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Neuroprotective effects of epigallocatechin gallate in cell culture and animal models of Parkinson's disease

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Neuroprotective effects of epigallocatechin gallate in cell culture and animal models of Parkinson's disease

by

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ABSTRACT

The role of iron has gained attention in Parkinson's disease (PD) because of its complicated interplay with other pathological mechanisms such as oxidative stress and inflammation. Epigallocatechin gallate (EGCG) might be a good candidate for the treatment of PD due to its antioxidant, iron chelating and anti-inflammatory properties. The overall objective of my project is to determine the neuroprotective effects of EGCG in both in vitro and in vivo models of PD and to investigate whether the protective effect is via regulation of iron homeostasis.

In my first project, I investigated the role of hepcidin in 6-hydroxydopamine (6-OHDA)-induced apoptosis in a cell culture model of PD. We down regulated hepcidin using siRNA interference techniques in N27 dopaminergic cells and compared with control siRNA transfected cells to investigate the role of hepcidin in 6-OHDA-induced neurodegeneration. We measured cell viability, cell apoptosis by caspase-3 activity and DNA fragmentation, intracellular free iron, and protein damage. We found hepcidin knockdown protected N27 cells from 6-OHDA-induced neurotoxicity by reducing intracellular free iron, protein oxidation, and decreasing caspase-3 activity and DNA fragmentation.

In the second project, we determined whether EGCG protected from hydrogen peroxide (H_2O_2)- and tumor necrosis factor alpha (TNFα)-induced oxidative stress and inflammation in N27 cells. We found EGCG pretreatment significantly prevented H_2O_2- and TNFα-induced apoptosis by normalizing cell viability and caspase-3 activity. The observed neuroprotection may be through the inhibition of oxidative stress and inflammation, which was possibly mediated by hepcidin and ferroportin.

In my third project, we determined the neurorescue effect of EGCG (25 mg/kg, oral administration) against 1-methyl-4-phenyl-1,2,3,6- tetrahydropyridine (MPTP, 20 mg/kg, IP)-induced neurodegeneration. The neurorescue effect of EGCG was assessed by motor behavior tests, neurotransmitter analysis, oxidative stress indicators, and iron related protein expressions. We found EGCG significantly rescued MPTP-induced neurotoxicity by increasing the rotational latency, increasing dopamine, and reducing serum protein carbonyl concentrations. In addition, the protection of EGCG may have been associated with the regulation of iron efflux protein ferroportin in the substantia nigra.

Overall, my project demonstrated that EGCG has potential therapeutic value for the treatment of PD and the protective effect might be associated with its ability to alter iron regulated proteins, hepcidin and ferroportin and reduce oxidative stress.

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CHAPTER 1 GENERAL INTRODUCTION

Introduction

Parkinson's disease (PD) is a progressive and disabling neurodegenerative disorder afflicting the elderly. It is characterized by a number of motor symptoms such as tremor, rigidity, slow movement, and postural instability and non-motor symptoms including cognition impairment, depression and insomnia.

The medications and therapies including the precursor of dopamine levodopa, the monoamine oxidase B inhibitor, dopamine receptor antagonist, catechol-o-methyl transferase inhibitor can only relieve the symptoms but not able to cure or reverse the progression of the neurodegenerative process (Singh *et al.*, 2007). Moreover, serious side effects of drugs including psychiatric symptoms, cognitive impairment and dyskinesia further limit their use in PD patients (Guridi *et al.*, 2012). Therefore, there is a need to identify the pathogenic mechanisms leading to disease development and find the neuroprotective therapies to slow down or even reverse the progression of PD.

Recent findings indicate that mitochondrion dysfunction, oxidative stress, abnormal protein accumulation, neuroinflammation, excitotoxicity are considered as key molecular mechanisms contributing to the cell death in both sporadic and familial PD (Thomas and Beal, 2007; Dexter and Jenner, 2013). These factors may interact with each other and result in snowball effects triggering or exacerbating the neurodegenerative process. The role of iron has increasingly gained attention in PD due to its complicated crosstalk with other pathological mechanisms including its ability to induce oxidative stress and neuroinflammation, promote protein aggregation, and exacerbate neurodegeneration

(Mounsey and Teismann, 2012). Iron is an essential element in the human body and possesses key physiological functions by participating in the electron transfer trough the oxidation-reduction reactions due to its existence of two oxidation states. It is a component of vital proteins such as hemoglobin, cytochromes and involved in numerous biological processes including oxygen transport, mitochondrion respiration and DNA biosynthesis (MacKenzie *et al.*, 2008). However, excessive iron is deleterious due to the generation of reactive oxygen species (ROS) such as highly reactive hydroxyl radicals via the Fenton reaction and the initiation of DNA oxidation, protein damage and lipid peroxidation (Kalinowski and Richardson, 2005). Consequently, iron uptake, storage, utilization is highly regulated by specialized proteins to prevent the participation of free iron in Fenton reaction-mediated damage. Hepcidin is a small peptide produced mainly in the liver in response to inflammation, iron accumulation and oxidative stress (Ganz, 2005). Hepcidin binds to the cellular iron exporter ferroportin (Fpn) and induces a conformational change and lysosomal degradation, which leads to decreased iron efflux (Myhre *et al.*, 2013). By this mechanism, hepcidin regulates both systematic and intracellular iron metabolism. Recent studies have demonstrated there is a wide distribution of hepcidin and Fpn in the human brain and they are co-localized in neurons and astrocytes, suggesting they have roles in brain iron homeostasis (Wang *et al.*, 2010; Sun *et al.*, 2012; Raha *et al.*, 2013).

