microscope (Zeiss Axiovert 200M Apotome) using the 20X objective lens. Scale bars = 100μm).

4.12.3 MAP2- Immunofluorescence of CAD cells, acute exposure (24 hours) - to differentiated cells; Sham vs Treated (100µM)

Immunofluorescence CAD cells (MAP2) to acute exposure of oxidative stress dose (100µM) shows far significant loss of processes in differentiated cells, however, not as profound as chronic exposure.

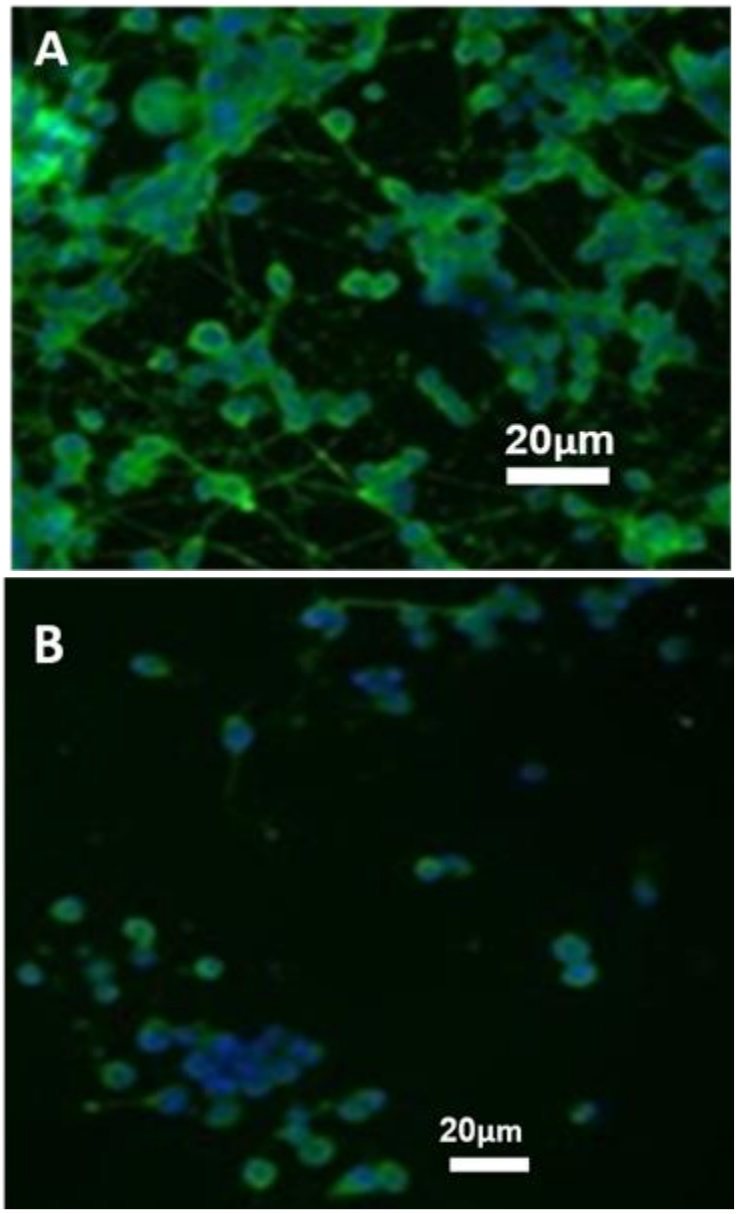


Fig.3.4.18: MAP2 localised to neuronal processes in Differentiated (acute - 24 hours exposure) CAD cells. Immunofluorescence shows that MAP2 expression was localised to neuronal processes of differentiated CAD cells (and very markedly expressed in Sham treated cells- left panel) but a significant reduction in the expression of MAP2 in treated cells (100µM- lower panel). DAPI was used as a nuclear stain (DAPI: 1:1000; incubated for 10mins). While MAP2 was stained with Alexa-Fluor 594 (1:500; incubated for 25mins).

Images were obtained on a widefield fluorescence microscope (Zeiss Axiovert 200M Apotome) using the 20X objective lens. Scale bars = 100μm)

4.12.4 MAP2- Immunofluorescence of CAD cells- at different stages of development Sham vs Treated (10µM & 100 µM).

Immunofluorescence (MAP2) of acute exposure of low dose Vd to mature, maturing and matured cells

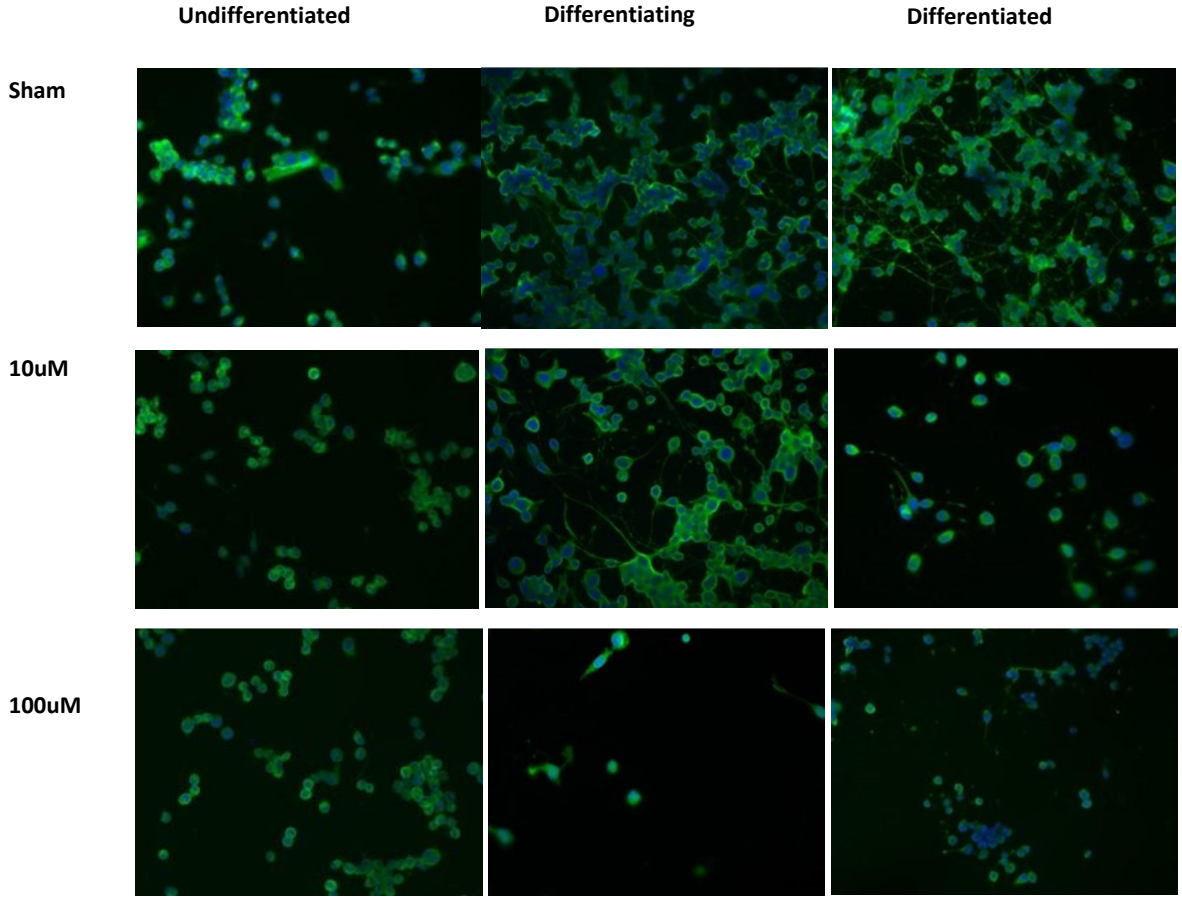


Figure 4.19: Immunofluorescence (IF) labelling of three stages of development of CAD cells.

showing processes in maturing (differentiating) and matured (differentiated) CAD cells, using MAP2 differentiation marker as primary antibody and Alexa fluorophore, as secondary. A clear absence of processes in undifferentiated CAD cells is evident- this was used as a negative control (following sham treatment). There was a marked loss of processes following chronic treatment (differentiating cells-100µM) and moderate loss in acute exposures (differentiated cells-100µM) when compared with sham acute treatments. This marked loss is not seen in differentiating & differentiated cells exposed to sub-toxic dose (10µM). Images were obtained on a widefield fluorescence microscope (Zeiss Axiovert 200M Apotome) using the 20X objective lens. Scale bars = 100µm).

4.13 Effect of vanadium on the nuclear shape (DAPI-staining)

ER stress has been reported in several neurodegenerative diseases. Chronic ER stress leads to over activation of unfolded protein response (UPR) and thereby triggering apoptosis. UPR activation is a common feature observed in most neurodegenerative diseases. Perri, et al (2016) reported a strong association between ER stress and UPR in most neurodegenerative disorders. The effect of Vd (acute and chronic exposures) on different stages of development on dopaminergic neurons was investigated. A strong functional indicator of ER stress is nuclear shape change. A round-shaped nucleus is an index of a healthy cell, while a stressed cell nucleus is bean-shaped (unpublished observation).

4.13.1 DAPI- Immunofluorescence of CAD cells- on the nuclear shape, chronic exposure (differentiating cells); Sham vs Treated (100µM vanadium)

Nuclear shape morphology is an index to establish ER stress spherical nuclear is indicative of a healthy morphology whilst, crescent shape is indicative of stress cells.

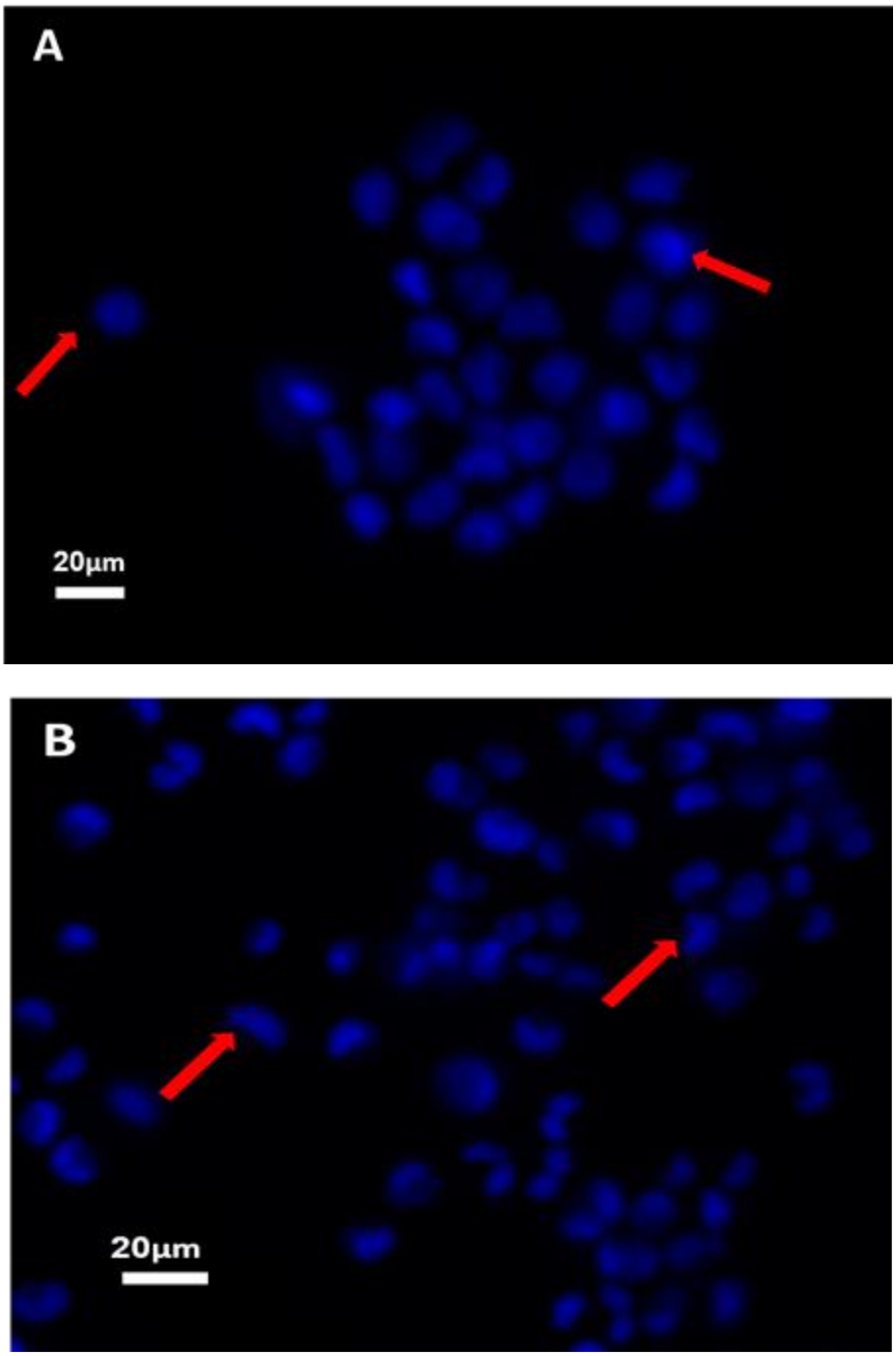


Figure 4.20: Immunofluorescence labelling of nuclear shape in differentiating (chronic exposure to vanadium-100µM) CAD cells; sham vs treated.
Results show mostly circular nuclear shape in sham-treated (differentiating) cells (A). In contrast, most of the treated cells (B) showed alterations in nuclear shapes in most cells. DAPI was used as a nuclear

stain (DAPI: 1:1000; incubated for 10mins). Images were obtained on a widefield fluorescence microscope (Zeiss Axiovert 200M Apotome) using the 20X objective lens. Scale bars = 100μm).

4.13.2 DAPI- Immunofluorescence of CAD cells- on the nuclear shape, acute exposure (differentiated cells); Sham vs Treated (100µM)

Acute treatment of matured cells with oxidative stress dose (100µM) shows a higher number of crescent-shaped nuclear in relation to sham-treated cells.

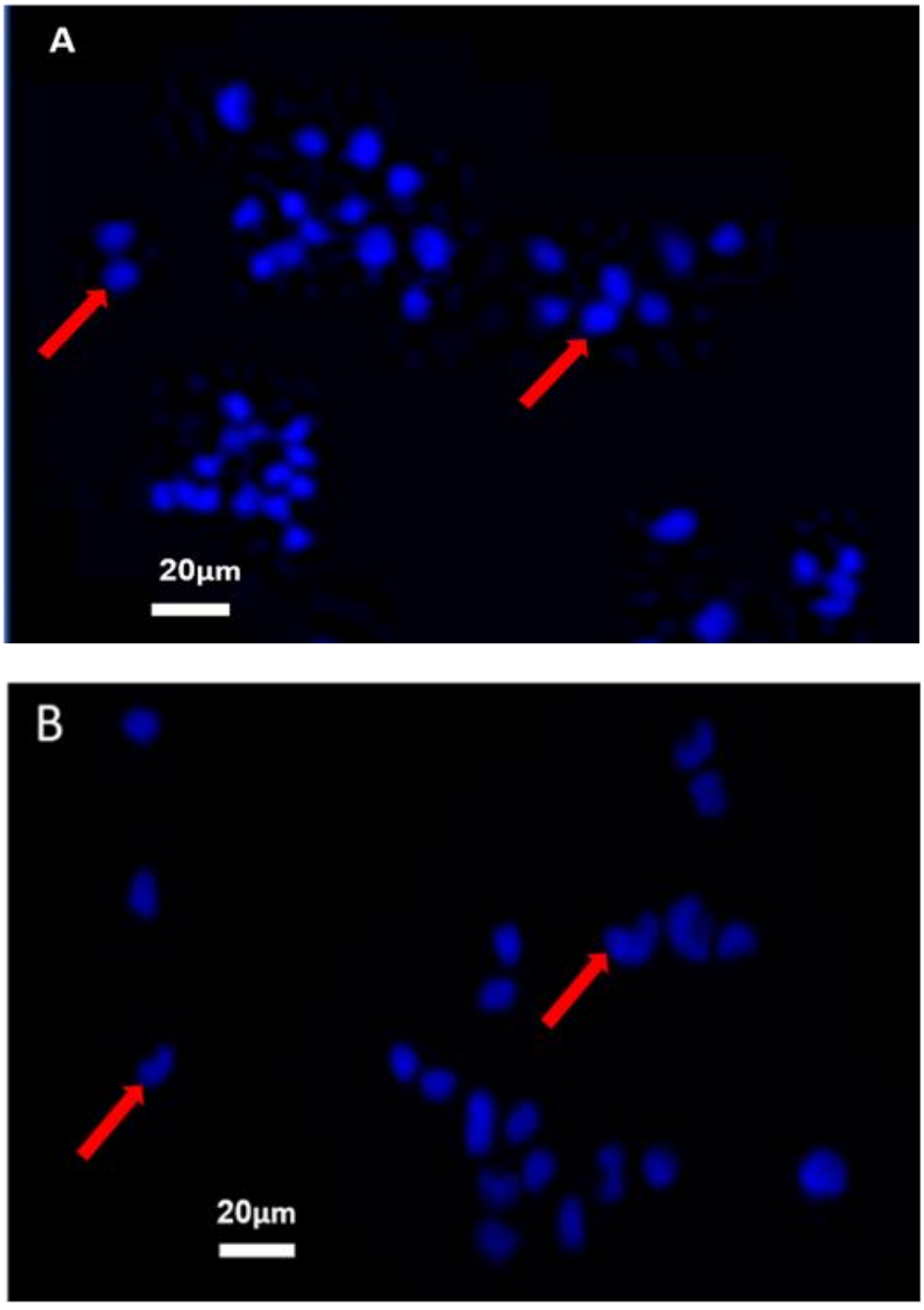


Figure 4.21: Immunofluorescence labelling of nuclear shape in differentiated (acute-24h, exposure to vanadium;100µM) CAD cells; sham vs treated.
Results show mostly circular nuclear shape in sham-treated (differentiated cells (A). In contrast, most of the treated cells (B) showed alterations in nuclear shapes. DAPI was used as a nuclear stain (DAPI:

1:1000; incubated for 10mins). Images were obtained on a widefield fluorescence microscope (Zeiss Axiovert 200M Apotome) using the 20X objective lens. Scale bars = 100μm).