Although it is not clear whether iron overload is a cause or an effect of PD, accumulating evidence suggests that the disruption of iron homeostasis leading to nigral iron elevation is an important feature in PD pathogenesis (Ayton and Lei, 2014). Postmortem studies have shown that increased levels of iron deposits in the substantia nigra (SN) and globus pallidus are present in parkinsonian brains (Gotz *et al.*, 2004; Rhodes and Ritz, 2008), which was also

observed in living patients measured via MRI and ultrasound techniques (Kaur and Andersen, 2004). The process of iron accumulation in PD may be caused by the imbalance of iron regulation mechanism and increased reactive free iron pool, such as divalent metal transporter-1 (DMT-1), transferrin receptor (TfR) or lactoferrin receptor-mediated increased iron uptake, ceruloplasmin and Fpn-facilitated decreased iron export, or ferritin or neuromelanin regulated-altered iron storage (Mounsey and Teismann, 2012; Weinreb *et al.*, 2013; Le, 2014). 6-hydroxydopamine (6-OHDA) has been shown to induce neurotoxicity by upregulating DMT-1 and hepcidin and downregulating Fpn which lead to intracellular iron overload conditions in a cell culture model of PD (Chen *et al.*, 2015a)

Based on the correlation between iron dysregulation and PD, use of iron chelators might be a potential treatment for preventing the onset or slowing down the progress of the disease by reducing excessive free iron in the brain. Studies have shown that a number of iron chelators such as clioquinol or deferoxamine (DFO) exert neuroprotection in animal models of PD (Kaur *et al.*, 2003; Fine *et al.*, 2014). However, disadvantages associated with these iron chelators such as its poor ability to cross blood brain barrier and severe adverse effects impedes its further investigation in clinical studies.

Natural iron chelators derived from food and plants have attracted increasing interest because of their low-toxicity over long term use, affordability and general acceptance. The major tea polyphenol epigallocatechin gallate (EGCG) is a potent neuroprotective agent for the treatment of neurodegenerative disorders. The beneficial effects of EGCG may be associated with its antioxidant, iron chelating and anti-inflammatory properties (**Figure 1-1**). In a recent study, consumption of green tea (3 cups/day) for 3 months improved PD patients' antioxidant status (Chen *et al.*, 2015b).

Our long-term goal was to identify the effect of a natural compound with fewer side effects than traditional therapies to slow down the progression of PD. The overall objective of this project was to investigate the neuroprotective effect of EGCG in both in vitro and in vivo models of PD and determine whether the protection is through altering iron related proteins and maintaining intracellular iron homeostasis. *Our central hypothesis was that EGCG exerts neuroprotection in both cell culture and animal models of PD, and the protection is due to its inhibition of oxidative stress and inflammation through the alteration of iron related proteins and maintenance of intracellular iron homeostasis (**Figure 1-1**).*

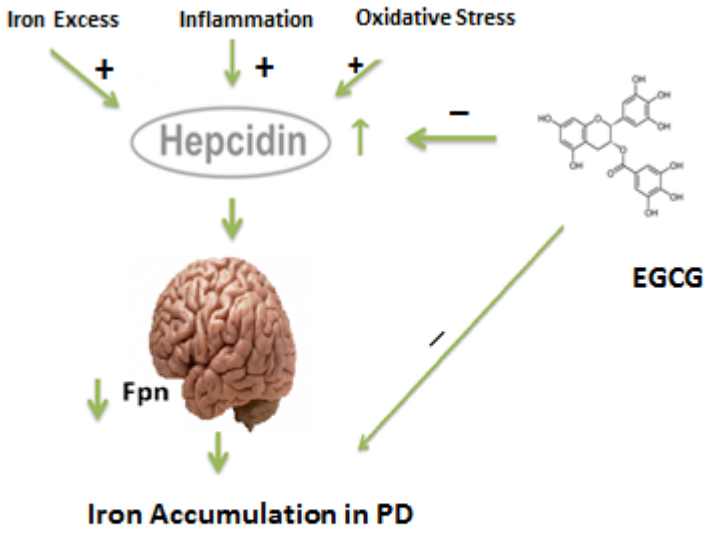

Figure 1-1 Central hypothesis. EGCG: epigallocatechin gallate; Fpn: ferroportin.

Specific aim 1: To determine the role of hepcidin in 6-OHDA-induced cell death by knocking down hepcidin expression in rat dopaminergic neuronal cell line (N27 cells).

Specific aim 2: To determine whether EGCG exerts neuroprotective action against hydrogen peroxide (H_2O_2)- and tumor necrosis factor alpha (TNFα)-induced neurotoxicity through regulating iron related proteins hepcidin and Fpn in N27 cells.

Specific aim 3: To determine the neurorescue effects of EGCG in 1-methyl-4-phenyl-1,2,3,6- tetrahydropyridine (MPTP)-induced PD in animals and to examine the involvement of iron-related proteins in that protective effect.

To address the specific aim 1, we downregulated hepcidin using siRNA interference and evaluated the role of hepcidin in 6-OHDA-induced neurodegeneration in N27 cells. We found that hepcidin knockdown protected N27 cells from 6-OHD-induced apoptosis by possibly regulating iron exporter Fpn and subsequent reducing cellular iron burden and oxidative damage. In the second study, we determined whether EGCG protected N27 cells from H_2O_2- and TNFα-induced neurotoxicity. We found that EGCG protected against both TNFα- and H_2O_2-induced neuronal apoptosis, and that neuroprotection may be through the inhibition of oxidative stress and inflammation, which is possibly mediated by hepcidin and Fpn. In the third study, we assessed the neurorescue effect of EGCG against MPTP-induced neurodegeneration. We found that EGCG restored MPTP-induced functional and neurochemical deficits and the neurorescue effects might be associated with regulating iron exporter Fpn in SN and reducing oxidative stress. All these findings suggest that iron regulatory proteins hepcidin and Fpn play important roles in the pathogenesis of PD and the neuroprotection of EGCG might be through the regulation of these proteins and reduction of brain iron overload and oxidative stress conditions.

Dissertation organization

This dissertation contains five chapters, including a general introduction, three research papers, and a general conclusion. Chapter 1 is a general introduction. Chapter 2 is a literature review relevant to the projects. Chapter 3 is the first manuscript "Hepcidin Plays a Key Role in 6-OHDA-Induced Iron Overload and Apoptotic Cell death in a Cell Culture Model of Parkinson's Disease" submitted to Parkinson's Disease Journal. Chapter 4 is the second manuscript "Epigallocatechin Gallate Protects against tumor necrosis factor alpha- and Hydrogen Peroxide-Induced Apoptosis in a Cell Culture Model of Parkinson's Disease" which will be submitted to the International Journal for Vitamin and Nutrition Research. Chapter 5 is the third manuscript "Neurorescue Effect of Epigallocatechin Gallate in an Animal Model of Parkinson's Disease" which will be submitted to the International Journal of Food Science and Nutrition. Chapter 6 is a general conclusion. Tables, Figures and legends in each chapter are placed at the end of each chapter. The list of references is cited and included at the end of each chapter. Some illustrations in the literature review are made using the template provided by motifolio.com.

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CHAPTER 2 LITERATURE REVIEW

Parkinson's disease

Parkinson's disease (PD) is the second most common neurodegenerative disorder that affects about 1.5% of the global population (Blesa and Przedborski, 2014). There are approximately 1.5 million individuals in the US suffering from PD and this number is expected to rise dramatically in the coming decades due to an increased aging population (Abdullah *et al.*, 2015). Parkinson's disease was first defined by James Parkinson in his paper entitled "Essay on the Shaking Palsy" and described as a neurological syndrome consisting of rest tremor, slowness, shuffling gait, flexed posture, festination, falls, soft speech, dysphagia, and saliva trickling from the mouth (Goetz, 2011; Fahn, 2015). Subsequently, for the next one century and a half, scientists continue to pursue the causes and pathogenesis of the disease, to identify the common symptoms and risk factors, and search for the treatments.

Parkinson's disease today can be characterized by the primary motor symptoms of tremor, rigidity, slowness of voluntary movement, and postural instability (Cronin-Golomb, 2013). These symptoms result from the loss of tyrosine hydroxylase positive neurons in the substantia nigra pars compacta (SNpc) which leads to 70% to 80% of dopamine deficiency in the striatum, where their projections are located (Pickrell *et al.*, 2011). Dopamine is an important catecholamine that controls locomotion, learning, working memory, cognition and emotion by binding to the specific membrane receptors on the neurons (Drozak and Bryla, 2005). The major pathway for dopamine biosynthesis starts from dietary tyrosine. Tyrosine can also be synthesized by hydroxylation of phenylalanine. Tyrosine is then converted to L-3,

4-dihydroxyphenylalanine (L-DOPA) by the rate limiting enzyme tyrosine hydroxylase in dopaminergic neurons and further converted to dopamine by the enzyme aromatic amino acid decarboxylase (AADC) (**Figure 2-1**).

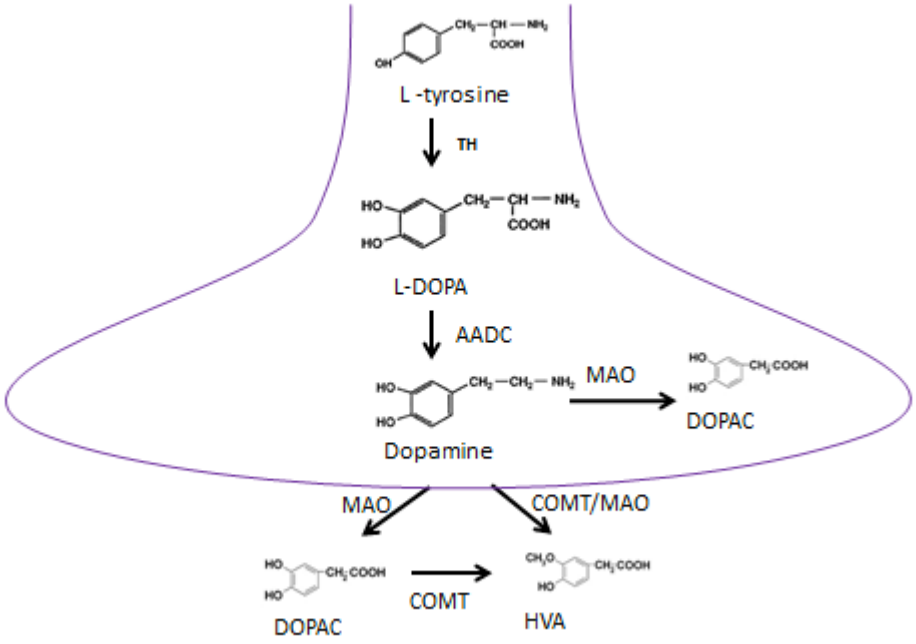

Figure 2-1 Biosynthesis and metabolism of dopamine. TH: tyrosine hydroxylase; AADC: aromatic amino acid decarboxylase; COMT: catechol-o-methyltransferase; MAO: monoamine oxidase; L-DOPA: L-3,4-dihydroxyphenylalanine; DOPAC: 3,4-dihydroxyphenylacetic acid; HVA: homovanillic acid. Figure adapted from (Elsworth and Roth, 1997).