4.13.3 DAPI- Immunofluorescence of CAD cells- on the nuclear shape, acute exposure (differentiated cells); at different stages of development Sham vs Treated (10 µM & 100 µM)

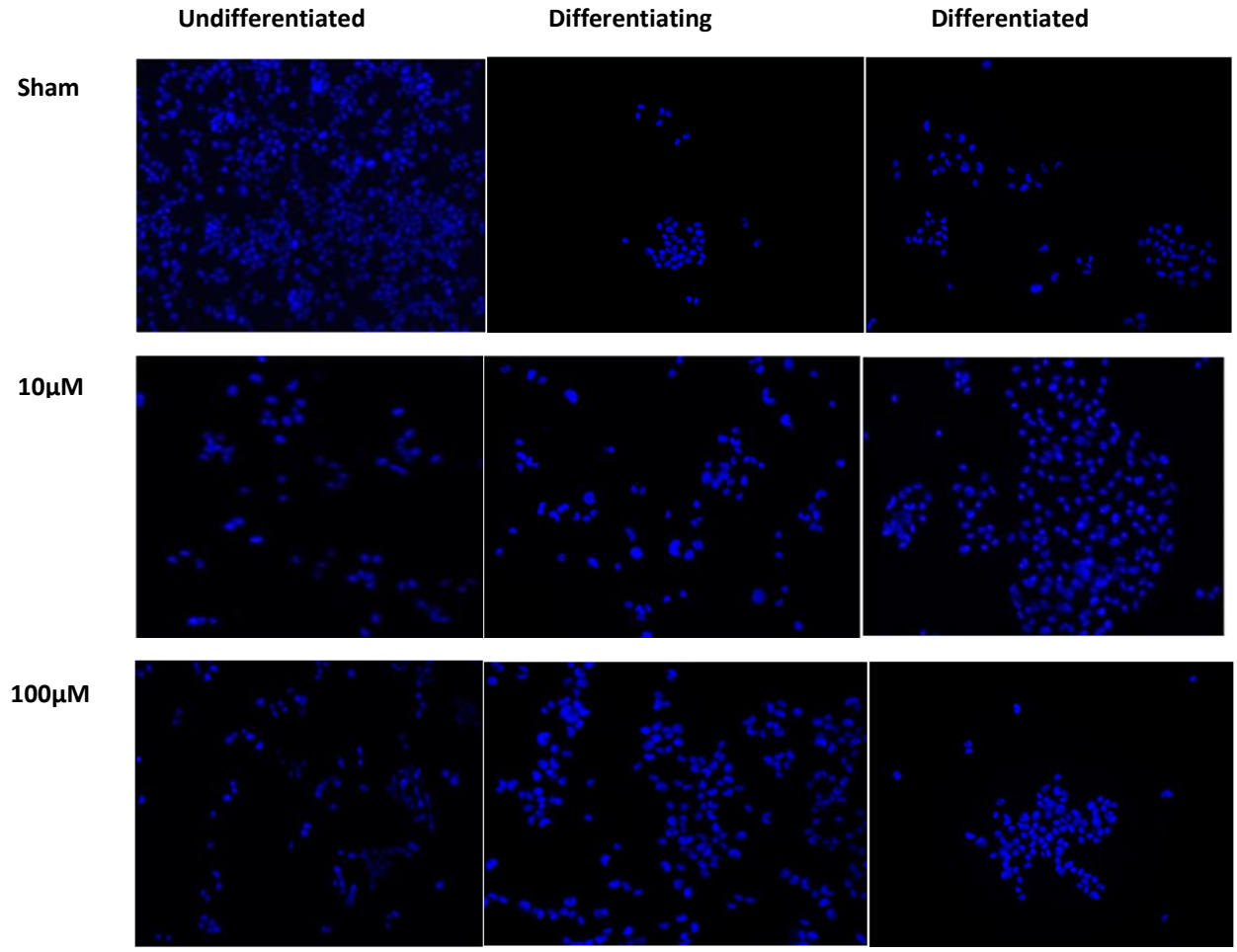


Figure 4.22: Effects of vanadium on the nuclear shape of three stages of development of CAD cells.

Results show the effect of acute exposure of Vd (10µM & 100µM) in immature (undifferentiated), maturing (differentiating) and matured (differentiated) CAD cells; sham vs treated. Oxidative stress dose had a significant effect on nuclear shapes (clearly shown in Fig.3.19 and Fig.3.20). DAPI was used as a nuclear stain (1:1000; incubated for 10mins). Images were obtained on a widefield fluorescence microscope (Zeiss Axiovert 200M Apotome) using the 10X objective lens. Scale bars = 100µm)

4.14 PDI

The biochemical function of the enzyme, Protein Disulphide Isomerase (PDI) is adapted to form, breakdown and rearrange disulphide bonds that support in promoting the formation of native conformation in a misfolded or unfolded protein. Protein misfolding within the ER triggers ER stress. thus, the upregulation of PDI. PDI is upregulated in various tissues during disease and interestingly, its protective and detrimental effects have been described (Hertz and Mollereau, 2014). PDI is upregulated in dopaminergic neurons and Lewy bodies of patients with PD (Con, et al., 2004; Uehara, et al., 2006; Varma and Sen, 2015; Fernandes, et al., 2016). The effect of low and oxidative stress doses of Vd of PDI expression will help elucidate the molecular mechanism underpinning disease pathology associated with sporadic PD associated with this heavy metal.

4.14.1 PDI Immunofluorescence: Effect of chronic exposure vanadium on differentiating cells Sham vs Treated (100µM)

The protein disulphide isomerase (PDI) is located around the nucleus. Chronic exposure of differentiating CAD cells to oxidative stress dose of Vd (100µM) shows morphological differences in shapes between the treated and sham group Figs 4.23 and 4.24 below.

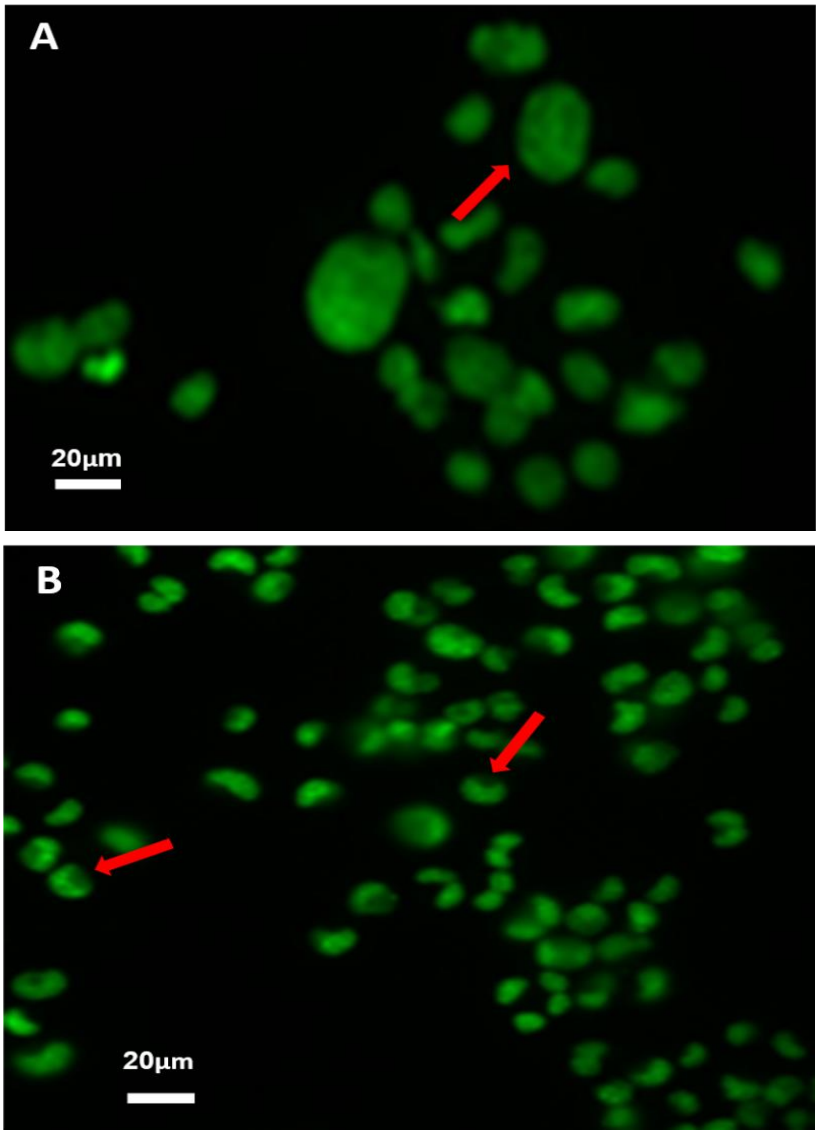


Figure 4.23: Immunofluorescence labelling of PDI expression in differentiating (chronic exposure) CAD cells; sham vs treated (100µM Vd).

Results show the perinuclear localisation of PDI expression in sham-treated differentiating cells (upper panel) which are mostly round- shape compared to the treated cells. In contrast, most of the treated cells (chronic treatment-100µM Vd; lower panel) are bean-shaped; an indication of stressed-cells. The immunofluorescent signal labels PDI in differentiated CAD cells. Polyclonal PDI was stained with Alexa Fluor 488 (1:500; incubated for 25mins); Primary AB PDI: 1:200 (incubated for 2 hours). Images were obtained on a widefield fluorescence microscope (Zeiss Axiovert 200M Apotome) using the 20X objective lens. Scale bars = 100µm).

4.14.1.1PDI Immunofluorescence: Effect of chronic exposure vanadium on differentiated cells Sham vs Treated (100µM).

Results of acute treatment of differentiated CAD cells to oxidative stress dose Vd (100µM) shows a distinct morphological difference of the perinuclear location of PDI in the treated vs the sham group.

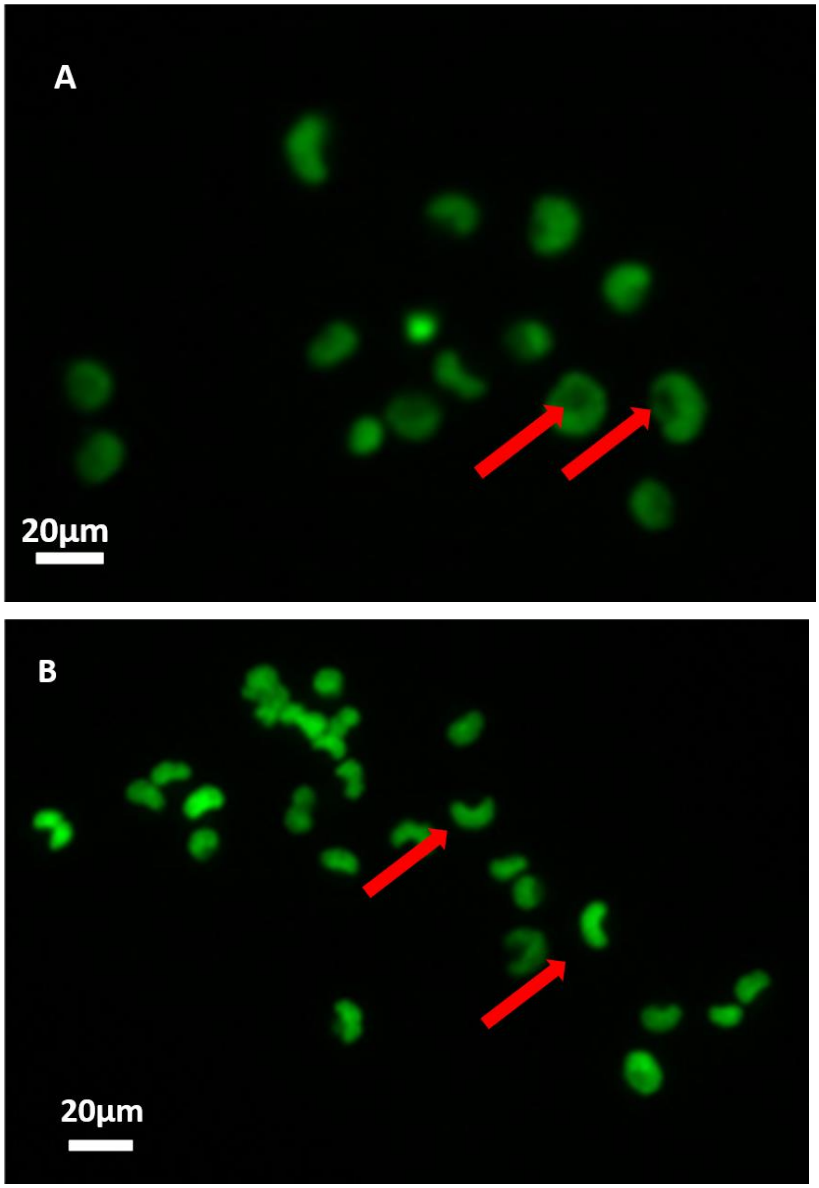


Figure 4.24: Immunofluorescence labelling of PDI expression in differentiated (acute 24 hours exposure) CAD cells; sham vs treated (100µM).

Results show the perinuclear localisation of PDI expression with more rounded shaped cells in sham-treated differentiated cells (upper panel). In contrast, most of the treated cells (lower panel) are bean-shaped; an indication of stress-cells. The immunofluorescent signal labels PDI in differentiated CAD cells. Polyclonal PDI was stained with Alexa Fluor 488 (1:500; incubated for 25mins); Primary AB PDI: 1:200 (incubated for 2 hours). Images were obtained on a widefield fluorescence microscope (Zeiss Axiovert 200M Apotome) using the 20X objective lens. Scale bars = 100µm).

4.14.1.2PDI Immunofluorescence: Effect of vanadium on the nuclear shape at different stages of development Sham vs Treated

Cross-sectional representation of PDI expression at different stages of development for both low dose and oxidative stress doses of Vd**.**

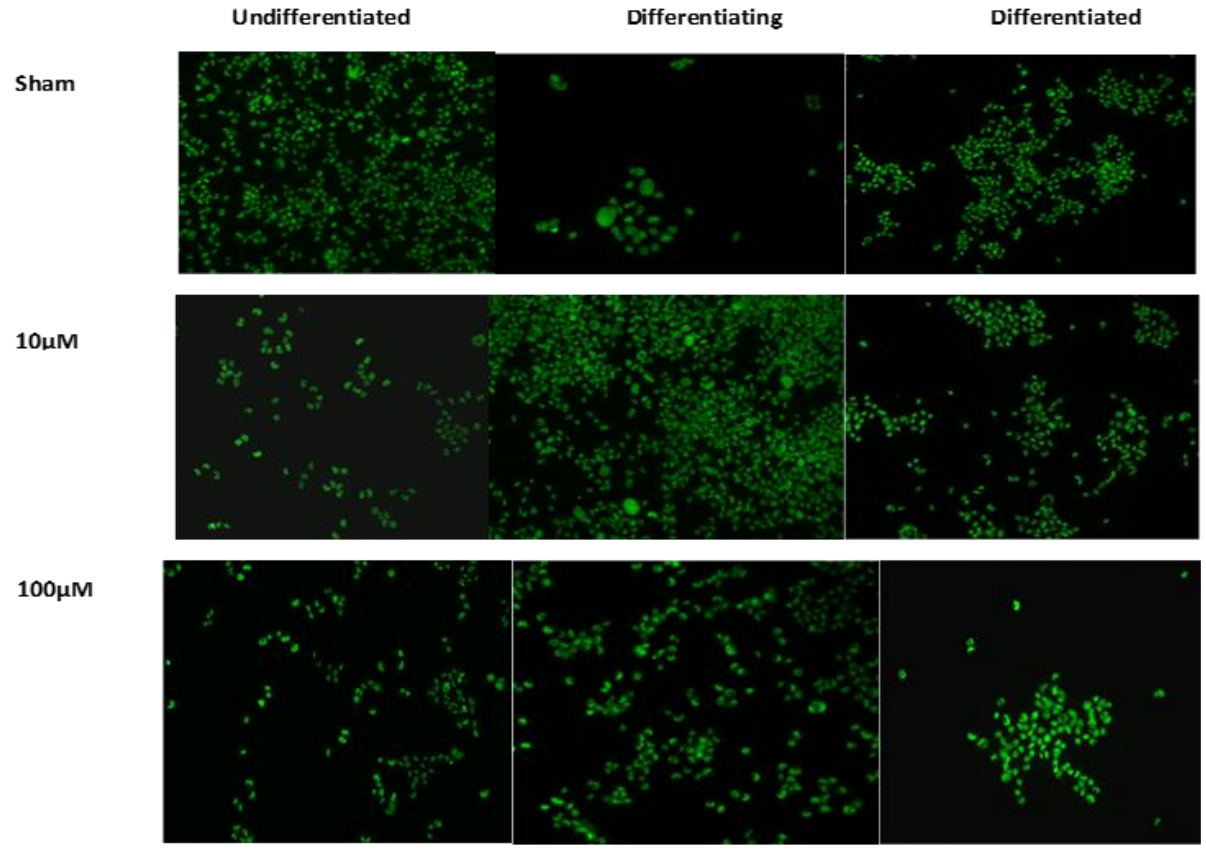


Figure 4.25: Effects of vanadium upon PDI expression in different stages of development of CAD cells exposed to vanadium (10µM & 100µM) -undifferentiated, differentiating, and differentiated CAD cells.

The immunofluorescence signal indicating PDI signal (green), shows a circular pattern around the nucleus in the control group (clearly shown in Figs. 3.22 and 3.23 above) as compared with sham vs treated (10µM & 100µM. Polyclonal PDI was stained with Alexa Fluor 488 (1:500; incubated for 25mins); Primary AB PDI: 1:200 (incubated for 2 hours). Images were obtained on a widefield fluorescence microscope (Zeiss Axiovert 200M Apotome) using the 20X objective lens. Scale bars = 100µm).

4.15 Discussion

This chapter was devoted to investigating the effect of sub-toxic Vd (an exogenous metal) which has been proposed as a potential causal agent of PD. The aetiology of PD has been broadly categorised into familial (genetic) or sporadic (xenobiotics) (Dauer, et al., 2003). Familial causation of PD occurs through the recessively inherited loss-of-function mutations in parkin, DJ-1, and PINK1 which leads to mitochondria dysfunction and accumulation of reactive oxygen species (ROS) (Springer, et al., 2011). However, the dominantly inherited missense mutations in α - synuclein and LRRK2 affects protein degradation pathways.

This leads to the aggregation of protein (proteinopathies) and accumulation of Lewy bodies. PD can also potentially arise as a result of exposure to environmental neurotoxins. These results functional impairment of the mitochondrial (Corti, et al., 2005; Rossignol and Bradstreet, 2008; Ekstrand and Galter, 2009) and release of ROS (Patten, et al., 2010) leading to a plethora of cellular responses including apoptosis and disruption of protein degradation pathway and misfolding of proteins.

As a first step towards determining suitable concentrations for Vd for subsequent experiments on neuronal functionality and differentiation, a dose-response effect of Vd on CAD cells was performed using acute (24 hours) and chronic (6 days) exposure times on metabolic activity (MTT) and cytotoxicity (LDH). From the experiments, neuronal metabolic status was resistant to applied Vd in undifferentiated, differentiating, and differentiated CAD cells, with IC50 values >1000µM. However, on chronic exposure, undifferentiated cells were relatively more sensitive to Vd (6 days exposures) with IC50 values of ca.30µM (from day 3 of exposure) in contrast to differentiated CAD cells with IC50 values of 500µM.

Based on these results, 10µM of Vd was shown to have no significant effect upon metabolic rate or cytotoxicity, while 100µM of Vd only reduce metabolic rate for undifferentiated CAD cells (from day 3 of chronic exposure) and no significant effect on differentiating and differentiated CAD cells (6 days of exposure) – but remained non-cytotoxic for differentiated CAD cells as shown in the LDH result in Fig 3.6.

Vd-induced oligodendrocyte cytotoxicity is modulated by iron content (Todorich, et al., 2011). Therefore, the metal content in undifferentiated and differentiated CAD neurons was investigated. A significant difference was observed in metal content with a significant variation in the concentration of metals in undifferentiated and differentiated CAD cells (Fig.4.4). The concentrations of Fe, Mg and Mn were significantly higher in undifferentiated CAD cells compared to differentiated cells. However, Ca^{2+} levels were significantly higher in differentiated CAD cells with over 300% difference in Ca^{2+} content compared with that in undifferentiated cells. Mg (226.85 ppb) was about 350% higher in undifferentiated cells compared to differentiated cells (66.31 ppb). The concentration of Fe^{2+} in undifferentiated cells (14.79 ppb) was over 3-fold higher than differentiated cells (4.8 ppb).

There is a growing concern on the role of redox-active transition metals in the etiopathology of neurodegenerative disorders, such as PD, AD and Amyotrophic Lateral Sclerosis (ALS). These metals are crucial in most biological reactions, notably Fe. However, excessive tissue accumulation of Fe can be cytotoxic, particularly due to the fact that perturbation in metal homeostasis leads to a plethora of cellular disturbances characterised by oxidative stress and an increase in the production of free radicals. The resultant oxidative stress leads to the production of reactive oxygen species (ROS) and concomitant molecular damage, resulting in critical failure of biological functions and ultimately cell death. It is noted that Vd capacity to disrupt iron homeostasis and resultant depletion of iron from intracellular iron store underpins its role in ROS generation and subsequent cytotoxicity (Ghio, et al.,2015). Ghio and his colleagues (2015) reported that Vd can reduce the non-heme iron in the mitochondria and nuclear fraction in the bronchial epithelial cells with a resultant increase in the non-heme iron of the supernatant.