After synthesis, dopamine is stored in specialized storage vesicle in the cytoplasm. It can be released into the synaptic cleft by the mechanism of exocytosis, or taken back into the nerve terminals (Elsworth and Roth, 1997). The major end metabolites of dopamine are acidic metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) or homovanillic acid (HVA) (Elsworth and Roth, 1997). When an excess amount of cytosolic dopamine exists outside of the synaptic-vesicle, dopamine is easily metabolized to produce intracellular reactive oxygen

species (ROS) through auto-oxidation or by enzymes such as monoamine oxidase (MAO) (Miyazaki and Asanuma, 2008).

Outside of nigrostriatal pathways, other major dopaminergic pathways such as mesolimbic, mesocortical have also been identified in the mammalian brains (**Figure 2-2**).

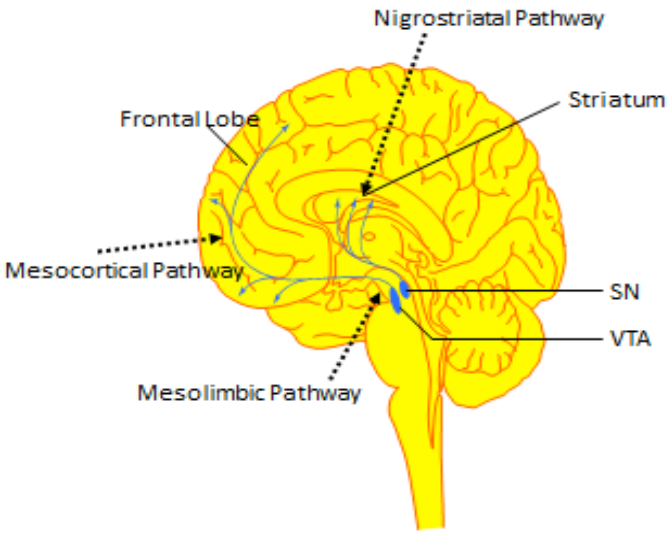

Figure 2-2 Major dopaminergic pathways. SN: substantia nigra; VTA: ventral tegmental area. Figure made using the template from Motifolio drawing toolkits (www.motifolio.com).

These pathways play a key role in various vital central nervous system functions such as voluntary movement, feeding, reward, attention, working memory and learning (Beaulieu and Gainetdinov, 2011). Traditionally PD is defined according to its motor deficits, which reflects the impaired nigrostriatal pathway and degree of dopamine depletion in the striatum, particularly in the putamen. Recent studies have also identified the associated non-motor symptoms including olfactory dysfunction, cognitive impairment, psychiatric symptoms, sleep disorders, autonomic dysfunction, pain and fatigue, which occurs not only in the advanced disease but also in early states and considered as a key determinant of quality of

life (Chaudhuri and Schapira, 2009; Kalia and Lang, 2015; Miller and O'Callaghan, 2015). These cognitive impairment and neuropsychiatric symptoms indicate that other dopaminergic pathways are also affected during the course of the disease. For example, loss of mesocortical dopaminergic neurons may make the system more vulnerable to stress, as dopamine release in the cortex inhibits stress activated neurons in the nucleus accumbens (Hemmerle *et al.*, 2012). In addition, these non-motor symptoms may also be related to the involvement of the non-dopaminergic systems and other neurotransmitters such as serotonin and norepinephrine (Bonnet *et al.*, 2012). The decreased activity and number of the serotoninergic neurons in the dorsal raphe nucleus and loss of noradrenergic neurons have been observed in PD patients, which may explain the development of depression during the disease course (Hemmerle *et al.*, 2012).

Apoptosis is typically identified by characteristic cell morphology such as cell shrinkage, membrane blebbing, compartmentalization, nuclear condensation, and DNA fragmentation, which have been demonstrated in PD patients, and *in vitro and in vivo* models of PD (Venderova and Park, 2012). Neurons undergo apoptosis based on information from external or internal stimuli (**Figure 2-3**). The external pathway is termed as death receptor pathway mediated by the activation of death receptor via cytokines such as tumor necrosis factor alpha (TNFα) (Singh and Dikshit, 2007). Increased levels of cytokines such as TNFα and interferon ɣ have been reported in both patients and experimental models of PD, which may cause the activation of extrinsic pathways and induce apoptosis (Singh and Dikshit, 2007). The intrinsic pathway is activated in response to a number of stress conditions and involved in a series of events including mitochondrial potential change, increased oxidative stress, alteration in pro- and anti-apoptotic proteins, cytochrome c release, which eventually leads to

apoptosis via caspase activation. Intrinsic pathways of apoptosis have been well investigated in PD, and increased oxidative stress, decreased mitochondrial complex activity, altered expressional levels of pro and anti-apoptotic proteins such as BAX and BCL-2, cytochrome c release and caspase cascade activation have been demonstrated in PD pathogenesis (Singh and Dikshit, 2007; Levy *et al.*, 2009; Venderova and Park, 2012).

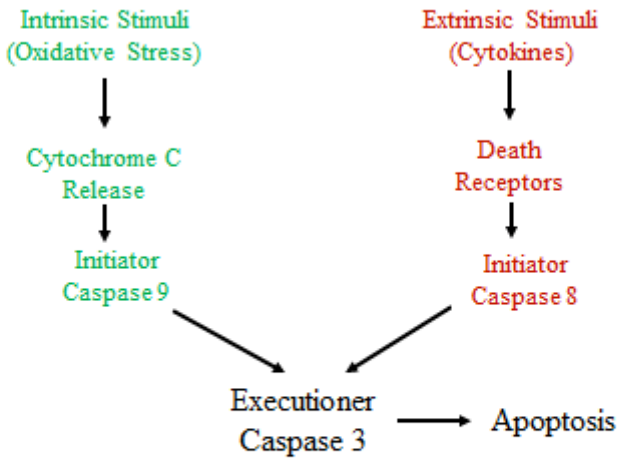

Figure 2-3 Intrinsic and extrinsic apoptotic pathways.

Another pronounced pathological feature of PD is the abundant expression of intracytoplasmic eosinophilic inclusions known as lewy bodies of dopaminergic neurons in the SNpc, and other regions of the central and peripheral autonomic system (Olanow and Brundin, 2013). Lewy bodies can also be found in the normal aging brains and in patients' brains with other neurodegenerative disorder such as Alzheimer's disease (Lotharius and Brundin, 2002). The major component of Lewy bodies is alpha-synuclein (α-synuclein). The exact function of α-synuclein is not known but may involve in the vesicle trafficking during the neurotransmitter release and protect nerve terminals from injury (Kalia *et al.*, 2013; Recasens and Dehay, 2014). The conversion of α-synuclein from a soluble monomer to

pathological oligomers and insoluble fibrils result in the disruption of membrane structure, mitochondrial dysfunction, impairment of protein clearance pathway, and enhanced oxidative stress, all of which can lead to neurodegeneration (Kalia *et al.*, 2013; Roberts and Brown, 2015).

Risk factors of Parkinson's disease

A number of factors including male gender, advanced age, genetic predisposition, environment exposure (pesticide exposure, prior head injury, rural living, beta blocker use, agricultural occupation, well water drinking) have been proposed to increase PD risk (Shulman *et al.*, 2011; Kalia and Lang, 2015) (**Figure 2-4**). On the other hand, reduced risk of PD is associated with tobacco and coffee use, calcium channel blocker use, and non-steroidal anti-inflammatory drug use (Noyce *et al.*, 2012; Kalia and Lang, 2015).

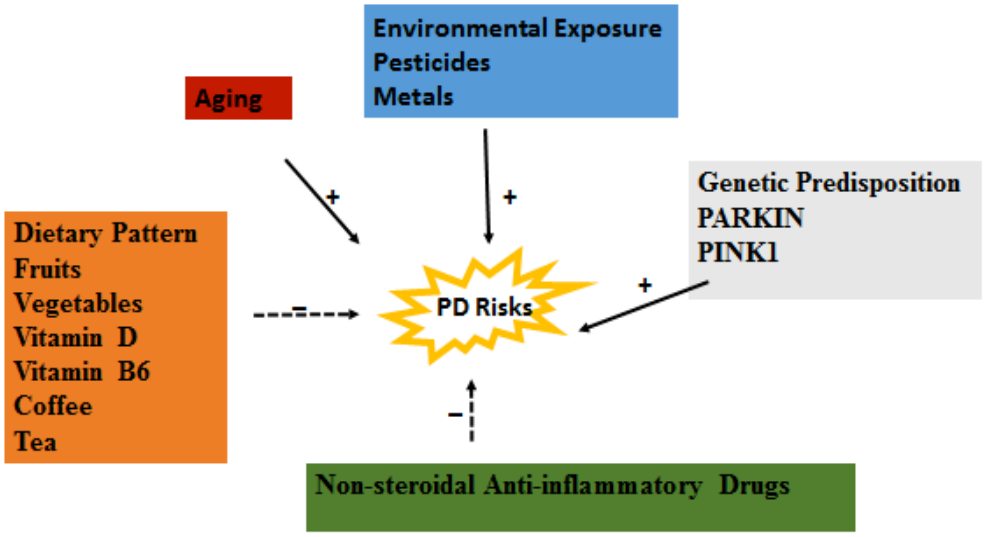

Figure 2-4 Potential risk factors in PD.

1. Aging

Age is considered as the most potent risk for PD with an average of onset of approximately 50 to 60 years (Beitz, 2014). A meta-analysis of world wide data indicates a steadily increased prevalence of PD with age (all per 100,000) : 41 in 40 to 49 years; 107 in 50 to 59 years; 173 in 55 to 64 years; 428 in 60 to 69 years; 425 in 65 to 74 years; 1087 in 70 to 79 years; and 1903 in older than age 80 (Pringsheim *et al.*, 2014). This trend has important implications for public health and scientists to predict that the number of PD is expected to double by year 2030 due to the increased aging population (Dorsey *et al.*, 2007; Kalia and Lang, 2015). Aging is characterized by a progressive decline of physiological functions and an increased susceptibility to certain diseases, and an increased risk of death (Gemma *et al.*, 2007). Research shows that aging affects many cellular processes including mitochondrial dysfunction, increased free radical production, increased genomic instability, shortened telomeres associated with reduced cell survival, reduced efficiency of chaperones, declined proteasome activity, imbalanced autophagy recycling (Hindle, 2010). All these processes can result in the accumulation of unrepaired cellular damage and weakened cellular compensatory mechanisms, leading to the acceleration of neurodegeneration. In addition, dopaminergic neurons in the substantia nigra (SN) age more rapidly than the majority neurons in other brain regions due to their particular vulnerability to the accumulated aging effects such as mitochondrial dysfunction and altered protein degradation pathway (Surmeier *et al.*, 2010; Reeve *et al.*, 2014). Recent research suggests that PD can be considered as the result of the slow neurodegenerative action of aging, which is accelerated by the repeated damage to dopaminergic neurons that accumulates over the life course (Rodriguez *et al.*, 2015).