The associated iron deficiency created by Vd results in the increase expression of DMT-1 and triggers the production of oxidants such as superoxide (Ghio, et al.,2015). There is a strong indication that a strong relationship exists between Vd and Fe. For example, Vd toxicity is markedly reported in cells known to utilize a great deal of iron, such as the Oligodendrocytes Progenitor Cells (OPC) (Todorich, et al., 2011). Though essential for cellular respiration as a component of cytochromes protein in the mitochondria-electron transport chain and metabolism, Fe can undergo univalent redox reactions. Its oxidized and reduced forms - ferric

and ferrous iron respectively in aberrant levels has been implicated in most cases of neurodegenerative diseases – hence, its level is tightly regulated.

Fe chelation, therefore, as a possible therapy to mitigate against these effects has been proposed. As highlighted above, Vd-induced toxicity is exacerbated in the presence of Fe in oligodendrocyte progenitor cells (Todorich, et al., 2011). The effect of iron chelation (DFO) on Vd insults in cells was, therefore, explored. To select the appropriate concentration of DFO to use for the experiments and to ensure that DFO had no significant effect on CAD cells viability, an acute (24 hours) treatment of CAD cells with a wide range of concentrations was performed. DFO does not elicit any apparent neurotoxic effect (up to 250µM). Based on the dose-response curves, 10µM Vd was selected as a non-toxic, non-stress dose and 100 µM as non-toxic, mild-stress dose, respectively, for the subsequent molecular, cellular, and functional analyses. Both the synthetic (DFO) and natural Fe chelator (*Aloysia citrodora Palau*) significantly and efficiently protected against chronic (6 days) exposure to Vd (200µM) and induced significant mitochondrial stress (approx. 50%), which favours the case for iron chelation therapy (Fig. 4.6).

Functional effects of sub-toxic concentrations of Vd (10µM) have a neuroprotective effect against Vd-induced toxicity following chronic exposure. Oxidative stress dose (100 µM) had no apparent effect on mitochondrial stress and is non-cytotoxic confirmed by LDH release assay (Fig. 3.6) in immature cells (undifferentiated). These findings correlate with that reported by Fatola, et al (2019) where they noted that at concentrations commonly used as a nutraceutical, Vd is non-toxic; due to the low absorption rate of dietary Vd. However, Rehder (2015) adduced the metabolic regulatory functions of Vd to its resemblance to phosphate in structure - which mirrors the functional crosslink between vanadate and phosphate-dependent enzymes, in which the protein binding domains for phosphate is blocked by vanadate. This interaction (vanadate-phosphate antagonism) couple with the influence of Vd on DNA as well as its involvement on ROS generation has been identified as Vd's mode of action in therapeutic application.

Intracellular calcium mobilisation is a useful method to assess the functional effects of Vd. Calcium changes can reflect effects upon voltage-gated calcium channels, intracellular calcium stores in the ER and mitochondria organelles. The non-toxic dose of Vd (10µM) had no clear effect on the cell body intracellular calcium levels or elevation due to KCl–induced depolarisation in undifferentiated CAD cells (See Fig 3.7). However, a sub-toxic, yet oxidative stress dose of Vd (100µM) caused a modest rapid inhibitory spike followed by a protracted inhibition, with limited effects upon depolarisation response. In differentiated CAD cells, a contrasting effect was observed between the cell body and cell process (Fig 3.9). Vd (10µM) again showed no effect alone or KCl-induced depolarisation on the cell body. In contrast, a sharp but transient large increase in intracellular calcium in the process upon depolarisation was attenuated by 100µM Vd.

As noted above, 10µM of Vd has no apparent effect on mitochondrial stress and is non-cytotoxic while 100µM does elicit mild mitochondrial stress in undifferentiated but is non-cytotoxic. Differential levels of Fe in undifferentiated cells (14.79 ppb) and differentiated cells (4.8 ppb) correlate with sensitivity to Vd. As earlier mentioned, a strong relationship exists between Vd and Fe which explains the susceptibility of undifferentiated CAD cells to Vd.

Fe has been shown to aggravate the cytotoxic effect of cells in culture (Todorich, et al., 2011). This is made possible by the capacity of Vd to disrupt Fe homeostasis and further depletion of Fe from intracellular store - which is the mechanism that underlies its role in the generation of reactive oxygen species and eventual cytotoxicity (Ghio, et al., 2015). Fe deprivation in culture has been shown to result in growth arrest (Pittelkow, et al.,1986) which can be reversed by exogenous addition of Fe. Also as previously explained, the capacity of univalent iron to undergo redox reactions when reduced (ferrous) makes it deleterious to cells.

The possible depletion of intracellular Fe store by Vd and the higher Fe content (in situ) in undifferentiated cells may cause the readily available Fe, (in the ferrous oxidative state) free to participate in Fenton chemistry. This constitutes a significant source of oxidative stress. The ferrous iron subsequently catalysed conversion of hydrogen peroxide into a hydroxide ion and a hydroxyl free radical with the concurrent oxidation of ferrous iron to ferric iron (Dunford,

1987). As noted, (Fig 4.6) above, Iron chelation protects against toxic Vd concentration this in contrast to Cu (previous chapter) where iron chelation exacerbates toxicity.

In contrast to sub-toxic doses, spiking with a toxic dose (500μM) elicited a modest increase in calcium signal in both the cell body and processes but did not suppress the depolarisation response in the cell body or process. However, whilst the signalling response in the cell body was restored to the baseline, at this dose, it was further repressed in the processes (post-depolarisation) - an indication that this may have a serious implication of synaptic plasticity and transmission.

Whilst 10μM of Vd had no apparent effect on mitochondrial stress and is non-cytotoxic and 100 μM does elicit mild mitochondrial stress in undifferentiated but is non-cytotoxic. These (concentrations 10μM and 100μM) have effects on differentiation and ER stress. The effect of Vd on differentiation was elucidated with the differentiation marker (MAP2). Microtubule associate protein, MAP2 is a protein that belongs to the MAP family, involved in microtubule assembly, an essential step in neuritogenesis. It serves to stabilize microtubule (MT) growth by cross-linking with intermediate filaments and other MTs. Its equivalent in mouse and rats are the neuron-specific cytoskeleton that is enriched in dendrites, implicating a role in determining, as well as stabilising, dendrite shape during neuron development. As previously stated (in Section 3.10), four isoforms of MAP-2 had been delineated which are restricted to the somatodendritic compartment, viz; high molecular weight MAP2a (~280 KD) and MAP2b (~270 KD) and the low molecular weight MAP2c (~70 KD) and 2d (~68 KD) have been identified in the neurons (Dehmelt, et al., 2005).

MAP2a which are often localised in the dendrites appears at the end of the differentiation process and it is absent in axons. Whereas MAP2b is found early in the differentiation process, therefore, any xenobiotics that interferes with embryogenesis may adversely affect the expression of MAP2b, hence, the differentiation process. MAP2b is often located in the dendrites (Doll, et al., 1993). There is a marked increase concentration of MAP2b during the maturation process but is absent in the axons. MAP2b are found in early development but are replaced.

The only two detectable isoforms in differentiated CAD cells were MAP2c and 2d (latter most prominent) which is surprising. In differentiated CAD cells, following acute exposure (24 hours) (10µM) of Vd, caused an up-regulation of MAP2c and 2d, which, however, was not statistically significant. In contrast, 100µM Vd caused a marked decrease in MAP2c expression, while chronic exposure of differentiating CAD cells to a toxic dose (1000µM) showed some expression of MAP2d but completely lacked the expression of 2c, 2b and 2a (not shown). MAP2c are juvenile forms of MAP2 and are found in both dendrites and axons. Their levels diminish during neuronal development. Besides, their presence during embryogenesis are transitional, and it is eventually replaced by MAP2a in the matured neurons (Banker and Goslin, 1990). The significant decrease in the expression of MAP2c and the absence of MAP2a and MAP2b suggest that acute exposure of matured CAD cells to Vd affected the further development of neurites in these matured CAD cells.

Low molecular MAPs (2C and 2D) are expressed in developing neurons, mostly prenatally (Melkova, et al., 2019). The downregulation of MAP2D and absence of 2C, 2B and 2A following chronic exposure of CAD cells to Vd to oxidative stress dose (100uM) suggest that this concentration of Vd interferes with microtubules dynamics (which is crucial in intracellular cargo trafficking along the axons and dendrites (Pellegrini, et al., 2017).

Goslin and Banker (1990) posited that MAPs and tau that are specifically expressed in the neurons are pivotal molecules to produce dendrites and axonal processes. Caceres and Kiosk (1990) further pointed out that if the expression of MAP2 is suppressed, it inhibits the development of exploratory neurites, but suppression of tau prevents the differentiation and maintenance of the axonal process. This was further confirmed in a related study by Caceres, et al (1991; 1992). However, upregulation of MAP2c and 2 days by sub-toxic dose (10µM) of Vd, may be associated with a possible neuroprotective effect. This correlates with findings on metabolic function, where oxidative stress dose (10µM) had no apparent effect on mitochondrial stress and is non-cytotoxic. Findings from immunofluorescence investigations on MAP2 - result (Fig.4.12 *cf* Fig. 4.13 and Fig. 4.13) also confirms that this concentration does not affect the neuronal processes in both chronic and acute exposures.

4.15.1 PDI expression and ER stress response

Protein disulphide isomerase (PDI) is a versatile redox chaperone of the endoplasmic reticulum (ER). It is detected as a signal around the nucleus because of the proximity of the ER to the nucleus, and both organelles are closely associated/interact in the process of protein expression and maturation. Immunofluorescence result obtained provides qualitative information on the effect of 10μM and 100μM exposure on CAD cells to establish the presence or otherwise of possible Vd-evoke stress. In our investigation, we seek to explore the effect of sub-toxic dose (10uM) and oxidative stress dose (100uM) of Vd on the Redox regulation of PDI.

In reducing condition, treatment of differentiating cells (chronic – Fig. 3.13) and differentiated cells (acute – Fig. 3.14) with sub-toxic dose Vd (10μM) resulted in a downregulation in the expression of PDI (signal). Reduced condition (in the presence of DTT) affect the conformation of PDI, restricting it to exist only reduced configuration. As PDI catalyses redox reaction, it gets reduced and oxidized respectively. This forces the protein to change conformation (Galligan and Petersen, 2012). Evidence implicates PDI in increasing the levels of ROS, hence inducing oxidative stress and apoptosis (through its chaperone activity) rather than the disulphide interchange activity (Fernandes, et al., 2009; Wantabe, et al., 2014). Paes and his colleague (2011) noted that only oxidized PDI triggers the production of ROS. Reduced PDI inhibits the production of ROS (Paes, et al., 2011).

Similarly, in non-reducing condition, there was down-regulation of PDI protein expression following acute exposure of differentiating cells (chronic exposure- 6 days) to the sub-toxic dose of Vd (10μM - Fig. 3.15). This implies, that the expression of PDI in both reducing and non-reducing condition remains unchanged (compare to the control). This suggests that the sub-toxic dose, in both acute and chronic exposure, did not induce ER stress.

In contrast, oxidative stress dose of Vd (100μM) resulted in an up-regulation of PDI protein expression in both acute (differentiated cells – 24 hours) and chronic exposure to Vd reducing and non-reducing conditions (Figs 3.13; 3.14 and 3.15). In moderate ER stress, there is an upregulation in PDI protein and concurrently/ concomitant modification of PDI. However, in severe stress, a notable increase in PDI expression was evident with oxidative dose (100uM) in differentiating cells. There was a significant increase in PDI protein expression. In addition to the increase, PDI expression observed in both differentiating and differentiated cells (under reducing and non-reducing conditions). Furthermore, exposure to acute and chronic oxidative

stress dose resulted in a double band (upper and lower) PDI signal expression (under both conditions) (Figs 3.13; 3.14 and 3.15).

PDI has been implicated in both the physiology and pathophysiology of neurodegenerative conditions (Andreu, et al., 2012). It is upregulated in various tissues in disease conditions (Andreu, et al., 2012), in response to hypoxia in the brain (Tanaka, et al., 2000), in dopaminergic neurons and Lewy bodies of patients with Parkinson disease (Uehara, et al., 2006); to prevent neuronal apoptosis (Tanaka, et al., 2000). Most studies suggest that the stimulation of PDI during ER stress in neurodegenerative diseases reduce the load of misfolded proteins and therefore, protective. This protective effect restores proteostasis and increases neuronal viability. A metabolic disorder that underlies most neurodegenerative diseases is protein misfolding. Since protein misfolding within the ER triggers ER stress and the associated up-regulation of PDI. Thus, ER stress is being connected to these diseases (Hetz and Mollereau, 2014). From the foregoing, the upregulation of PDI expression (in both acute and chronic exposure to Vd) is suggestive that this dose/concentration causes ER stress. This is reflected in the immunofluorescence result with PDI and nuclear shape (Figs 3.19 and 3.20 respectively) where the nuclei displayed an aberrant crescent-formed shape. The nuclear shape serves as an indicator for the health of the cells (Chazot and Karakesisoglou - unpublished).

Furthermore, double bands can be seen in the PDI protein expression in cells (differentiating and differentiated cells) (Figs 3.13; 3.14 and 3.15). The presence of reducing agents like DTT can alter the conformation of PDI and can influence the running of the protein in a gel. The Western blots undertaken in both reducing and non-reducing conditions showed the that the different PDI conformations were still present but altered - which was depicted in the double-band signal seen for PDI on the membrane (Figs 3.13; 3.14 and 3.15). This presence of double band is an indication of post-translational modifications of PDI.

Aberrant post-translational modification of PDI associated with the state of the disease has been reported. Examples of this redox post-translation include S-nitrosylation, carbonylation and S-glutathionylation. Various publications have shown that PDI shows S-nitrosylation as post-translational modifications in patients suffering from neurodegenerative diseases (Forrester, et al., 2006; Uehara, et al.,2006; Nakamura and Lipton, 2019). Several events can lead to post translation (modification) of PDI. First, is as a result of metabolic activity at the cellular level which could result in accumulation of high levels of reactive nitrogen species (RNS), hydrogen peroxide and reactive oxygen species (ROS) in cells. These events provoke

nitrosative or oxidative stress. Secondly, nitrosative stress can. This leads to post-translation modification of PDI by the addition of NO to active site cysteine residues, resulting in S-nitrosylation. S-nitrosylation of proteins under pathological conditions is an abnormal, irreversible process that is linked to protein misfolding, ER stress and apoptosis. Thirdly, proteins resident in the ER is particularly vulnerable to post-translation modification due to the presence of critical redox-regulated cysteines.

Since PDI is the major enzyme responsible for modification of protein disulphide bonds, the loss of function of PDI could result in increased cellular protein misfolding and thus increase ER stress (Nakamura and Lipton, 2011; Wang et al., 2012)

S-nitrosylated PDI has been detected in several neurodegenerative diseases (Nakamura and Lipton, 2011; Wang, et al., 2012). S-nitrosylation of PDI has been detected in postmortem brain tissues of patients with Alzheimer's disease, PD (Uehara, et al., 2006) and lumbar spinal cord tissues of ALS patients (Honjo, et al., 2010; Walker, et al., 2010). In essence, S-nitrosylation reduces both its chaperone and isomerase activity (Uehara, et al., 2006).

These unfolded (Vitale and Boston, 2018; Cola Emanuela, 2019), However, in a persistent chronic state, it can further attenuate UPR and cause neuronal cell death. Hence, aberrant modifications of PDI lead directly to harmful effects as well as a loss of the normally protective properties of PDI.

Overall, low sub-toxic levels of Vd have a range of morphological, cellular, functional, and molecular effects, which dose-dependently range in severity (between 10µM and 100µM), including morphological changes, cellular stress, reduced process excitability. The next chapter is designed to explore the behavioural (motor activity), and biochemical effects as well as ageing (lifespan) of a sub-toxic low dose (1µM) Vd upon wild-type and PD Drosophila fly models.

Chapter 5: Interplay between iron and vanadium in PD in an *in vivo* model

In the preceding chapter, we explored the interplay between Vd and iron in an *in vitro* model - Catecholaminergic a-differentiated (CAD) cells. This chapter is devoted to investigating the effect of Vd in an *in vivo model* (Drosophila *melanogaster*). We investigated the effect of chronic exposure of Drosophila *melanogaster* (WT and mutant) to low dose Vd, particularly the effect on iron-induced oxidative stress, motor activity and life span in both wild-type and the Pink-1 mutant Drosophila PD model.

5.1 Introduction

The PD pathology is characterised by distinct cellular defects, notably abnormal protein aggregation, oxidative damage, mitochondrial dysfunction, and a selective loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc). The PD brain SNpc has also been found to have higher levels of iron than age-matched controls, which has been associated with mitochondrial dysfunction (Dexter, et al., 1991; Bharath, et al., 2002; Dauer and Przedborski, 200; Kaur and Andersen, 2004; Salazar, et al., 2008). Familial (genetic) inheritance accounts for 5 to 10 % of cases of PD while up to 90% are sporadic.

Recent studies have linked environmental factors interacting with a genetic predisposition in the causality of PD (Bjorklund, et al., 2018; Draoui, et al., 2020). Several studies have reported several PD genes that are activated by xenobiotic as evidence of a link between environmental toxicants and PD (Rybicki, et al., 1993; Shiba-Fukushima, et al., 2014; Kumudini, et al., 2014). Several epidemiological studies have particularly reported an association between PD and exposure to metals (Zayed, et al., 1990; Winkel, et al., 1995; Gorrel, et al., 1997; Kuhn, et al.,1998; Gorrel, et al., 1999; Miller, et al., 2009; Coon, et al., 2006; Taba, 2017). The cellular toxicity of exogenous heavy metals such as Vd has previously been shown to be exacerbated in the presence of iron in oligodendrocyte progenitor cells (Todorich, et al., 2011; Olopade and Conor, 2011; Fatola, et al., 2019). As a heavy metal nutraceutical, Vd has some therapeutic functions. It has been used at, low doses, in different health supplement formulations (Rehder, 2015). Environmental exposures to low dose Vd can have deleterious effects on the population over extended periods.

5.1.1 ROS/RNS, Oxidative Stress and Neurodegenerative Diseases

Reactive Oxygen Species (ROS) is a group of reactive molecules derived from oxygen (Bolisetty, et al., 2013). They are labile and highly reactive due to their unpaired valence electrons (Patten, et al., 2010). These include free radicals (superoxide, O_2), hydroxyl radical (-OH), or non-radical (hydrogen peroxide, H_2O_2). Due to its short-lived nature in the cell, O_2 - plays a key role in ROS production. It can be transformed into a more stable H_2O_2 form (by superoxide dismutase) or protonated to form H_2O_2. The resulting H_2O_2 can form a highly reactive hydroxyl radical –OH (Patten, et al., 2010). This can further be broken down into H_2O and O_2 by catalase, glutathione peroxidase, and other peroxidases (Song, et al., 2015).

The most reactive ROS that is mainly responsible for the cytotoxic effects in cells is –OH (Bolisetty, et al., 2013). Through a process of Fe^{2+}-mediated decomposition, -OH can be generated from H_2O_2 and O_2, which is catalysed by Fe ions by Fenton reaction (Zorov, et al., 2014). Reactive Oxygen Species (ROS) in cells have an endogenous and exogenous origin (Mani, 2015). Endogenous production of ROS is mediated enzymatically by mitochondrial and non-mitochondrial derived enzymes. Thus, comprising nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, (NOx), xanthine oxidase (XO), cytochrome p450 (from ER) and Flavin oxidase-from peroxisomes (Song, et al., 2015; Mani, 2015). ROS are predominantly produced from the mitochondrial respiratory chain and NOx system.

A significant driver of the pathogenesis of PD is oxidative stress (Jiang, et al., 2016). Oxidative stress results in mitochondrial dysfunction (Bonnard, et al., 2008; Hsu, et al., 2000; Hastings, 2009). There is an upregulation of ROS production and oxidative stress in patients with PD. Besides, the impairment of mitochondrial functionality has been reported in PD patients (Hald and Lotharius, 2005; Zhou, et al., 2008; Gan, et al., 2014). The increased oxidative stress is underscored by elevated iron levels (Gotz, et al., 1990; Hartley, et al., 1993), elevated lipid peroxidation (Boll, et al., 2008; Agil, et al., 2006; Sanyal, et al., 2009), nuclei acid oxidation (Zhang, et al., 1999; Kikuchi, et al., 2002; Moreira, et al., 2008; Sanders and Greenamyre, 2013), and low levels of the antioxidant, glutathione (GSH) in the dopaminergic areas.

5.2 List of Materials & Chemicals

Table 5.1: Materials and corresponding suppliers

Product	**Supplier**
WT (WT) flies - Dahomey; Drosophila *melanogaster*	Dr Ed Okello's lab- Institute of Cellular Medicine, Medical School, Newcastle University
PINK1 mutant flies – ($^{w-}$ PINK1 ^{B9}/FM7. GFP $^{w+}$) -Drosophila *melanogaster* PINK1	Dr Ed Okello's lab- Institute of Cellular Medicine, Medical School, Newcastle University
250ml flies' bottles	Scientific Laboratory Supplies
Applied Scientific Jazz-Mix Drosophila Food	Fisher Scientific (Loughborough, UK)
Carbon dioxide	Sigma Aldrich (Dorset, UK)
Fly pad	Blades Biological (Kent, UK)
Controlled room (12 hourly day-night cycle @25^{o}C)	Lab
Brush	Blades Biological (Kent, UK)
Filter using Whatman #1 paper	Fisher Scientific (Loughborough, UK)
Microplate reader - Thermo scientific multiskan FC.	Thermo Fisher Scientific Oy P.O Box 100, FI-01621 Vantaa, Finland
Refrigerated centrifuge (4^{o}C)- Eppendorf Centrifuge 5415R. Spec:CF3CH2F.	Eppendorf AG 22331 Hamburg, Germany
Liquid nitrogen	Biosciences Nitrogen Tank
Synergy H4 hybrid multi-mode microplate reader	BioTek Instruments, Inc., P.O. Box 998, Highland Park, Winooski, Vermont 05404-0998 USA.
Vortex (machine) Genie-2; Model:G-560E	Scientific Industries Inc. Bohemia, NY, 11716, USA
Plugs	Blades Biological (Kent, UK)

Table 5.2: Chemicals and corresponding suppliers

Product	Supplier
Absolute Ethanol	Fisher Scientific, UK
Ascorbic acid (vitamin C powder)	Sigma Aldrich (Dorset, UK)
Bovine Serum Albumin (BSA)	Sigma Aldrich (Dorset, UK)
Bradford Reagent	Sigma Aldrich (Dorset, UK)
Coomassie Brilliant Blue G-250	Sigma Aldrich (Dorset, UK)
DCFH-DA (2,7-Dichloroflouresceine diacetate)	Sigma Aldrich (Dorset, UK)
Deferoxamine (DFO) Mesylate salt	Sigma Aldrich (Dorset, UK)
Dibasic Phosphate buffer (K_2HPO_4)	Sigma Aldrich (Dorset, UK)
DTNB (Ellman's Reagent) (5,5-dithiobis-(2-nitrobenzoic acid)	ThermoFischer Scientific
Ethanol	Sigma Aldrich (Dorset, UK)
L-DOPA (3,4-Dihydroxy-L-phenylalanine)	Sigma Aldrich (Dorset, UK)
Methanol	Fisher Scientific, UK
Monobasic Phosphate buffer (KH_2PO_4)	Sigma Aldrich (Dorset, UK)
Phosphoric acid (H_3PO4)	Sigma Aldrich (Dorset, UK)
Potassium Phosphate Buffer	Fisher Scientific, UK
Reduced Glutathione- GSH	Abcam

5.3 Method

5.3.1 Drosophila *melanogaster* stocks and culture conditions.

Drosophila *melanogaster* PINK1 ($^{w-}$ PINK1^{B9}/FM7.GFP^{w+}) mutant and - Dahomey WT (WT) were used for this experiment. Flies were kept in an incubator with a 12 hour day-night cycle at 25^{o}C. Fly food was prepared by mixing 15.025g of instant medium (Applied Scientific Jazz-Mix Drosophila Food) with 46ml of deionized water (formula with this food: water ratio was found to have the best consistency) per bottle; 250mL. Following this, each bottle was slightly shaken to ensure even distribution of water. Each bottle was plugged with a foam plug and left to set for at least an hour at room temperature before transferring the flies (progenies) into them. Fresh food was prepared every two weeks and flies were flipped into new bottles.

5.3.2 Dosage and treatment

A low dose (oxidative stress dose) of Vd (1µM) was used. This concentration of Vd was calculated using the equation by Hong, et al (2011) to convert dosages used in human studies to effective and non-toxic doses that could be used in the Drosophila *melanogaster* experiments. To investigate the effect of chronic exposure of Vd on motor activities, lifespan, iron chelation and oxidative stress markers, 3 or 4 groups (depending on the experimental design) were used. These consisted of a control group (with no treatment), positive control (treated with L-dopa; 1.6mg) and a treatment group (Vd-1µM). Each group comprised of five replicates and each bottle had 10 flies. To prevent oxidation of L-Dopa, 20.8mg of ascorbic acid (equivalent to, 1.6µM) was added to each bottle containing L-Dopa (like the amount used in a study by Pendleton, et al. 2002).

At the start of each experiment, flies were allowed to lay eggs on food once the larvae appeared, the adult flies were released from the bottles, after 6 days the progenies were transferred to fresh food and redistributed onto treatments the next day (which is counted as test day zero). To establish the effect of synthetic iron chelation on motor activity and lifespan, a dose-dependent effect of DFO (0µM, 5µM, 10µM, and 20µM) was tested –in both WT and mutant flies. From the result of this experiment, we established an optimal concentration of synthetic iron chelator Deferoxamine (DFO) Mesylate salt required for the subsequent experiment as 5µM; WT & 20µM; PINK1.

5.3.3 Climbing assay and lifespan

The motor functions were tested against different treatment regimen with respect to the symptoms in PINK1 vs WT flies. This was measured by testing their climbing ability. Adult flies were placed in vials with treated foods. These could lay eggs in these vials. After eclosion, the adult flies were released, and male flies were selected from their progenies and transferred into new vials. These (selected male) were used for the various chronicity investigations - motor activity using a climbing test adapted from Pendleton, et al (2002), Nichols, et al (2012) and Cha, et al (2005), conducted with moderate modifications, survival test and biochemical assays. Days of the climbing assay (i.e. day zero) is taken as the day the progenies were transferred into the new vials.

For the motor activity, all groups were tested at random. Groups of 10-15 flies (depending on the experiments) were transferred into empty 100mL Pyrex graduated cylinder with a foam plug, and a height of 8cm horizontal line above the bottom of the cylinder was drawn on a paper as criteria. The flies were allowed 10 minutes to acclimatise. The flies were then gently tapped down and allowed to climb up past the 8cm mark (in 8 seconds) on the chart, and afterwards tapped down again. A digital camera was used to record the flies, at 30cm from the paper, and a timer was used to record the time. The total number of flies that crossed the 8cm mark was recorded as the “Escaped flies”. This was repeated twice more.

The climbing assay was performed at 10am, every two to three days. An average of the total number of flies that escaped was noted. The ability of surviving flies per day (%) was calculated by dividing the number of flies that climbed over the 8 cm mark by the total number of surviving flies multiply by a hundred. The survival rate per day was calculated by dividing the number of surviving flies by the number of flies on Day Zero (multiply by a hundred). Experiment duration was for two weeks (from the start of the experiment). The mean of each group was calculated using the data from the three replicates tests. An adjusted two-way multiple measure analysis of variance (ANOVA) with post-test Bonferroni correction was performed using GraphPad Prism, Version 7 (GraphPad Software, San Diego California USA).

5.3.4 Sample preparation for biochemical assays

At the end of exposure (14 days), the flies from each group of control and treated (Vd only; Vd + DFO) groups were anaesthetised in ice. The flies were then snapped frozen in liquid nitrogen and vortexed at high speed to separate the fly head from the body (since we are only interested in measuring the RONS in the head). The detached fly heads were transferred into pre-weighed Eppendorf tubes and weighed. It was then homogenised in 0.1M phosphate buffer, pH 7.0 (1mg: 10µL), centrifuged for 10 minutes at 4000g (temperature, 4^{o}C). The supernatants obtained were stored at -20^{o} C and used to determine the RON's level, protein, and total thiol content. The assays were performed in duplicates for each of the three replicates of the treatment groups (Vd only; Vd + DFO) treatment groups.

5.3.5 Measurement of DCFH oxidation for RONS – Kinetic assay

To determine the RONS level following chronic exposure to Vd and influence of Fe chelation, 2', and 7'-Dichlorofluorescein (DCFH) oxidation was measured as an index of oxidative stress according to the method of Perez-Severiano, et al (2004). From the frozen supernatant, five microliters (5µL, 1:10 dilution) of each supernatant from Vd only- and Vd + DFO- treated and control flies (for both WT and mutant) were transferred into a 96-well plate.

Subsequently, 5µL of 200 µM DCFH-DA (final concentration of 5µM) was added to the samples and the fluorescence product of DFH oxidation (that is, DCF) was measured for 10 minutes (at 30-secs intervals), using Synergy H4 hybrid multi-mode microplate reader (See Table 5.1) with excitation 488 and 525nm emission. All the experiments were conducted in duplicates for each of the three replicates of Vd ±DFO – treated and control flies (for both WT and mutant). The rate of DCF formation was expressed in percentage of the control group.

5.3.6 Total Thiol (T-SH) assay – WT and Mutant.

The total thiol content for both WT and mutant flies was determined using the method of Ellman (1959). The reacting mixture contained 170µL of 0.1 M potassium phosphate buffer (pH 7.4), 20µL of the sample as well as 10µL of 10mM DTNB. It was incubated for 30 minutes at room temperature, the absorbance was measured at 412nm and used to calculate the sample total thiol levels (in µmol/mg protein) using GSH as standard.

5.4 Results

These sets of experiments were designed to explore the effect of Vd on an *in vivo* PD model, Drosophila *melanogaster*- (PINK1 mutant) and WT. The aim was to determine the effects of low dose Vd on a mutant PD Drosophila *melanogaster* model as well as the interaction between Vd and iron in neurons. Furthermore, we investigated the ameliorating effect of an iron chelator and the effect of chronic exposure to low dose Vd on oxidative stress markers in the model.

5.4.1 Effect of chronic exposure of vanadium (1µM) to motor activity and lifespan in Drosophila *melanogaster*: WT and PINK1 Mutant

5.4.1.1 Time-dependent effect of chronic exposure of sub-toxic vanadium on the motor activity and survival of WT.

A progressive decrease in the motor activity of the WT types of flies with increasing age was observed. Besides, in the WT flies, chronic exposure to sub-toxic doses of Vd revealed no significant effect. A modest increase in motor activity was seen in flies treated with L-dopa ($p<0.05$) compared to the control group.

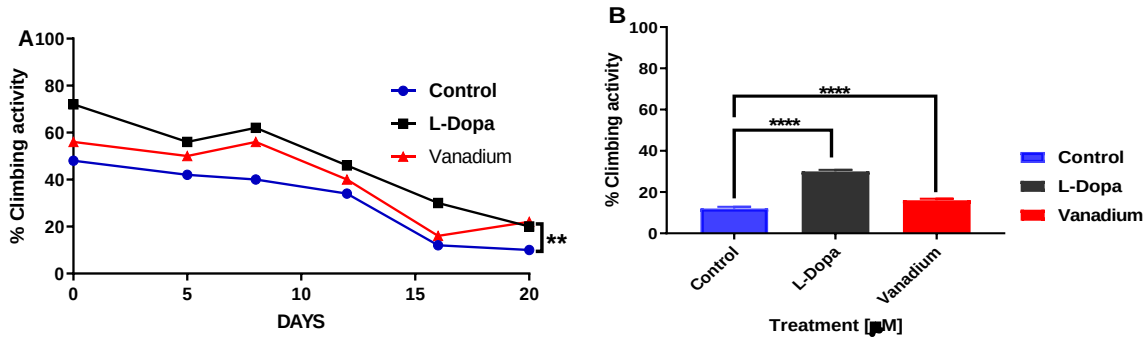


Figure 5.1: Effect of chronic exposure of vanadium (1µM) on the percentage of flies that escaped in the climbing ability test for WT flies.

A progressive decrease in the motor activity of the flies over time was observed across the three groups tested: saline (control blue line), L-dopa (black line) and Vd (1 µM) treatment groups. A modest increase in motor activity can be seen with flies treated with L-dopa ($p<0.0001$) as well as the group treated with Vd ($p< 0.01$) compared with the control group. There is no significant difference between the L-dopa and Vd treated group ($p<0.1$). A repeated measure one-way ANOVA (with Tukey's Multiple Comparisons Test) was performed. Panels B show significant contrasting effects on climbing ability after two weeks (day 16 for WT and Mutant, respectively), analysed with a one-way ANOVA (with Dunnett's Multiple Comparisons Test) was performed. (ns= $p>0.05$; *$p<0.05$; **$p<0.01$; ****$p<0.0001$)

5.4.1.2 Time-dependent effect of chronic exposure of sub-toxic vanadium on the motor activity and survival of PINK-1 mutant flies

Vd significantly exacerbated the existing locomotor deficits in mutant PINK-1^{89} flies ($p<0.01$) while L-dopa ameliorated this effect.

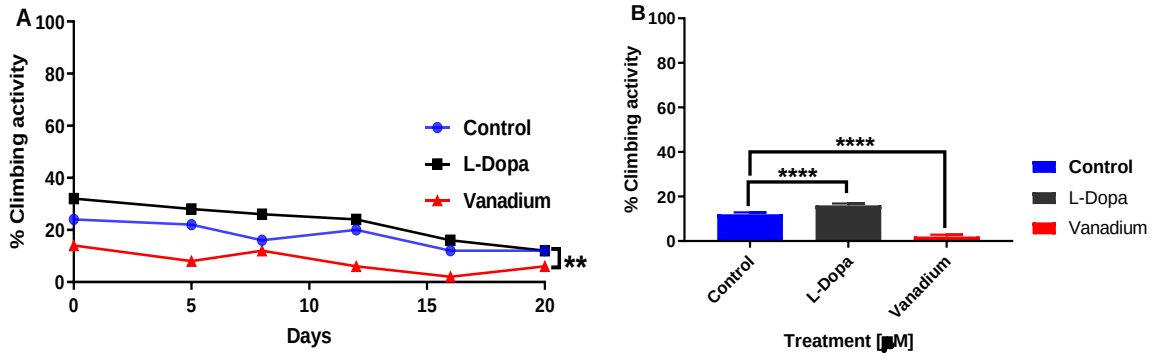


Figure 5.2: Effect of chronic exposure of vanadium (1 µM) on the percentage of flies that escaped in the climbing ability test for PINK1 flies.

A progressive decrease in the motor activity of the PINK1 flies over time was observed across the three groups tested: saline (control blue line), L-dopa (black line) and Vd (1µM) treatment groups. Whilst exposure of PINK1 mutant to Vd exacerbated the existing locomotor deficits ($p<0.001$), a significant increase in motor activity was observed with flies treated with L-dopa ($p<0.0001$) relative to the control group. There was no significant difference between the L-dopa and Vd treated group ($p<0.1$ A repeated measure one-way ANOVA (with Tukey's Multiple Comparisons Test) was performed. Panels B show significant contrasting effects on climbing ability after two weeks (day 16 for WT and Mutant, respectively), analysed with a one-way ANOVA (with Dunnett's Multiple Comparisons Test) was performed. (ns= $p>0.05$; *$p<0.05$; **$p<0.01$; ****$p<0.0001$).

5.4.1.3 Effect of chronic exposure to vanadium (1µM) on survival in WT flies

Similar to the effect on motor activity, chronic vanadium had no general effect on the survival of WT flies apart from a modest effect seen in the first week (Day 5). However, a significant increase in survival of flies treated with L-dopa ($p = 0.0016$) was observed (Fig. 4A).

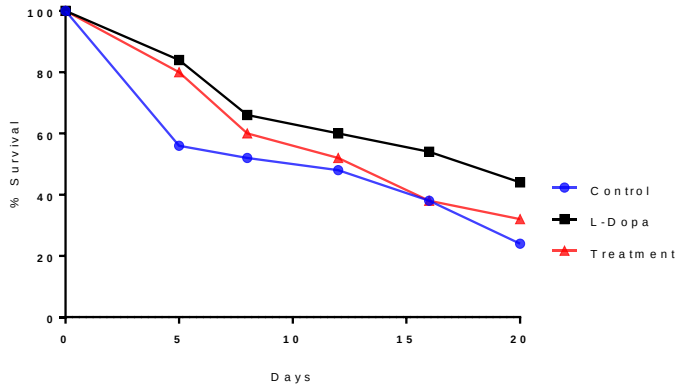


Figure 5.3: Effect of chronic exposure of vanadium (1µM) on the percentage of fly's survival in lifespan studies in WT flies.

A progressive decrease in the lifespan over time was observed across the three groups tested. Saline (control blue line), L-dopa (black line) and Vd (1µM) treatment groups. Results show a significant increase (and clear effect in the first week) in the lifespan of flies treated with L-dopa ($p<0.01$) and a slight, but insignificant, increase in lifespan in the group exposed to Vd (1µM) $p>0.05$, relative to the control.

5.4.1.4 Effect of chronic exposure to vanadium (1µM) on survival in PINK1 flies

In PINK1 mutant flies, a reduction in survival in the Vd treated group (p= 0.0349) relative to the control group was observed with the median survival for Vd treated group being 5 days compared to 8 days for control flies. There was no significant effect on survival in the L-dopa treated group relative to control, with median survival for both groups (WT and PINK1) being 8 days.

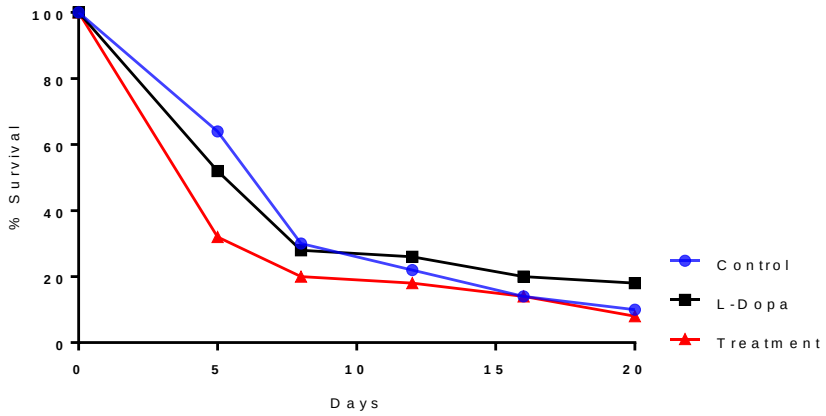


Figure 5.4: Effect of chronic exposure of vanadium (1µM) on the percentage of fly's survival in lifespan studies in PINK1 flies.

A progressive decrease in the life span over time was observed across the three groups tested. Saline (control, blue line), L-dopa (black line) and Vd treatment groups. Results show an increase in lifespan in flies treated with L-dopa, though not statistically significant ($p>0.05$) and a reduction in the life span in the Vd treated group ($p>0.05$) relative to the control. Data are presented as mean of SEM of three independent replicates carried out in duplicates: ns $p>0.05$

5.4.2 Dose Dependent effect of DFO- Motor Activity and lifespan in – WT and PINK1 mutant Drosophila melanogaster

The effect of iron chelation in an *in vivo* model was investigated to establish the effect of iron chelation on motor activity and lifespan (in both WT and mutant flies). The next sets of experiments were designed to establish the effect and optimum concentration of synthetic iron chelation (DFO) on motor activity and lifespan (in both WT and mutant flies). This is aimed at both establishing a possible beneficial or deleterious effect of DFO on DM and the optimal concentration useful for further *in vivo* assays.

5.4.2.1 Motor effect of different concentration of DFO in WT flies (DM)

PDF optimisation result shows that 5µM resulted in an improved and a significant increase in motor activity with WTs flies.