2. Genetic factors

Although the majority of PD cases are sporadic, about 10% patients report a positive family history (Klein and Westenberger, 2012). The first evidence for the existence of genetic forms of PD is based on the identification of association between A53T mutation in the gene encoding α-synuclein (SNCA) and inherited PD (Collier *et al.*, 2011). Current studies of the familial PD have discovered associated genes such as PAKIN, PINK1, DJ-1, LRRK2, VPS35, causing rare monogenic forms of the disease (Bonifati, 2014). Mutations in these associated genes have provided important insight into the molecular mechanisms involved in the disease pathogenesis such as mitochondrial or lysosomal dysfunction, protein aggregation, and autophagy-lysosomal pathway (Deas *et al.*, 2011; Giraldez-Perez *et al.*, 2014; Thomas *et al.*, 2014). For example, PARKIN gene is demonstrated to play an important role in mitochondrial function, including its ability to interact with mitochondrial transcription factor A to enhance mitochondrial biogenesis and its ability to maintain mitochondrial homeostasis through targeting damaged mitochondria for mitophagy (Thomas, 2009). VPS35 mutation contributes to approximately 1% of familial Parkinsonism and 0.2% sporadic PD. Studies have shown that VPS 35 is crucial for endosome-trans-golgi trafficking and membrane protein cycling, and involved in both early endosome receptor recycling in dendritic spines and lysosomal ATPase recycling from multivesicular bodies (Vilarino-Guell *et al.*, 2011; Lin and Farrer, 2014). In addition, genome wide association studies have identified more than 20 chromosomal loci modulating the risk of developing PD, supporting the extensive and complex genetic contribution to PD and implicating new proteins in the pathogenesis of the disease (Bonifati, 2014; Nalls *et al.*, 2014).

3. Environmental exposure

In recent years, exposure to environmental agents including pesticides, metals and microbial toxins has been recognized as possible risk factors for PD. Among these environmental toxicants, pesticides are considered as the most persistent contaminants (Kanthasamy *et al.*, 2005). The association between pesticides and PD first gained attention in 1980 upon the discovery that exposure to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a substance structurally similar to the herbicide paraquat resulted in chronic Parkinsonism and dopaminergic neurodegeneration in humans (Freire and Koifman, 2012). From that time, various studies including case reports, ecological studies, mortality studies and case-control and cohort studies search for the possible association between pesticides exposure and the increased risk of PD (Freire and Koifman, 2012; Pezzoli and Cereda, 2013). Among these pesticides, paraquat, rotenone and dieldrin have been found to be strongly associated with the increased risk of PD (Kanthasamy *et al.*, 2005; Berry *et al.*, 2010; Tanner *et al.*, 2011). Rotenone is a broad spectrum pesticide used in organic food farming based on its label as a natural product (Cicchetti *et al.*, 2009). Plants containing rotenone have been used for centuries by people to catch fish, and currently it is still used as a pesticide to remove invasive fish species in lakes (Goldman, 2014). Research has shown rotenone can easily cross blood brain barrier and accumulates at mitochondrial complex I where it inhibits complex I activity and induces oxidative stress. Rotenone can also enhance the amount of mitochondrial ROS production, induce α-synuclein aggregation, trigger endoplasmic reticulum (ER) stress, cause dopamine redistribution, activate microglial cells, and activate cytochrome C release and caspase dependent apoptotic cell death, which are all implicated in the pathogenesis of PD (Franco *et al.*, 2010). Recent case-control study nested in the

Agricultural Health Study also demonstrated a strong association between human exposure of rotenone and PD risk (Tanner *et al.*, 2011). Based on 110 PD cases and 358 controls, it was reported that PD developed 2.5 times as often in those who reported use of rotenone compared with nonusers, and a similar magnitude association was also observed even when exposure was truncated up to 15 years before PD diagnose. Paraquat is another most widely used herbicide worldwide. It is able to cross blood brain barrier and induces reduced motor activity and a dose-dependent loss of tyrosine hydroxylase positive striatal fibers and midbrain SNpc neurons after the systemic application to the mice (Blesa *et al.*, 2012). The neurotoxicity of paraquat is associated with its ability to increase lipid peroxidation, generate ROS, decrease antioxidant enzymes, impair mitochondrial function, and increase expression and aggregation of α-synuclein (Goldman, 2014). Epidemiological studies also suggest the role of paraquat in the development of PD in humans (Liou *et al.*, 1997; Tanner *et al.*, 2011). Dieldrin is an organochlorine insecticide that was commonly used on crops from 1950 to1970 and to control termites from 1972 to 1987 (Goldman, 2014). It is one of the more likely candidates for the development of PD. A postmortem study shows that dieldrin level was significantly higher in PD patients than those in control brains (Corrigan *et al.*, 1998). A nested case control study with serum samples collected during 1968-1972 and analyzed in 2005-2007 found an association between increasing dieldrin concentrations and increased odds of developing PD (Weisskopf *et al.*, 2010a). It was suggested that dieldrin damages dopaminergic neurons by inducing oxidative stress, aggregation and fibrillation of α-synuclein, disrupting the ubiquitin-proteasome system, and mitochondrion membrane potential, stimulating dopamine release leading to intracellular dopamine depletion, and activating caspases (Chhillar *et al.*, 2013).

Exposure to metals like manganese, copper, lead or iron can occur at workplaces in primary metal production or metal working activities like welding, galvanizing, grinding or through the diet or medications (van der Mark *et al.*, 2015). Although the evidence from epidemiological studies is limited and controversial, some studies suggest that prolonged exposure to metals might be a risk factor for PD (Wirdefeldt *et al.*, 2011). One study examined the levels of mercury in blood, urine and hair and reported an increased risk of PD associated increased mercury levels (Ngim and Devathasan, 1989). A recent study also reported that mercury exposure negatively affected dopamine transporters in the striatum of workers at risk of mercury vapor exposure (Lin *et al.*, 2011). Although the mechanisms remain unknown, it was suggested that the neurotoxicity of mercury is associated with its generation of oxidative stress through various routes, such as promoting lipid peroxidation, mitochondrial damage, and inducing superoxide production (Caudle *et al.*, 2012). Lead is a non-essential, toxic metal that has caused extensive environmental contamination due to its widespread use. Although environmental levels of lead have been significantly reduced over the past several decades, it is still a health concern especially for infants and young children in critical periods of neurodevelopment. A large control study based on 121 PD patients and 414 match controls found individuals who experienced the highest quartile of exposure were twice as likely to have PD as those in the lowest quartile of exposure (Coon *et al.*, 2006). There are several mechanisms that might explain the neurotoxicity induced by lead. Lead was found to increase midbrain oxidative stress and lipid peroxidation, and enhance fibrillation and accumulation of α-synuclein (Goldman, 2014). In addition, studies found that lead decreased dopamine synthesis, turnover, and uptake in the midbrain, increased spontaneous dopamine release, and reduced tyrosine hydroxylase activity and the number of

spontaneously active dopaminergic neurons (Jadhav and Ramesh, 1997; Tavakoli-Nezhad *et al.*, 2001; Weisskopf *et al.*, 2010b). Manganese is a heavy metal that is widely distributed in the environment including air, water, and food. It is an essential element for biological function and serves as a cofactor for several enzymes such as superoxide dismutase, and plays an important role in the neurotransmitter synthesis and metabolism (Caudle *et al.*, 2012). However, exposure to high level of manganese is associated with several neurological symptoms such as motor dysfunction and neuropsychological impairments, which resemble PD symptoms and are defied as Parkinsonism (Olanow, 2004). In addition, manganese exposure is also considered as a risk factor for PD. A few studies have reported an earlier age at onset of PD was associated with occupational exposure to manganese (Racette *et al.*, 2001; Ratner *et al.*, 2014). Excessive exposure to manganese was demonstrated to induce dopaminergic neurodegeneration by increasing oxidative stress, impairing ATP production, and causing protein aggregation as well as mitochondrial dysfunction (Chen *et al.*, 2014).

4. Nutrition/ Diet in PD

Recent research showed that lifestyle factors including nutrition/diet has an important influence on the risk of developing of PD during later life (Schulz and Deuschl, 2015). Some dietary factors are found to be involved in the etiology of neurodegeneration and increase the risk of PD, while other dietary factors may exert neuroprotection and is associated with a decreased risk of PD (Seidl *et al.*, 2014). An early prospective study found a positive association between dairy consumption and the increased risk of PD, particularly in men (Chen *et al.*, 2002). A meta-analysis by pooling the results of three prospective studies suggested that high dairy consumption can increase the risk of PD, especially in men, independent of calcium, vitamin D or fat intake (Chen *et al.*, 2007). Similarly, a recent

population based prospective cohort study based on 26173 participants in Greece also confirms the results and shows a strong positive association between PD incidence and the consumption of milk but not cheese or yogurt (Kyrozis *et al.*, 2013). A possible explanation for the positive association is that dairy products are contaminated with neurotoxic chemicals such as pesticides, or the potential effects of dairy products on circulating levels of uric acid (Chen *et al.*, 2007). High dairy consumption is associated with a lower circulating level of uric acid. Uric acid is suggested to have neuroprotective effect and high serum uric acid is associated with a significantly reduced risk of PD (Andreadou *et al.*, 2009).

The association between vitamin D deficiency and the increased risk of PD has recently been proposed. A recent systematic review and meta-analysis showed that patients with vitamin D insufficiency [25(OH)D level <75nmol/L] had an increased risk of PD (OR 1.5, 95% CI 1.1-2.0), and patients with vitamin D deficiency [25(OH)D level <50nmol/L] experienced a twofold increased risk of PD (OR 2.2, 95% CI 1.5-3.4) (Lv *et al.*, 2014). A recent genetic study conducted a comprehensive genetic analysis of vitamin D receptor (VDR) in PD and found VDR as a potential susceptibility gene supporting the essential role of vitamin D in PD (Butler *et al.*, 2011). The neuroprotective effects of vitamin D might be associated with its ability to stimulate the synthesis of nerve growth factor and glial cell line derived neurotrophic factor, sequester ROS and downregulate inducible nitric oxide synthase (iNOS) expressions (DeLuca *et al.*, 2013). Recent studies have also identified the association between B vitamins intake and PD risk. A hospital based case-control study in Japan examined the association between dietary intake of folate, vitamin B6, Vitamin B12 and riboflavin and the risk of PD (Murakami *et al.*, 2010). The result shows that low intakes of vitamin B6 but not of folate, vitamin B12 or riboflavin were associated with an increased risk

of PD. It is suggested that the neuroprotective role of vitamin B6 may be through its antioxidant capacities or its role in dopamine synthesis.