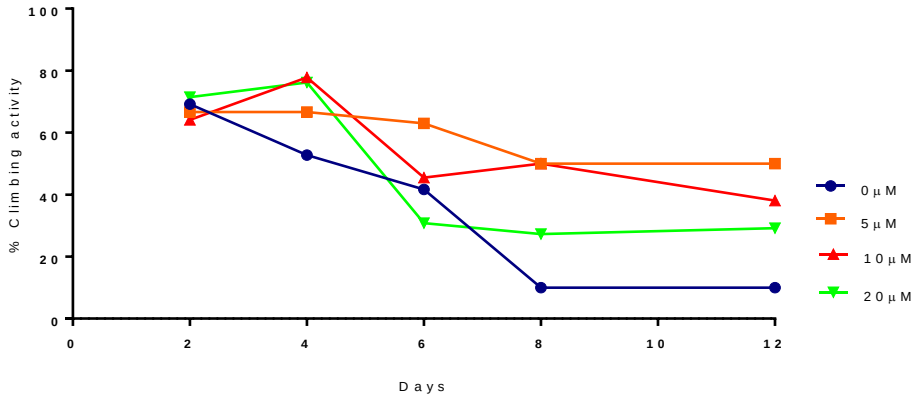


Figure 5.5: Dose-dependent effect of Iron Chelator (Deferoxamine Mesylate-DFO) following chronic exposure on Drosophila *melanogaster* WT.

Result of dose-dependent concentrations of DFO (0µM, 5µM, 10µM, and 20µM) on motor activity in WT DM shows a decrease in motor activity with time, but a relative increase in motor activity following treatment with DFO can be seen when compared with the control group. An improved (and significant) motor activity with DFO can be seen with the group treated with 5µM ($p<0.1$) when compared with control; as against 10µM ($p>0.05$); second week of treatment. However, the optimal effect of DFO is seen in the group treated with 20µM ($p>0.05$). All values are means (± SEM) from 5 separate experiments and n=10 for each experiment: with a Tukey's Multiple Comparisons Test (repeated measures two-way ANOVA).

5.4.2.2 Motor effect of different concentration of DFO in PINK1 flies (DM)

In contrast to WT flies, DFO optimisation in mutant shows that 20µM of DFO resulted in an optimum improvement in motor activity in the mutant.

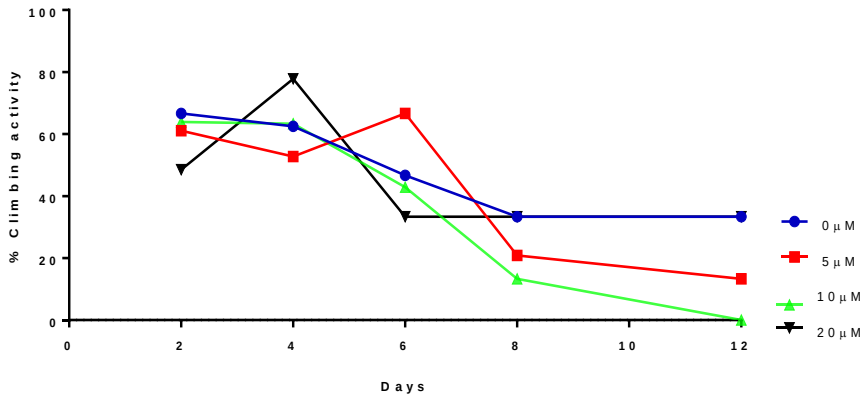


Figure 5.6: Dose-dependent effect of Iron Chelator (Deferoxamine Mesylate-DFO) following chronic exposure on Drosophila *melanogaster* PINK1.

Result of dose-dependent concentrations of DFO (0µM, 5µM, 10µM, and 20µM) on motor activity in PINK1 flies shows a decrease in motor activity with time. Improved motor activity (though not significant) can be seen with lower concentrations 5µM ($p>0.05$) and 10µM ($p>0.05$) in the first week when compared with the control group. The optimal (but not significant) effect of DFO was seen with the group treated with 20µM ($p> 0.05$) when compared with control. All values are means (± SEM) from 5 separate experiments and n=10 for each experiment: with a Tukey's Multiple Comparisons Test (repeated measures two-way ANOVA).

5.5 Effects of iron chelation on survival in both WT and mutant flies were investigated.

5.5.1 Effect of dose-dependent concentration of DFO on the survival of WT flies (DM)

There was an increased survival rate in flies exposed to a higher concentration of DFO (20 µM). This contrasted with the response from the motor activity where a low dose of DFO (5µM) improved motor activity.

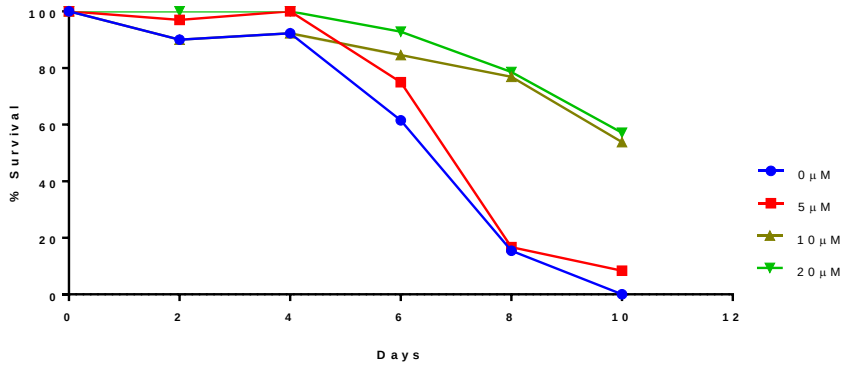


Figure 5.7: Dose-dependent effect of Iron Chelator (Deferoxamine Mesylate-DFO) following chronic exposure on Drosophila *melanogaster* PINK1.

Result of dose-dependent concentrations of DFO (0µM, 5µM, 10µM, and 20µM) on lifespan in PINK1 flies shows decrease lifespan with age. An enhanced lifespan effect can be seen in the group treated with 20µM ($p<0.1$) and 10µM, not significant, ($p>0.05$) when compared with the control group. The group treated with 5µM did not enhance lifespan ($p>0.05$) when compared with control. All values are means (± SEM) from 5 separate experiments and n=10 for each experiment: with a Tukey's Multiple Comparisons Test (repeated measures two-way ANOVA).

5.5.2 Effect of dose-dependent concentration of DFO on the survival of PINK 1 flies (DM)

There was an increased survival rate in flies exposed to a lower concentration of DFO (5µM). This contrasted with the response from the motor activity where a high dose of DFO (20µM) improved motor activity.

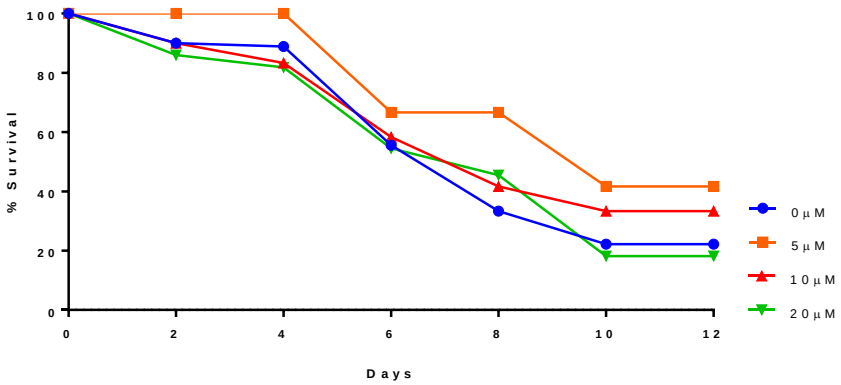


Figure 5.8: Dose-dependent effect of Iron Chelator (Deferoxamine Mesylate-DFO) following chronic exposure on Drosophila *melanogaster* PINK1.

Result of dose-dependent concentrations of DFO (0µM, 5µM, 10µM, and 20µM) on lifespan in PINK1 flies shows a decrease in lifespan with time. Significant enhancement of lifespan can be seen with flies treated with 5µM ($p<0.01$) DFO when compared with the control group. Also, 10µM DFO improved lifespan but not significant ($p>0.05$) when compared with the control, while 20µM DFO had a marked reduction in lifespan, though not significant ($p>0.05$) when compared with control. All values are means (± SEM) from 5 separate experiments and n=10 for each experiment: with a Tukey's Multiple Comparisons Test (repeated measures two-way ANOVA).

Having established the effect of Fe chelation on both the mutant and WT flies, an effective optimum concentration was selected. This concentration was used in exploring the effect of Fe chelation on motor functions and lifespan in chronic exposure to Vd in the presence and absence of Fe with 20µM and 5µM of DFO in WT respectively, as the optimum concentration with improved motor activity.

5.6 Effect of iron chelation on Drosophila *Melanogaster* on motor activity and Life span: In the presence of vanadium

5.6.1.1 Motor response to iron chelation following exposure to vanadium (1µM) in WT flies

To determine the effect of iron chelation on the VD - exacerbated motor deficits in PINK1 mutant flies, DFO was given to a sub-set of mutant flies (Vd +DFO). A significant improvement was observed in the motor activity in the presence of the iron chelator (DFO) + Vd on Day 10.

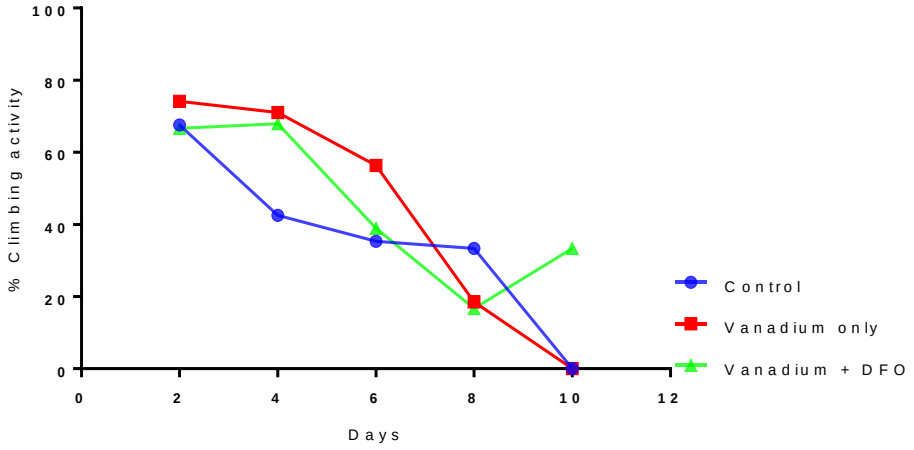


Figure 5.9: Effect of iron chelation on motor activity in WT (DM) following chronic exposure to Vd (1µM).

Result of iron chelation with DFO (20µM) on motor activity in WT flies shows a decrease in motor activity with time. In the first couple of days post-treatment, there was a modest (but insignificant) increase in motor activity followed by a continuous decrease in motor activity with Vd ($p>0.05$) and Vd + DFO ($p>0.05$) as compared with control and Vd + DFO treatment group ($p>0.05$). All values are means (± SEM) from 5 separate experiments and n=10 for each experiment: with a Tukey's Multiple Comparisons Test (repeated measures two-way ANOVA).

5.6.1.2 Chronic exposure of Drosophila melanogaster (PINK1 mutant) to low dose vanadium: influence of iron

Iron is an essential transition metal which is critical in many physiological functions in the cell and it is tightly regulated. As an enzyme co-factor, it protects the cell from oxidative damage. However, in the aberrant state, it is toxic and implicated in iron-mediated oxidative and carbonyl stress resulting in the alteration of internal homeostasis milieu. Vd toxicity is exacerbated in the presence of Fe (Todorich, et al., 2011).

This study was designed to investigate the possible benefits or otherwise of Fe chelation in an *in vivo* model- DM. A synthetic Fe chelator (DFO) was used is an iron-chelating agent.

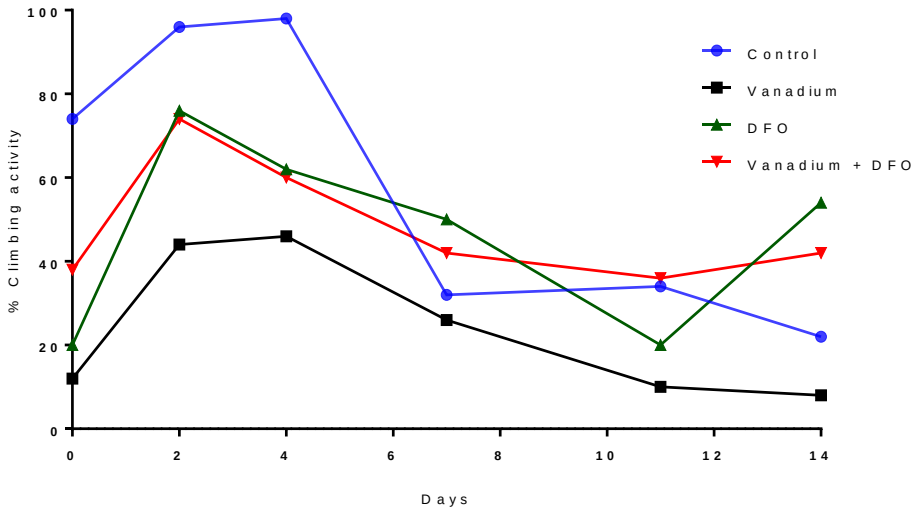


Figure 5.10: Chronic exposure of Drosophila *melanogaster* (PINK mutant) to low dose vanadium: influence of iron chelation.

Exposure of PINK1 mutant to Vd exacerbated the existing locomotor deficits in PINK1 flies ($p<0.01$) compare to the control. However, a moderate (but non-significant) improvement was observed in the motor activity in the presence of the iron chelator (DFO) + Vd ($p>0.05$) and DFO only ($p>0.05$) treated group ($p>0.05$) when compared with Vd only treated group. All values are means (± SEM) from 5 separate experiments and n=10 for each experiment: with a Tukey's Multiple Comparisons Test (repeated measures two-way ANOVA).

5.6.2 Effect of Fe iron chelation on life span in PINK1 mutant and WT.

5.6.2.1 Survival response to iron chelation following exposure to vanadium (1µM) in WT flies

Iron chelation did not enhance survival rate in WT flies following chronic exposure to Vd (1µM) in relation to Vd only group. However, a slight increase in survival rate was observed about the control group.

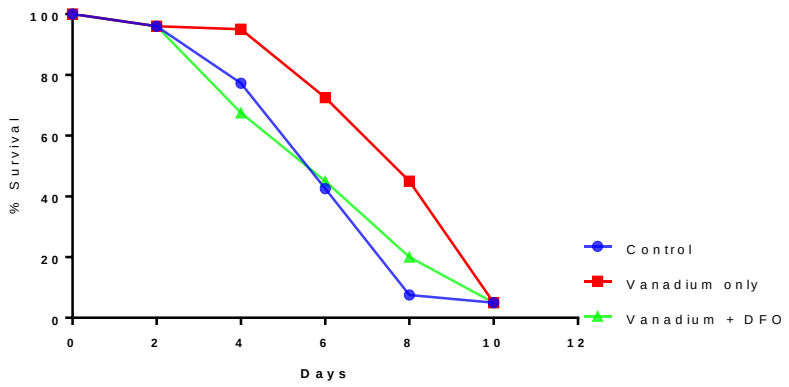


Figure 5.11: Effect of iron chelation on lifespan in WT flies (DM) following chronic exposure to vanadium (1µM).

Result of iron chelation using DFO (20µM) on lifespan in PINK1 flies shows a decrease in lifespan with time. A slight enhancement in lifespan was observed in Vd treated group in WT flies, but non-significant ($p>0.05$), when compared with control or in the presence of DFO ($p>0.05$). There was no significant difference in lifespan between the control group and the Vd + DFO treated group ($p>0.05$). All values are means (± SEM) from 5 separate experiments and n=10 for each experiment: with a Tukey's Multiple Comparisons Test (repeated measures two-way ANOVA).

5.7 Effect of vanadium and iron chelation on D. *Melanogaster* Brain oxidative stress markers (ROS, RONS and T-SH) - WT versus Mutant (PINK1)

Oxidative Stress (OS) plays a central role in the common pathophysiology of neurodegenerative diseases, such as PD. The brain is particularly vulnerable to the effects of ROS due to its high oxygen demand and owing to the presence of peroxidation-susceptible lipids cells. Oxidative stress predisposes to the degeneration of vulnerable neurons. The cause of this oxidative stress is multifactorial: mitochondria dysfunction, increased dopamine metabolism. Thus, leading to excess production of hydrogen peroxide and other ROS, increased reactive iron and a breakdown of the antioxidant defence pathways as well as a high oxidative intracellular environment in the dopaminergic neurons.

Mitochondrial dysfunction, alterations of the Ubiquitin Proteasomal System (UPS) and oxidative stress may lead to a cascade of events which act synergistically in the etiopathology of PD. The effect of chronic exposure of WT and PINK1 mutant flies to low dose (oxidative stress dose) Vd (1µM) was investigated. Oxidative stress markers (ROS/RONS) were measured.

5.7.1 Reactive Oxygen and Nitrogen Species (ROS) levels with Vanadium (1 µM): influence of iron chelation – WT

Measurement of ROS production in the WT flies, with a low dose of Vd, significantly reduced ROS generation after 14 days compared to the controls and iron chelation (DFO) completely reverses this Vd-induced reduction of ROS.

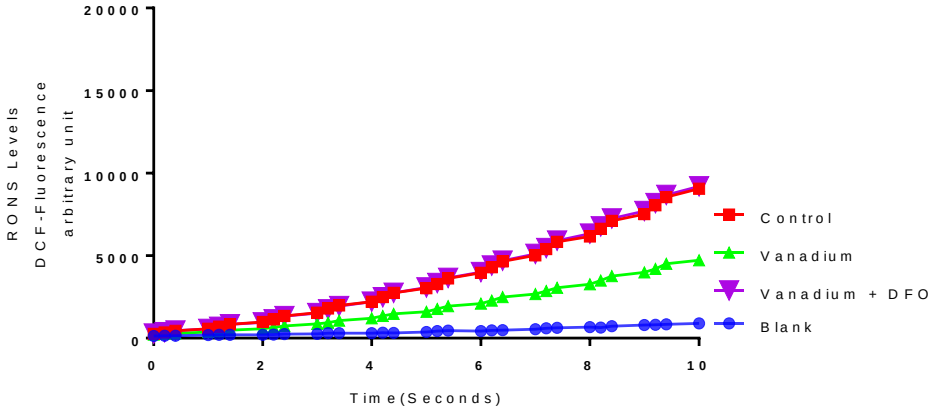


Figure 5.12: Kinetic assay of ROS levels in WT flies' brains after chronic exposure to vanadium.

Results showed that treatment with a low dose of Vd (1µM) decreased ROS generation after 10 days in WT flies when compared with the control ($p<0.0001$). In contrast, iron chelation enhances ROS generation (Vd + DFO), though not significant when compared with the control group ($p>0.05$). ROS level following iron chelation was markedly significant in comparison with the Vd only treated group ($p<0.0001$). All values are means (± SEM) from 2 separate experiments and n=50 for each experiment: with a Tukey's Multiple Comparisons Test (repeated measures two-way ANOVA).

5.7.2 Reactive Oxygen Species (ROS) levels with Vanadium (1µM): influence of iron chelation – PINK1

Measurement of ROS production in PINK1 mutant fly brains revealed that treatment with a low dose of Vd enhanced ROS generation after 14 days, compared with the control group, and that iron chelation (DFO) significantly reverses this.

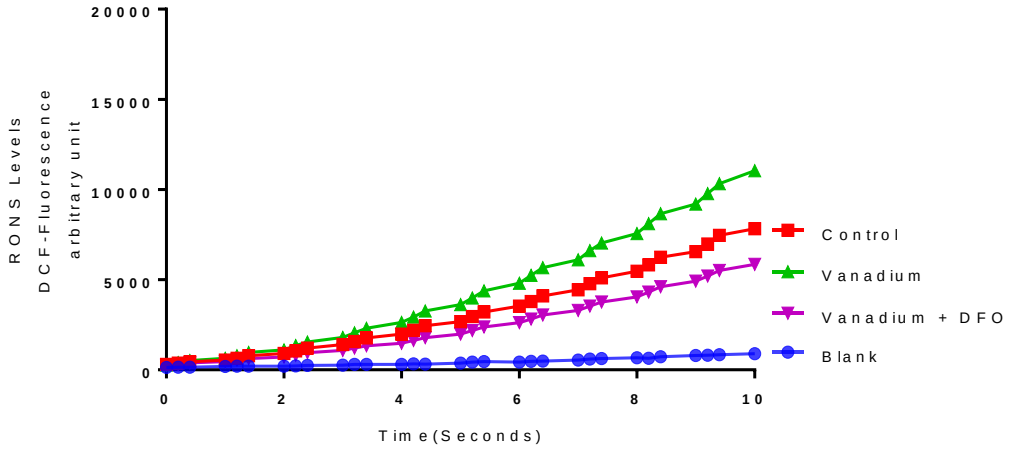


Figure 5.13: Kinetic assay of ROS levels in PINK1 flies' brains after chronic exposure to vanadium.

Results showed that treatment with a low dose of Vd (1µM) enhanced ROS generation after 10 days in PINK1 flies when compared with the control group ($p<0.01$). In contrast, iron chelation (Vd + DFO; 20µM) seems to decrease ROS generation, though not significant when compared with the control group ($p>0.05$). However, comparing the enhanced ROS levels following treatment with Vd only with Vd + DFO was significant ($p<000.1$). All values are means (± SEM) from 2 separate experiments and n=50 for each experiment: with a Tukey's Multiple Comparisons Test (repeated measures two-way ANOVA).

5.8 Reactive Oxygen and Nitrogen (RONS) levels in DM brains following treatment with low dose vanadium (1µM): Iron chelating effect (DFO: 5µM; WT and 20µM; PINK1).

Dysfunction of the mitochondrial respiratory system is a major source of RONS in the cell. Excessive RONS may induce dysfunction of the UPS resulting in protein aggregation. This explains the aetiology of the two PD neuropathological hallmarks - Lewy bodies formation and neuronal death. The RONS level was measured by investigating the effect of chronic exposure WT and mutant flies to Vd and Fe chelator (DFO). Following this, 2', 7'-Dichlorofluorescein (DCFH) oxidation was measured as an index of oxidative stress.

5.8.1 RONS generation in DM brain: influence of iron chelation (with DFO: 5µM) in WT.

Low dose Vd (1µM) had a beneficial effect on ROS/RONS generation after 14 days in WT flies as evident by the decrease in RONS level in reference to the control group. However, iron chelation, further increased ROS/RONS level. This contrasted with what was seen in the mutant flies.

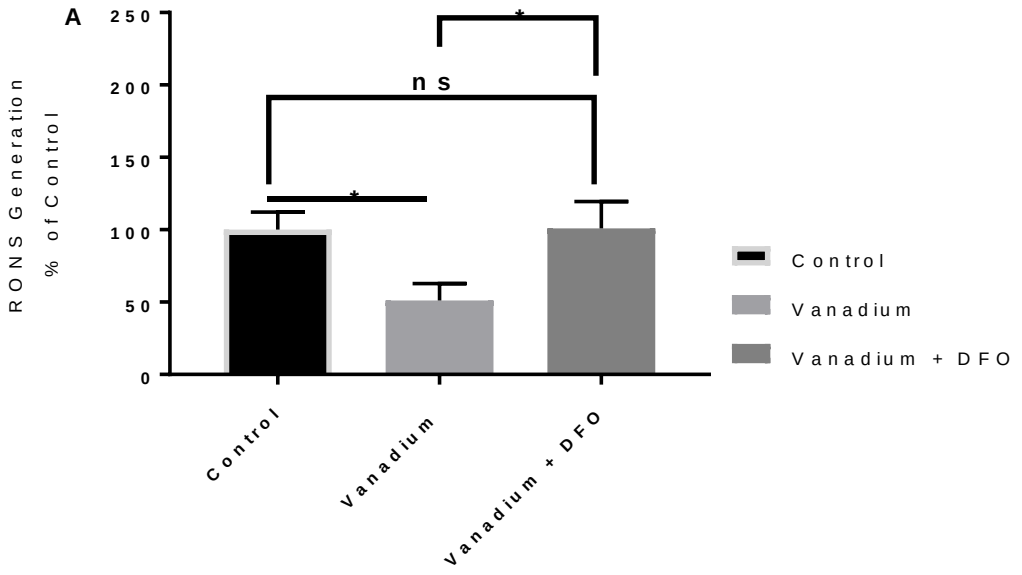


Figure 5.14: RON's generation % of control in WTs flies' brains: iron-chelating effect.

A significant decrease in RONS levels is seen following treatment with Vd after chronic exposure to Vd (1µM) when compared with the control group ($p<0.1$). In contrast, RONS level was not enhanced following iron chelation with DFO (20µM) when compared with the control group ($p>0.05$). All values are means (± SEM) from 2 separate experiments and n=50 for each experiment: with a Tukey's Multiple Comparisons Test (repeated measures two-way ANOVA). ns= $p >0.05$; *=$p<0.1$.

5.8.2 RONS generation in DM brains: influence of iron chelation (with DFO: 20µM) in PINK1

Low dose Vd (1µM) enhanced ROS/RONS generation after 14 days in mutant flies, in reference to the control group. This effect was completely reversed with iron chelation (DFO).

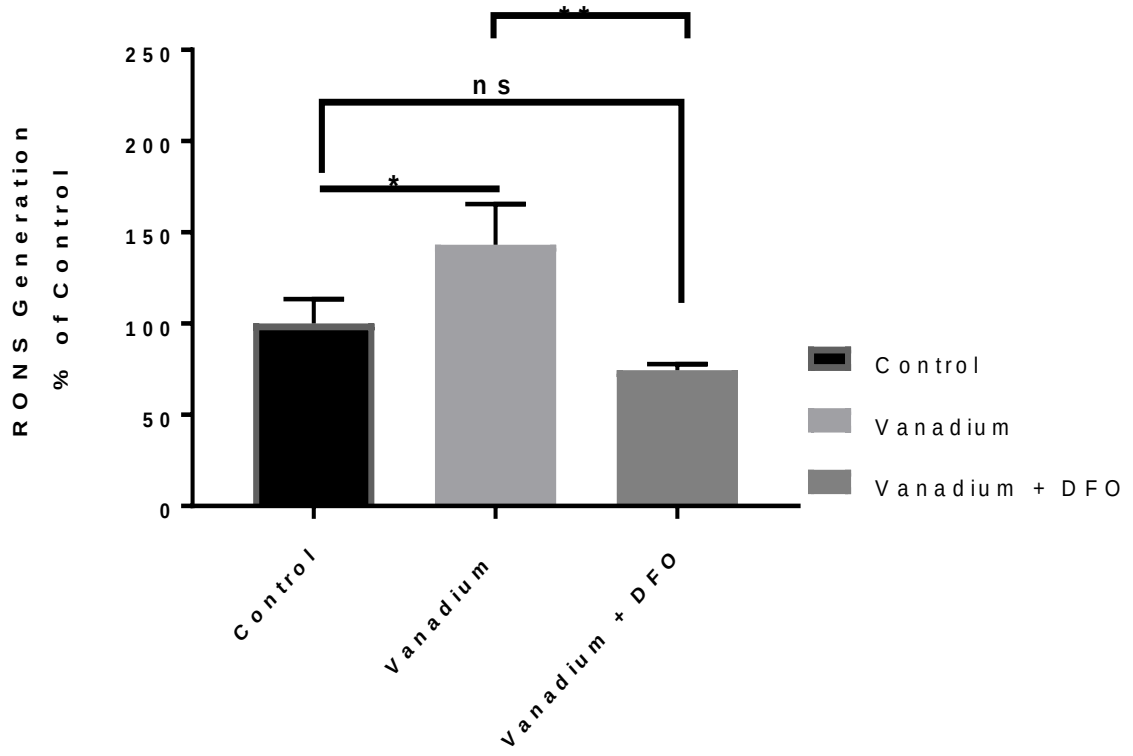


Figure 5.15: RONS generation % of control in PINK1 flies' brains: iron-chelating effect.

A slight, but significant increase, increase in RONS levels is seen following chronic treatment with Vd (1µM); $p<0.1$ and a non-significant decrease in RONS level with iron chelation (DFO; 5µM); $p>0.05$. All values are means (± SEM) from 2 separate experiments and n=50 for each experiment: with a Tukey's Multiple Comparisons Test (repeated measures two-way ANOVA). ns= $p>0.05$; *=$p<0.1$; **$p<0.01$

5.8.3 Total thiol levels

Total thiol (T-SH) levels in fly brains of both the WT and mutant was measured. Thiol groups are highly reactive and susceptible to oxidation that could cause a significant loss of biological activity. In a biological system, the regulation of thiol redox balance is critical for several metabolic signalling and transcriptional process in mammalian cells. The oxidation of free thiol groups (in proteins) produces modification with a structural-functional implication - impact on the protein function (that is, its ability to engage in protein-protein interaction). In the cell, there is a thiol redox buffering system that is in place to protect thiol groups from oxidation and to repair those that have been oxidised due to physiological or aberrant cellular metabolism.

5.8.3.1 Chronic effect of low dose of vanadium (1µM) on total thiol (T-SH) levels in the brains of D. *melanogaster*- WTs and PINK1 mutant.

5.8.3.2 Chronic effect of low dose of vanadium (1µM) on T-SH levels in the brains of WT flies: Influence of iron chelation

Low Vd (1µM) Vd elicited a reduction in T-SH in WT flies. This result is indicative of the beneficial effect of Vd and was consistent with the positive effect on motor activity in WT flies.

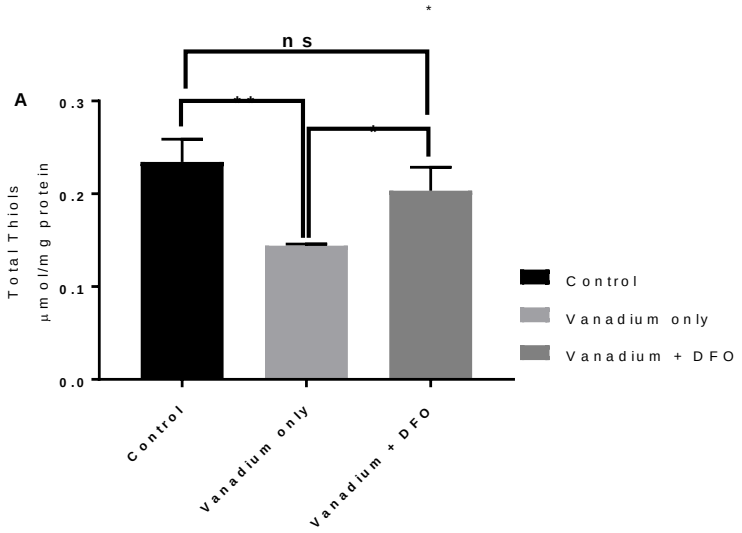


Figure 5.16: T-SH levels in WT flies' brains following chronic exposure to vanadium: iron chelating effect.

A significant decrease in T-SH levels is seen following chronic treatment with Vd (1µM) when compared with the control; $p<0.01$. The decreased T-SH level may be indicative of the positive effect of Vd treatment with WT DM. This ties in with observed positive effects on motor activity following chronic exposures to WT flies. A, non-significant, decrease in T-SH levels following iron chelation (Vd + DFO; 20µM). All values are means (± SEM) from 2 separate experiments and n=50 for each experiment: with a Tukey's Multiple Comparisons Test (repeated measures two-way ANOVA). ns= $p>0.05$; *=$p<0.1$; **$p<0.01$.

5.8.3.3 Chronic Effect of low dose of vanadium (1µM) on T-SH levels in the brains of PINK1 mutant flies: Influence of iron chelation

Low Vd (1µM) increased T-SH levels in WT flies. This result is indicative of exacerbation of the existing motor deficit in the mutant.

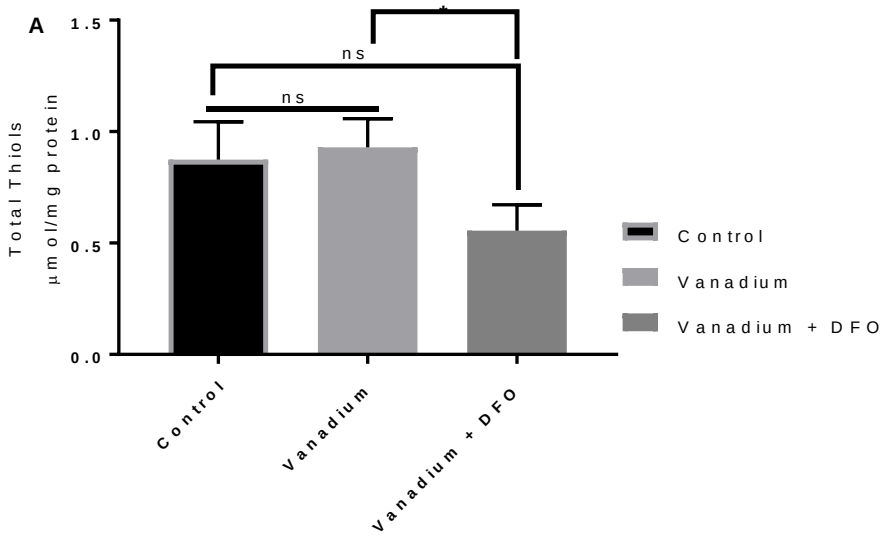


Figure 5.17: T-SH levels in PINK1 flies' brains following chronic exposure to vanadium: iron-chelating effect.

A slight (but non-significant) increase in T-SH levels is seen following chronic treatment with Vd (1µM) when compared with the control; $p>0.05$. A significant increase in T-SH levels following Iron chelation (Vd + DFO; 20µM) can be seen compared with the control group; $p>0.05$. T-SH levels ±DFO & Vd shows some significant difference ($p<0.01$). All values are means (± SEM) from 2 separate experiments and n=50 for each experiment: with a Tukey's Multiple Comparisons Test (repeated measures two-way ANOVA). ns= $p>0.05$; *=$p<0.1$.

5.8.3.4 Effect of low dose of vanadium (1 µM) on T-SH levels- WT vs Mutant: Effect of iron chelation.

Comparing the total thiol (T-SH) levels in the WT and PINK-1 mutant control flies, we observed a surprisingly high T-SH baseline level in the mutant compared to the WT flies. Chronic low dose Vd (1µM) elicited a reduction in T-SH in WT flies, but no significant effect upon PINK-1 mutant flies. Iron chelation (DFO) reversed the effect of Vd on TS-H levels in the WT flies to control levels, but significantly reduced the T-SH levels in PINK-1 mutant flies compared to control and Vd-treated PINK-1 mutant flies.

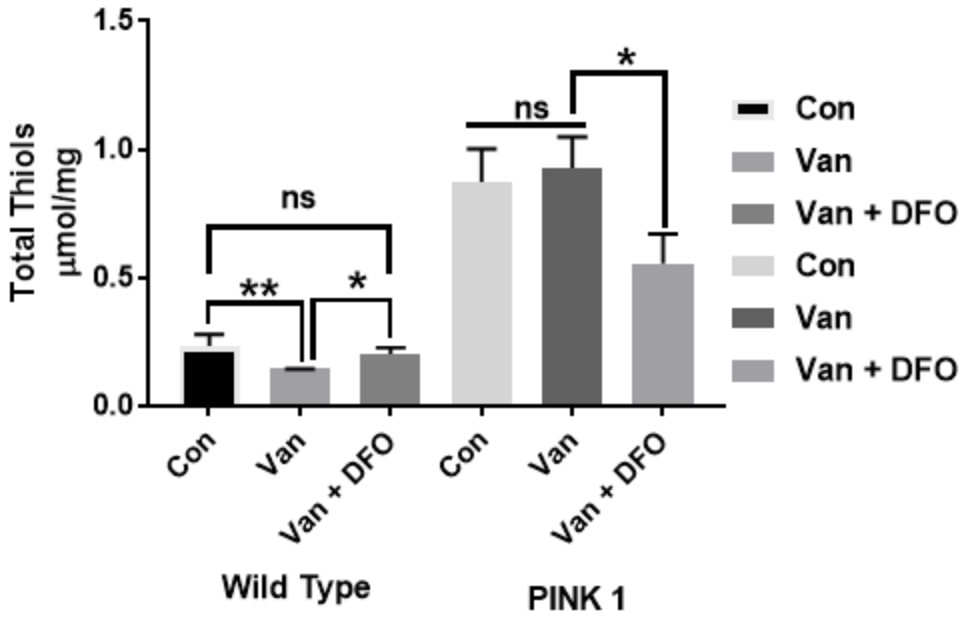


Figure 5.18: The influence of iron chelation following chronic exposure of Drosophila *melanogaster* (WT and PINK mutant) to low dose vanadium upon T-SH levels (after day 14): Influence of iron chelator.

T-SH levels in WT flies is lower than with those seen in PINK1 mutant flies. Vd elicited a reduction in T-SH in WT flies, but no significant effect upon PINK-1 flies. Iron chelation (DFO) reverses the effect of Vd on TSH levels in the WT fly to control levels (*p<0.05), and significantly reduced the T-SH levels in PINK-1 flies (*p <0.05) compared to control and Vd-treated PINK-1 flies. All values are from 5 separate experiments, n=10 flies for each experiment, analysed with a repeated measure two-way ANOVA. *p <0.05.

5.9 Discussion

The *in vivo* effect of chronic exposure of low concentration of Vd to WT and PINK1 mutant flies was investigated. PINK1 mutation causes mitochondrial bioenergetics deficit resulting in energy depletion, shortened lifespan, and degeneration of select indirect flight muscles and dopaminergic neurons (Course, et al., 2018). These outcomes were consistent with results obtained in the control values with PINK1 mutant (See Figs: 5.6; 5.8; 5.10; 5.15; 5.17 and 5.18) that showed that the mutant flies had a motor deficit; evident in the observed decrease in motor activity (Fig.5.2); shortened lifespan compared to WT (Fig.5.3), increase in ROS production (Figs 5.15 and 5.17) as well as an increase in total thiol levels compare to the WT (Fig. 5.12 and 5.16).

Mitochondrial dysfunction, oxidative stress, and alterations in the ubiquitin-proteasomal system (UPS) have been implicated in the death of specific neurons in the etiopathogenesis of PD (Branco, et al., 2010). These three events work in synergy to cause the devastating symptoms associated with PD. Arduino and his colleagues (2010) reported that mitochondrial dysfunction, oxidative stress and/or proteasome system dysfunction potentiated alpha-synuclein aggregation in sporadic models of PD.

Gallacher and Schapira (2009) noted that oxidative stress and free radical generation are linked to PD pathogenesis. In addition to these factors, a plethora of mechanisms are involved, among which include mitochondrial dysfunction, glutamate-mediated excitotoxicity, inflammation, oligodendrocyte interaction, neurotrophic factors, UPS impairment, autophagy and apoptosis are contributive factors underpinning PD pathology.

Oxidative stress is critical because it compromises the integrity of vulnerable neurons. Hence, it is an important player in neuronal degeneration. The source of oxidative stress has been attributed to mitochondrial dysfunction, impairment of antioxidant defence pathways, a highly oxidative intracellular environment within dopaminergic neurons, increased dopamine metabolism which leads to an excessive increase in hydrogen peroxide and other ROS and increased reactive iron (Arduino, et al., 2009; Medeiros, et al., 2016).

Reactive iron has gained notoriety in its contribution to ROS generation (Bondy and Kirstein, 1996; Bondy, et al., 1998; Wu, et al., 2012; Zhang, et al., 2018; Yang, et al., 2019). The presence of reactive iron has been shown to exacerbate Vd toxicity in oligodendrocyte progenitor cells (Todorich, et al., 2011). Thus, exposure of D. *melanogaster mutant* to chronic low dose Vd, exacerbated the motor deficits seen in the mutant (Fig.5.2) causing a further reduction in lifespan (Fig. 5.4) compared to the control values.

Findings from this present study showed that low dose Vd significantly increased RONS generation in mutant cells, with a possible deleterious effect on neuronal cell functionality. Increased RONS level implies disruption in antioxidant balance and redox status, evidenced by an increase in DCFH oxidation and an increase in the total thiol content (See Fig.5.17) in the mutant flies exposed to low dose Vd. Total thiol (T-SH) is a composite (but major) part of the total body antioxidants and they play significant roles in the defence against ROS.

Decreased levels of thiol have been reported in several disorders, such as chronic renal failure (Hultberg, et al., 1995; Himmelfarb, et al., 2000), cardiovascular disorders (Ashfaq, et al., 2006), stroke and neurodegenerative diseases (Mungli, et al., 2009; Prakash, et al., 2009). The observed increased in thiol levels, in our investigations, in the mutant flies (Fig. 5.17) may be an adaptive response. In oxidative stress, T-SH level is increased, the physiological response will be to synthesize GSH (an antioxidant) to combat the thiols levels, which will invariably lead to further increase in thiol levels.

In PD, oxidative stress has been associated with the activation of N-methyl-D-aspartate (NMDA)-type glutamate receptors, with increased production of reactive nitrogen species (RNS). The increased RNS is due to over activation of neuronal NO synthase (nNOS) (Garthwaite, et al., 1988; Bredt, et al., 1991). Mitochondrial defects generate ROS (superoxide anion (O^{2-}). This rapidly reacts with free radical (NO) and in turn creates peroxynitrite radical ($ONOO^{-}$) (Beckman, et al., 1990; Lipton, et al., 1993).

Nakamura and Lipton (2009) noted that the excess RONS formation by these two processes induce UPS impairment and/or misfolding of molecular chaperons. This interphase (mitochondrial-UPS) provides another point of intersection between the mitochondria and the UPS functioning as an underlying mechanism in PD pathology. It is reported that mitochondria dysfunction and production of excessive ROS, adversely affect UPS activity (Nakamura and Lipton, 2009).

Whilst, chronic exposure of mutant flies to low dose Vd worsens the motor deficit and decreased lifespan, in contrast, chronic exposure of WT D. *melanogaster mutant* to low dose Vd improved motor activity (Fig.5.1), indicating a beneficial health effect. Besides, improved lifespan effect was noted when compared with the control (Fig. 5.3). Vd reduced RONS generation in WT. This beneficial effect on WT was consistent with the effect of Vd on motor activity and lifespan in WT.

Furthermore, low dose Vd significantly reduced the total thiol level in WT flies. The observed beneficial motor effect was attenuated by DFO (Figs 5.1; .5.12). Besides, the decreased RONS generation in WT was reversed in the presence of DFO back to control level (Fig. 5.14) suggesting that iron chelation is preventing the beneficial effect of Vd or interacting with Vd to modulate the beneficial effects seen in WT flies. This finding is consistent with previous findings in culture with CAD cells (Chapter 3).

We also evaluated the effects of chronic exposure of low dose Vd on total thiols content in the brains of flies. Total thiols (protein and non-protein thiols) are important targets of RONS and useful biochemical indicators of oxidative stress (LoPachin and Barber, 2006; 2007). Membranes are also susceptible to free radicals resulting in lipid peroxidation and decreased concentration of –SH groups in membrane proteins Staroń, et al (2012).

In our investigation, the levels of T-SH groups were significantly reduced in WT flies, but slightly (but insignificant) increased in the mutant (Fig.5.17) flies, following chronic exposure to low dose Vd. The potential increase seen in mutant flies may be due to increased free radicals/RONS seen in the mutant. Comparing the levels of T-SH in both WT and mutant (Fig. 5.18). It is evident that the oxidative stress in the mutant is much higher compared with

the WT flies (Fig.5.18). The modest increase in total thiol level following chronic exposure to Vd was reversed by DFO in mutant flies.

It has been established that Fe chelation has a protective effect against oxidative-induced neurodegeneration. Fe chelation blocks/reverses the motor deficit of Vd as seen in mutant flies (Fig. 5.10). Crichton and Ward (2006) noted that redox-active metals (such as Fe, Cu, and Mn) play key roles in the nervous system. As metalloenzymes, they are extremely important in key metabolic pathways within the nervous tissue. For instance, Fe is an integral part of the enzyme Tyrosine hydroxylase, and crucial in the formation of dopa from tyrosine. However, if it is present in excess within localised regions of the CNS, it is often dangerous and associated with neurodegeneration (Folarin, et al., 2019).

Chelation therapy has been highlighted as a step to limit this deleterious effect. The reversal effect of DFO on the motor deficit seen in the mutant (following chronic exposure to Vd), therefore, may be an index of the probable benefit of iron chelation as a therapy in PD. Also, the mutant flies were protected against increased RONS production by DFO (Fig. 5.15). This is again suggestive of the probable beneficial effect of Fe chelation therapy in PD.

As previously stated, exposure to environmental toxicants generate reactive oxygen and nitrogen species (RONS), which often results in oxidative damage to cellular components (Schulz, et al., 2014; Liguori, et al., 2018; Madkour, et al., 2020). The results from this investigation indicated that exposure to chronic low dose Vd induces RONS-mediated antioxidant imbalance in flies' brains. Though ROS are vital in several biochemical processes, their overproduction can induce oxidative damage to cellular biomolecules such as DNA, carbohydrate and lipids leading to lipid peroxidation (Bennett, et al., 2001).

It is suggested that Vd exerts its effects at the level of the mitochondrial by inducing the generation of oxidative stress, through the Fenton-like reaction (Fatola, et al., 2019). The ROS data suggests that Fe and Vd are interacting at the level of the mitochondria. It has been reported that Vd can disrupt iron homeostasis and cause depletion of iron from intracellular stores, which underpins its role in the generation of reactive oxygen species and eventual cytotoxicity (Ghio, et al., 2015; Fatola, et al., 2019).

Ghio, et al (2015) reported that Vd is capable of inducing iron deficiency and increasing the expression of DMT-1 thereby precipitating the production of oxidants such as superoxide. They noted that the resultant superoxide is the attempt of the cell to restore the intracellular level of functional iron via ferri-reductase activity (Ghio, et al., 2003).

Liu, et al (2009) demonstrated in cell culture systems, where they induced loss of PINK1, that there is an existing relationship between mitochondrial dysfunction and proteasomal impairment. Furthermore, they noted that proteasomal function impairment is due to a reduction of mitochondrial-dependent ATP synthesis (Liu, et al., 2009), claiming that mitochondrial compromise is the primary event in mitochondria dysfunction. A consequence of this is alpha-synuclein aggregation. The resulting alpha-synuclein aggregates, in turn, can reduce significantly the proteasomal activity. Thus, resulting in a vicious cycle. Vd's ability to exacerbate the existing deficits in PINK1 model of D. *Melanogaster* flies underscores the fact that Vd exerts its influence at the level of the mitochondria and its modulation of Fe homeostasis further highlights this.

In conclusion, substantial evidence indicates that environmental toxicants can induce oxidative stress by altering cellular redox balance, and the activities of antioxidant enzymes such as GST, leading to excessive production of free radicals (Franco, et al., 2009). In turn, free radicals can damage lipids, proteins, carbohydrates, and nucleic acids, thereby, inhibiting their normal functions (Davies and Goldberg, 2000). It has been recently reported that the induction of lipid peroxidation in the brain following Vd administration underscores the role oxidative stress plays in the neurotoxicity of the metal (Folarin, et al., 2018; Igado, et al., 2011; García, et al., 2004).

Overall, strong evidence is provided that low dose Vd exacerbated the existing motor deficit in D. *melanogaster* mutant, reduced their lifespan, increased ROS generation total thiol levels. However, iron chelation reversed this motor deficit in mutant flies, which is indicative of a probable beneficial effect of iron chelation as a therapy in patients with familial PD. In contrast, chronic exposure of WT flies to low dose Vd caused an enhancement in motor activity that was attenuated by DFO, improved lifespan, and a reduction in the generation of RONS. The observed increase in total thiol levels was reversed by DFO.

Chapter 6: Overall Summary and Conclusions

This thesis was designed to address the following hypothesis:

Sub-toxic doses of heavy metals (Cu and vanadium) will differentially modulate downstream signalling, cytoarchitecture, and neuroplastic events, through mitochondrial oxidative stress pathways in healthy and PD neurons.

For many years, heavy metals have been implicated in the aetiology of brain diseases, including PD. Most studies to date have focussed on the toxicological effects of heavy metals. This study was designed to investigate the sub-toxic pharmacological effects of an endogenous (Cu, iron) and exogenous (Vd) upon neurons using an *in vitro* and *in vivo* approach.

6.1 Chapter 3: *In-vitro* effect of sub-toxic concentration of Cu relevance to PD

Evidence is provided in that sub-toxic Cu have a range of pharmacological effects upon monoaminergic neurons, including reduced mitochondrial functionality in both differentiating and differentiated dopaminergic neuronal cells, bi-directional modulation Ca2+ signalling in neurons and depolarisation-induced intracellular calcium levels, a significant reduction in the expression of MAP2, and an increased sensitivity of both immature and mature neurons in the presence of Fe chelators.

6.1.1 Metabolic functions

With respect to metabolic function, 10µM Cu had no effect on mitochondrial viability for both acute and chronic exposures. In contrast, whilst 40µM had no significant effect upon undifferentiated cells, it reduced mitochondrial viability for chronic differentiating and acutely treated differentiated cells. At both 10µM and 40µM, there was a suggestion of neuroprotection in undifferentiated CAD cells. The protective effect of this sub-toxic dose is suggestive of the physiological role of Cu (with this dose) in biological systems, a fine-tuned Cu homeostasis is essential in brain development. Cu deficiency or excess results in severe neuropathologies.

The mitochondria contain two notable Cu-dependent enzymes (cytochrome c oxidase and superoxide dismutase 1) that require adequate Cu supply (Zischka and Einer, 2018). The observed positive benefit is suggestive that this dose is within physiological and optimum required levels. Strong evidence is provided that the intracellular redox ability of Cu, as a co-factor of mitochondrial cytochrome c oxidase as well as the detoxifying capacity of Cu/Zn superoxide dismutase (SOD1) against the reactive oxygen species (ROS) underpins the role of Cu in these enzymes to manage the biochemical challenge and a safe Cu-mediated reduction of oxygen and ROS respectively (Rae, et al., 1999). This function is made possible by Cu chaperones that enhances the activity of the Cu –dependent enzymes in the mitochondria. Although the dominant risk factor associated with neurodegenerative diseases is increasing age, several animal and human studies have reported a rise in the levels of brain Cu (Barnham and Bush 2008). However, Barnham and Bush (2008) reported that from middle age onwards, Cu levels drop markedly, with a consequence loss of Cu-dependent enzyme activities- cytochrome c oxidase, superoxide dismutase I and ceruloplasmin.

The critical metabolic functions of Cu probably explain why the sub-toxic dose did not affect mitochondrial viability. It sub-serves a key cellular function. It has, however, been noted that the mitochondria can accumulate high Cu amounts before they ultimately break down (Zischka, et al., 2011; Lichtmannegger, et al., 2016; Borchard, et al., 2018). This explains why acute exposure of Cu (10µM and 40µM) to undifferentiated cells were seen to be neuroprotective as against chronic exposure (differentiating cells).

6.1.2 Signalling

As a trace element, Cu is essential for neurotransmission (Zischka and Einer, 2018).
On signalling, treatment with low dose Cu (40µM) showed a bidirectional modulation of calcium levels in neuronal processes (inhibitory effect) and cell body (an elevation of calcium signal) in differentiated cells. This has implications for synaptic plasticity and excitotoxicity. The inhibitory effect suggests that calcium ions which are vital in neurotransmitter release are blocked. These conclusions correlate well with studies that showed that Cu levels are distributed diffusely in neurons, but higher in the processes relative to other metal ions, such as zinc (Colvin, et al., 2015). Notably, Hatori, et al (2016) noted an increase in cellular demand for Cu during neuronal differentiation.

6.1.3 Cell Maturation

In terms of neuronal cell maturation, Cu had a significant effect on MAP-2 expression in differentiated CAD cells. Sub-toxic Cu negatively modulates differentiation by selectively reducing MAP-2a isoform, with no significant effect upon MAP-2b isoform, for both 10µM and 40µM concentrations of Cu. Since MAP-2 is associated with microtubule assembly, cargo trafficking plays a vital role in determining as well as stabilising the shapes of dendrites during neuronal development. The observed differential decreased expression of MAP2a in differentiated cells in response to Cu (40µM) may relate to the observed differential modulation of overall metabolic activity and calcium signalling in the neuronal processes, as well as the apparent suppression of depolarisation induced calcium in the processes (but not in the cell body). This may again have implications for synaptic plasticity and neurotoxicity. Recent evidence suggests that PD pathogenesis might be underscored by early cytoskeleton dysfunction (Pellegrini, et al., 2017).

6.1.4 Chapter 4: *In-vitro* effect of low and oxidative stress doses of Vanadium and the influence of iron with relevance to PD.

In comparison to Cu, sub-toxic Vd (an exogenous heavy metal) also to have a range of pharmacological effects upon immature and mature neurons. There was resistance in terms of neuronal metabolic status to acute exposure to applied Vd at different stages of cell culture development, downregulation of differentiation markers with low dose Vd exposure (10µM), but an up-regulation with oxidative stress Vd dose (100µM), together with morphological changes, cellular stress indicators (nuclear shape) and reduced process excitability.

Notably, in contrast to Cu, a neuroprotective effect against Vd-induced metabolic viability following chronic exposure in the presence of iron-chelators was observed. The levels of Fe in undifferentiated and differentiated cells correlated with sensitivity to Vd, with levels being significantly higher in undifferentiated cells in comparison to differentiated cells. Both natural and synthetic iron chelator significantly and efficiently protected against chronic Vd-induced mitochondrial stress, which favours the case for iron chelation therapy. This was in sharp contrast to results obtained with Cu, where iron chelation exacerbates toxicity

6.1.5 Signalling

In terms of cell signalling, sub-toxic Vd dose (10µM) had no clear effect on the intracellular calcium levels in cell bodies. However, an oxidative stress dose (100µM) caused a modest rapid inhibitory spike followed by a protracted inhibition.

6.1.6 Cell Maturation and Differentiation

Despite being sub-toxic doses, both 10µM, as well as 100µM Vd, had effects on differentiation. Acute exposure of differentiated CAD cells to 10µM resulted in up-regulation of MAP2c and 2d (though not statistically significant). However, an oxidative stress sub-toxic dose (100µM) caused a marked decrease in MAP2c expression. Evidence suggests that the low molecular weight MAP2c are expressed prenatally, in developing neurons. While the high molecular weight isoforms (MAP2a & MAP2b) are specified in the cell bodies and dendrites of matured neurons (Melkova, et al., 2019).

The expression of low molecular weight (MAP2c) is indicative of the non-toxic effect of this dose (10µM), whilst the absence of MAP2a and 2b is suggestive of a possible disruption of MAPs and development of the neuronal cytoskeleton. The expression of MAP2c in cells exposed to sub-toxic dose is suggestive that this dose did not affect the differentiation process/neurite formation. It was demonstrated in Chapter 3, that 10µM Vd did not affect neurite formation, in contrast to the oxidative stress dose (100µM), which showed a marked loss of processes following chronic treatment in differentiating cells, and a moderate loss in acute exposures to differentiated cells when compared with sham treatments.

6.1.7 Cellular and ER Stress Response

In terms of cellular ER stress responses, chronic exposure (differentiating cells) to 10µM Vd displayed a very moderate decrease in PDI expression, but an up-regulation of PDI signal with an oxidative stress dose (100µM). In ER stress, PDI is upregulated to attenuate the accumulation of misfolded proteins and decrease ER stress. However, S-nitrosylation compromises this stress response, due to post-translation modification. The presence of double protein bands in PDI expression in both acute and chronic exposures to oxidative stress dose is indicative of post-translational modifications of PDI.

It has been reported that PDI shows S-nitrosylation as post-translational modifications in patients suffering from neurodegenerative diseases (Benhar, Forrester, & Stamler, 2006; Uehara, et al., 2006; Wu, et al., 2014). Protein misfolding (an indication of ER stress) is associated with dendritic spine loss. There is evidence in our findings with immunofluorescence result of MAP2, where oxidative stress dose (100uM) caused a significant loss of processes in differentiating cells undergoing chronic exposure to Vd. Although PDI can be upregulated to attenuate the accumulation of misfolded proteins and decrease ER stress, S-nitrosylation post-translation modification compromises this stress response.

6.2 Chapter 5: Interplay between iron and vanadium in PD in an *in vivo* model

6.1.8 Behavioural and Biochemical effects- RONS and T-SH levels

Sub-toxic Vd (1μM) exposure had a range of differential behavioural and biochemical effects on wild-type and PD PINK-1 *Drosophila* fly models. It exacerbated the existing motor deficit in PINK-1 flies, concomitantly increased RONS production, augmented total thiol levels and abrogated lifespan. This is in contrast to the wild-type flies. The observed significant increase in RONS levels in PINK-1 fly's brains was, despite a slight increase in T-SH content, might be an adaptive response to a low dose of Vd. Indeed, the adaptive response is seen in organisms exposed to low to moderate stress levels (Santos, et al., 2018). Notably, iron chelation with DFO (Deferoxamine) hampered RONS production and reversed motor deficit in the mutant flies.

6.1.9 Behavioural effects/Oxidative stress markers: influence of Iron chelation

Chronic exposure of wild-type flies to low dose Vd caused an improvement in motor activity, reduction in RONS generation and extension of lifespan. Nonetheless, the observed beneficial motor effect (in wild-type) and increased T-SH content was attenuated by DFO, suggesting that chelation of iron ion interferes with the beneficial effect of Vd or possibly interacted with Vd, modulating the effects seen in wild-type flies.

Iron regulation is crucial in the etiopathogenesis of PD. Early iron deficiency, in humans, has been shown to cause impairment of dopamine (DA) metabolism, as well as DA clearance,

transporter density, and dopamine receptor (D1 and D2) densities (Mohamed, 2015). Iron deficiency has been associated with delayed motor development (Lozoff, et al., 1991; Lozoff, et al., 2000) and impaired physical growth (Gambling, et al., 2004; Shahbazi, 2009). Iron chelation in the wild-type flies may have reduced the bioavailability of free iron required for optimum physiological functions. It has been posited that early life deficiency of iron results in impaired cognition, neurodegenerative diseases, and specific movement disorders such as PD (Powers, et al., 2003) and restless leg syndrome (Earley, et al., 2000).

6.1.10 T-SH levels and Iron Chelation

It was interesting to note that there was a significant differential in T-SH levels in both WT and mutant flies – and this was quite striking. This is adduced to the notable increase in oxidative stress observed in mutant compared to the wild-type flies. An important implication arising from this thesis is the case for iron chelation as a therapeutic strategy. This was indicated (by the outcome of our findings) in the reversal of the increase in total thiol level seen in the mutant flies in the presence of DFO. The Fe chelation blocks/reverses the motor deficit of Vd as seen in mutant flies - an indication that there may be high Fe content in mutant flies or deregulation of Fe homeostasis. Thus, the beneficial effect was seen with iron chelation.

Further studies may be required to establish the basal Fe levels in both wild-type and PINK1 mutant flies. Vd is capable of disrupting iron homeostasis and causing the depletion of iron from intracellular stores, which has been associated with Vd role in the generation of reactive oxygen species (Fatola, et al., 2019).

Chapter 7: General Conclusions and Future Directions

7.1 General Conclusions

Strong evidence arising from this thesis is the neuroprotective effect of Cu on mitochondria functionality with low dose Cu (10μM) on undifferentiated CAD cells. Same concentration did not affect mitochondrial functionality in both acute and chronic exposure to differentiated and differentiating CAD cells. In terms of neuronal cell maturation, low dose Cu caused a decrease in MAP2a protein expression. Also, there was no elevation in calcium signalling with a low dose of Cu.

Overall, strong evidence is provided that low dose Vd exacerbated the existing motor deficit in D. *melanogaster* mutant, reduced their lifespan, increased ROS generation total thiol levels. However, iron chelation reversed this motor deficit in mutant flies. This is indicative of a beneficial effect of iron chelation - as a therapy in patients with familial PD. In contrast, chronic exposure of WT flies to low dose Vd caused an enhancement in motor activity (which was attenuated by DFO), improved lifespan, and a reduction in the generation of RONS. The observed increase in total thiol levels was reversed by DFO.

7.2 Limitations

The consequence of oxidative stress in the brain is neuronal cell death and thus, dopamine depletion. The research design was originally set out to establish dopamine levels in the fly brain. However, due to time constraint and funding limitation, these sets of the experiment could not be completed. The role of Cu and Vd on Tyrosine Hydroxylase activity would have been useful to explain the role of these metals on dopamine levels. For similar reasons, these were not done. In addition to the qualitative investigations of the morphology of CAD cells from the IF data, it would have been useful to quantify the data from the IF experiment.

7.3 Future Directions

1. It has been noted that Vd is capable of disrupting iron homeostasis and cause the depletion of iron from intracellular stores. This has been associated with Vd role in the generation of reactive oxygen species (Fatola, et al., 2019). Further studies with

DM as a model may require a need to establish the basal Fe levels in both wild-type and PINK1 mutant flies.

2. Accumulation of iron in the brain is an event related to PD. However, the proportion in which Cu or Cu proteins are responsible for iron dyshomeostasis in PD is unknown.
    a. It will be worthwhile to consider the role of Cu transporters in PD. It has been established that alpha-synuclein binds to Cu and acts as ferrireductase. This invariably increases the availability of iron for the generation of free radical, with deleterious implications in the brain, especially in the putamen and caudate regions, due to the presence oxidative labile dopamine (Dusek, et al., 2015; Guiney, et al., 2017; Patel, 2018).
    b. The activity of ceruloplasmin (Cu-dependent ferroxidase) has been continuously reported to be reduced in samples from PD patients. Theoretically, the decreased function of ceruloplasmin would aggravate the above-mentioned situation of alpha-synuclein. Hence, the level of ceruloplasmin in the PD model fly versus WT as well as in animal models of PD should further be explored.
    c. Knowledge about Cu compartmentalisation in the brain will help to establish promising therapeutic strategies aimed at enhancing the positive role of this metal in Parkinson's Disease.
3. The outcome of oxidative stress in the brain is neuronal cell death and hence, dopamine depletion, with a consequence of dopaminergic neuronal degeneration. It would be useful to explore the effect of Vd on brain dopamine levels in
    a. Mutant and WT fly under acute and chronic states exposures to low dose (sub-toxic), oxidative stress dose and toxic doses of Vd.
4. Biochemical analysis of PD samples reveals a systemic defect of complex I. Exposure to complex I inhibitor (rotenone) results in pathological, biochemical, and behavioural idiosyncratic features of PD. This mechanistic pathway (rotenone-induced toxicity) is suggestive of oxidative stress pathway, due to the blocking of the respiratory chain and the selective destruction of dopaminergic neurons. It would be notable to explore the effect of low dose, oxidative stress, and high/toxic doses of Vd on complex I activity in the presence and absence of iron (using rotenone as a positive control).
5. Besides, the disorder in Complex I causes aggregation of α-synuclein and this contributes to the demise of dopaminergic neurons. Other mitochondria complexes (II

and IV) are also implicated. For example, the production of Nitric oxide (NO) - either from the mitochondria or inducible NOS within the cell cytosol - inhibits components of the mitochondria respiratory chain, complexes I, II and IV (precisely in a reduced state of GSH), which cause a cellular energy-deficient state. It will be worthwhile to investigate the effect of sub-toxic, oxidative stress dose and toxic doses of Vd on Complexes II and IV as well α-synuclein expression.

6. Future studies may seek to understand the beneficial effect of antioxidants and molecular chaperone (Heat shock proteins) that are induced in response to environmental, physical, and chemical stresses following exposure to sub-toxic, oxidative stress and toxic doses of Vd.

Summary Fig and pathways of Vanadium and Cu mode of action

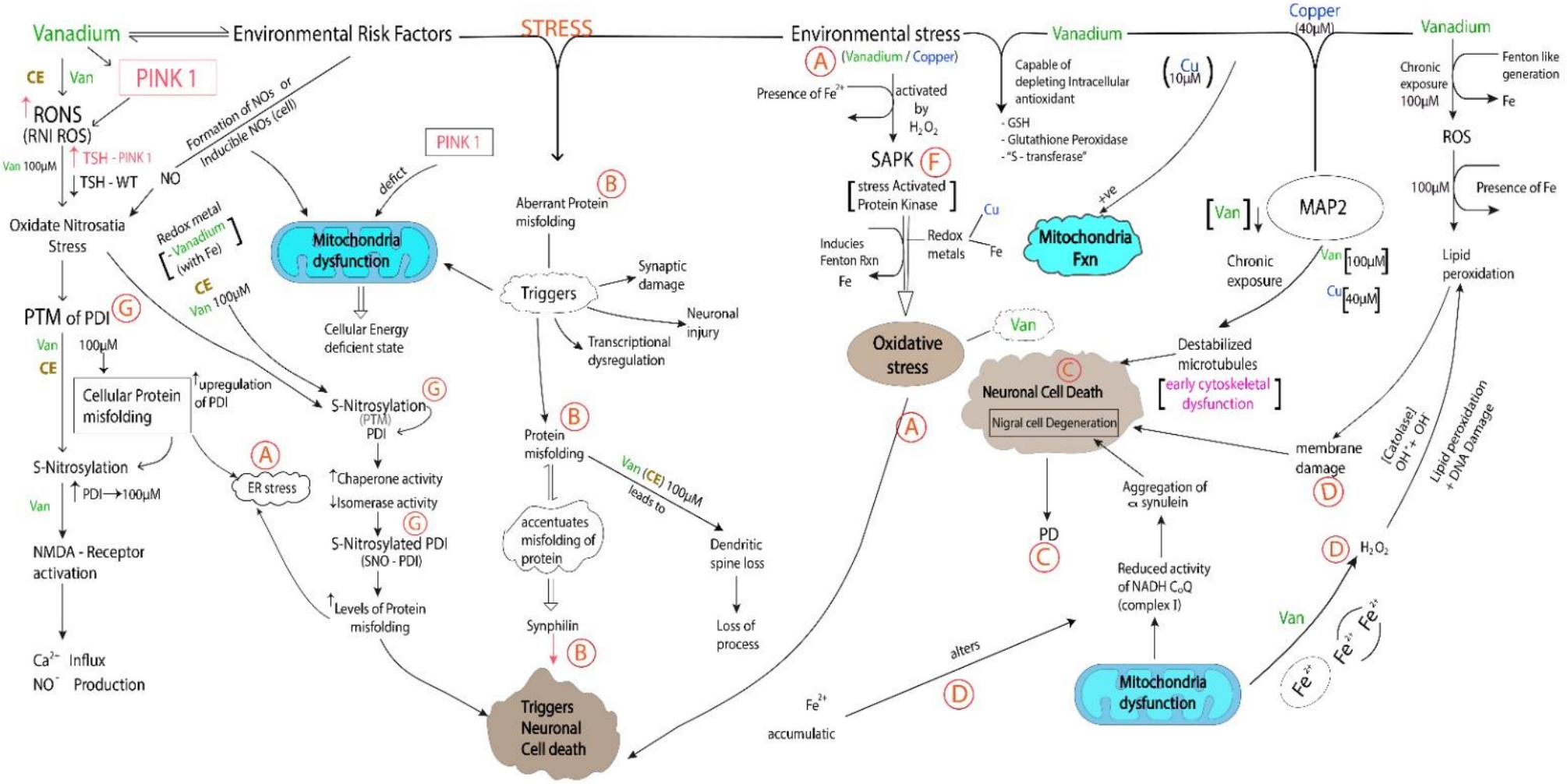


Figure 7.0.1: Summary Fig and pathways of Vanadium/ Cu mode of action

7.4 Summary Fig.

KEY: ER – Endoplasmic Reticulum; PTM- Post Translational Modification; CE- Chronic Exposure; T-SH – Total thiol; PDI- Protein Disulphide Isomerase; RONS- Reactive Oxygen & Nitrogen Specie; ROS- Reactive Oxygen Species; WT- WT DM; PINK1 – Mutant DM; Fe – Iron; Ca^{2+} - Calcium ions; NO – Nitric Oxide; Cu – Cu, Van – Vanadium; MAP2; Microtubule Associated Protein; SAPK – Stress Activated Protein Kinase; GSH- Glutathione;

Sub-toxic doses of Vd (10uM) and Cu (10uM) had a positive effect on mitochondrial functionality under acute exposure. 10µM Vd displayed a very moderate decrease in PDI expression and less sensitivity of differentiated cells to both acute and chronic exposure.

In the condition of ER stress, the Apoptosis pathway is induced because PDI is unable to maintain cellular homeostasis (A).

(This evident in chronic exposure to oxidative stress dose of Vd (100uM), by the up-regulation of the expression of PDI (under both reducing and non-reducing conditions). In sub-toxic dose, however, this was not apparent).

Oxidative stress results in the accumulation of insoluble aggregates of misfolded protein in neurons (B).

Ultimately leading to a decrease in neuronal cell viability and cell death (neurodegeneration) (C).

Cumulative evidence supports oxidative stress “hypothesis” for initiation of nigral dopamine neuron loss - apoptosis. Cellular metabolism of dopamine generates H2O2 (D) which is reduced to hydroxyl radicals in the presence of Fe - which promotes neuronal damage (E).

(It was reported in this thesis that Vd toxicity is exacerbated in the presence of iron, which was ameliorated in the presence of both synthetic and natural iron chelator. Also, a previous study (by our partner) reported that lipid peroxidation is induced by Vd in the presence of Vd.

Stress Activated protein Kinase (SAPK) is activated by oxidative stress (F) and propagate stress signals to pathways. [JNK / SAPK & P38 / SAPK - Not Shown]

This study argues that under chronic exposure (to low dose Vd 100uM), oxidative stress pathway way may be activated as evident in this thesis by the increase in PDI expression and post-translation modification of the PDI signal, as well as an increase in RONS, cellular

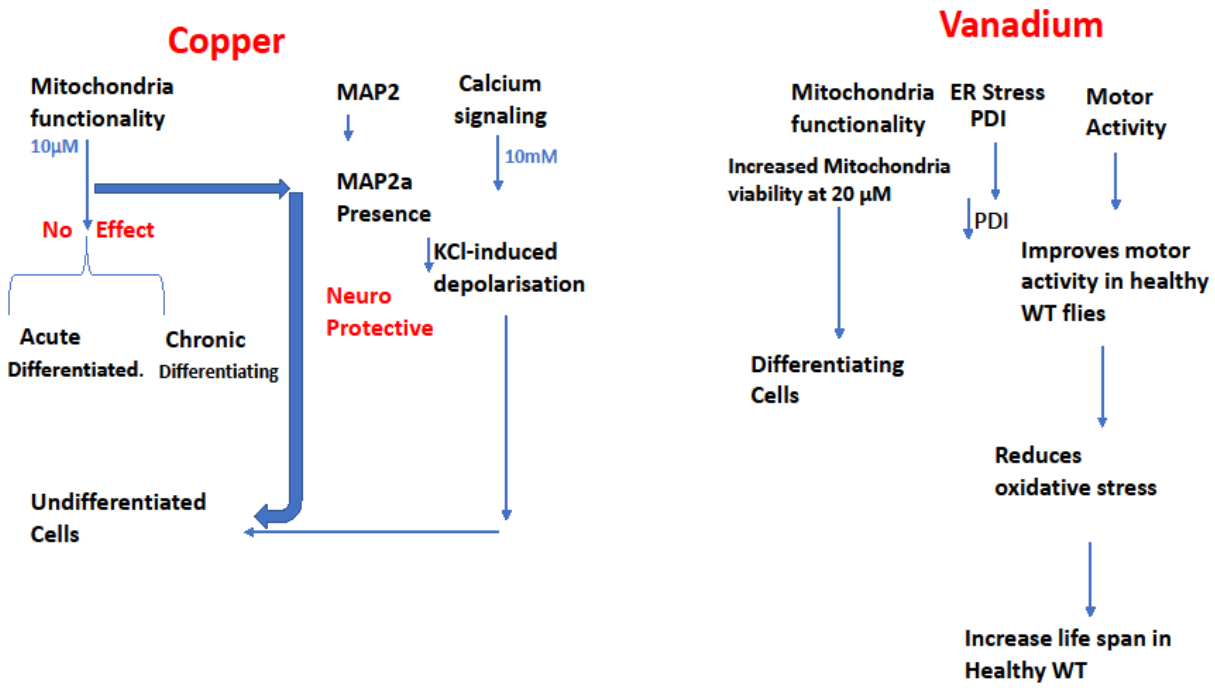
stress indicators (nuclear shape), reduced process excitability, and neuronal cells morphological changes.

Post-translational Modification (PTM) (G) attenuate unfolded protein response (UPR) and cause neuronal cell death. Hence, aberrant modification of PDI leads directly to harmful effect and the loss of the usually protective properties of PDI.

S-nitrosylation compromises this stress response due to post-translation modification. This was evident with low dose Vd in differentiating cells (chronic exposure). An increase RONS, T-SH and PDI expression was apparent in our *in vivo* studies with the mutant fly.

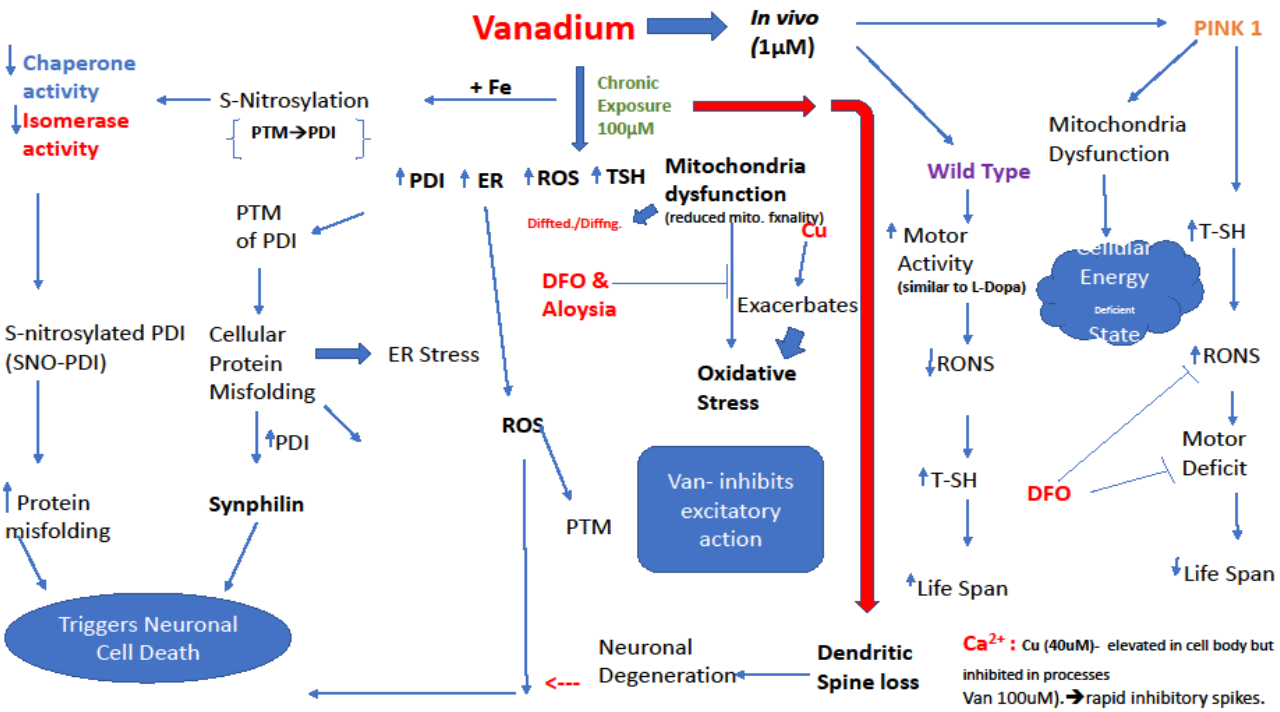
Vd toxicity has been previously reported as a possible link in the pathogenesis of PD, yet a link between chronic low dose Vd and ER stress in PD has so far not yet been reported.

Positive Effects of sub-toxic concentration of heavy metals relevance to PD



Low dose Cu (10µM) had no significant effect upon metabolic rate or cytotoxicity on differentiated and differentiating CAD cells. It had a neuroprotective effect on undifferentiated cells and decreased KCl-induced depolarisation. It positively enhanced the expression of MAP2 in differentiated cells. Low dose of Vd (20 µM) increased mitochondria viability in differentiating cells (following chronic exposure), decreased PDI expression, improved motor activity in, increased survival rate and reduces oxidative stress in WT flies.

Summary pathway of the interplay between iron and vanadium in PD in an *in vivo* model



Summary pathway of the sub-toxic effects of Vd and its interplay with Fe on PD-like phenotypes in CAD cells, and Pink-1^{B9} *Drosophila melanogaster* models of PD, shows that that undifferentiated CAD cells were more susceptible to vanadium exposure than differentiated cells and this susceptibility was modulated by iron. In pink-1 flies, exposure to chronic low dose of vanadium exacerbated the existing motor deficits, reduced survival, and increased the production of reactive oxygen species (ROS), increase PDI expression, ER stress and T-SH; leading to post translational modification (PTM) and ultimately triggers neuronal cell death. Iron chelation (with *Aloysia citrodora* L, a natural iron chelator, and Deferoxamine Mesylate a synthetic iron chelator) significantly protected against the PD-like phenotypes in both WT and mutant flies: which favours the case for iron-chelation therapy as a viable option for the symptomatic treatment of PD.

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Appendix 1 - List of Publications

Peer-reviewed original publications

1. Kennedy, D., Okello, E., Chazot, P., Howes, M.J., Ohiomokhare, S., Jackson, P., Haskell-Ramsay, C., Khan, J., Forster, J. and Wightman, E., 2018. Volatile terpenes and brain function: Investigation of the cognitive and mood effects of mentha× Piperita l. Essential oil with *in vitro* properties relevant to central nervous system function. *Nutrients*, *10*(8), p.1029.

2. Yahiaoui, H., Howes, M.J., Ohiomakhare, S., M'Hamedi, A. and Chazot, P.L., 2017. Algerian Anacyclus pyrethrum aqueous extract: novel antioxidant and neuroprotectant activity of a chemically profiled aqueous extract. *South Asian Journal of Experimental Biology, 7(6), pp.262-270.*

3. Megdad, H., Ohiomokhare, S., M'Hamedi, A. and Chazot, P.L., 2019. Phytochemical and antioxidant properties of Algerian Medicago sativa L. water, ethanol and chloroform extract. *South Asian Journal of Experimental Biology, 9(3), pp.80-86.*

4. Samuel Ohiomokhare1, Francis Olaolorun1,2, Amany Ladagu1,2, Funmilayo Olopade2, Ed Okello3, James Olopade2, Paul L Chazot1. The Pathopharmacological Interplay Between Vanadium and Iron in Parkinson's Disease Models (in press)

Appendix 2 - List of Conferences

Poster communication

1. Ohiomokhare SO, Chazot PL. Sub-toxic pharmacological effects of copper upon CAD monoaminergic neurons in culture - BPS Conference, December 2015. Accepted for publication in 2016 in online $PA_{2.}$

2. Ohiomokhare SO; Chazot PL. Chronic vanadium neurotoxicity: synthetic and natural product iron chelator protection *in vitro; Away research day, Durham January 2016*

3. Ohiomokhare SO; Chazot PL. Endogenous and environmental heavy metals: sub-toxic pharmacological effects upon calcium signalling in cultured monoaminergic neurons – *Wolfson Research Institute of Health and Wellbeing ESR Conference;* June 2016. Published in Conference Programme.

4. S Ohiomokhare, P Chazot - BNA Festival of Neuroscience Chronic exposure to low dose Vanadium exacerbates the motor deficits in the *Drosophila melanogaster* PINK mutant model of PD. A Festival of Neuroscience, Birmingham - April 2017.

5. Ohiomokhare SO. Endogenous and Environmental heavy metals: sub-toxic pharmacological effects upon calcium signalling in cultured monoaminergic neurons. - ESR Conference, Durham University June 2018.

6. Ohiomokhare SO. Vanadium: The good, The bad and the ugly. Parkinson's Society, UK meeting, Newton Aycliffe, Durham, 2018.