Growing evidence suggests dietary patterns can play a protective role in PD. Dietary patterns represent a combination of food, which is considered as a more powerful predictor of health outcomes than a single nutrient (Gao *et al.*, 2007). A prospective study of dietary patterns and risk of PD found dietary patterns including a high intake of fruits, vegetables, legumes, whole grains nuts, fish and poultry, a moderate intake of alcohol and a low intake of saturated fat was inversely associated with PD risk (Gao *et al.*, 2007). Another dietary pattern analysis based on a multicenter hospital-based case-control study conducted in Japan also reported a dietary pattern with high intakes of vegetables, seaweed, pulses, mushrooms, fruits and fish may be associated with a decreased risk of PD (Okubo *et al.*, 2012). In addition, tobacco use, coffee or tea drinking were also found to be associated with a lower risk of PD (Migliore and Coppede, 2009). A pooled analysis of tobacco use and risk of PD showed a dose dependent reduction of PD risk associated with cigarette smoking and potentially with other types of tobacco use (Ritz *et al.*, 2007). A recent meta-analysis including 61 case-control and 8 prospective cohort studies also supports the inverse association between cigarette smoking and the risk of PD (Li *et al.*, 2015). Although the exact mechanisms for the protective effects of smoking on risk of PD is not known, it is suggested that the major component nicotine in tobacco exerts neuroprotection by stimulating nicotinic acetylcholine receptors or inhibiting α-synuclein fibrillation (Li *et al.*, 2015). A linear dose relationship for decreased PD risk with tea and caffeine consumption were found in a meta-analysis and the strength of protection reached a maximum at approximate 3 cups/day for coffee consumption (Qi and Li, 2014). It is suggested that the

major bioactive compounds caffeine or polyphenol in tea and coffee may offer neuroprotection against the underlying dopaminergic neuron degeneration and prevent the onset of PD.

5. Epigenetics in PD

Epigenetics is the study of heritable changes in gene expression or function without changes in DNA sequence. Primary epigenetic modifications include DNA methylation, post-transcriptional modifications of histone and non-coding RNA mediated changes of gene expression (Feng *et al.*, 2015). Recent research suggests that environmental factors such as heavy metals, pesticides or nutrition may affect PD risks through the epigenetic changes (Kwok, 2010). Studies have found that heavy metals such as arsenic, cadmium, chromium, lead, mercury, coppers, and nickel can induce changes in DNA methylation patterns either at the global or the individual gene level, or cause global histone modification, or miRNA expression (Ding and Zhu, 2009; Cheng *et al.*, 2012; Ho *et al.*, 2012). These epigenetic changes may modify the expression of critical genes and influence the phenotype of offspring and increase the risk of disease development at the later life (Kim *et al.*, 2009). The epigenetics modification is also observed in pesticides mediated neurodegeneration. Dieldrin was found to increase histone acetylation to promote apoptosis in dopaminergic neurons (Song *et al.*, 2010). In addition, dieldrin exposure during gestation and lactation was found to lead to persistent alteration of the developing dopaminergic system and induce dopamine dysfunction in an animal model of PD (Richardson *et al.*, 2006). Epigenetics mechanism also linked the nutrition and the risk of PD. Nutrients such as folate, vitamin B12, methionine, choline and betaine are demonstrated to affect DNA methylation and histone methylation through altering one-carbon metabolism (Choi and Friso, 2010). Since the impaired one-

carbon metabolism and altered DNA methylation potential was observed in PD (Coppede, 2012), B vitamins might represent a promising preventative and therapeutic approach through exerting epigenetic regulations.

6. Experimental models of PD

Different experimental models have been developed to understand the PD etiology, pathology, and test the neuroprotective strategies. 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridineis a commonly used neurotoxin for inducing both rodent and primate models of PD (Duty and Jenner, 2011). It was discovered accidently in 1982, when young drug addicts mysteriously developed parkinsonian syndrome after intravenous injection of this compound. 1-methyl-4-phenyl-1,2,3,6- tetrahydropyridineis is currently considered as the gold standard for toxin based animal models of PD since it replicates almost all hallmarks in PD except the formation of lewy bodies (Blesa *et al.*, 2012). 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridineis can cross the blood brain barrier easily due to its highly lipophilic property and is metabolized to its major toxic metabolite 1-methyl-4-phenylpyridinium (MPP^+) by monoamine oxidase B in astrocytes. 1-methyl-4-phenylpyridinium can enter neurons by the dopamine transporter, inhibit mitochondrial complex I activity, leading to increased oxidative stress and decreased ATP production and apoptosis (**Figure 2-5**). 6-hydroxydopamine (6-OHDA) is a neurotoxin that has been used widely in an animal model of PD (**Figure 2-5**). It is a hydroxylated analog of dopamine and has high affinity for their catecholaminergic transporters like dopamine transporter (Le *et al.*, 2014). Once in the neurons, it is accumulated in cytoplasm and undergoes auto-oxidation, producing a large amount of free radicals such as hydrogen peroxide (H_2O_2), and inducing dopaminergic neuronal damage (Blandini *et al.*, 2008). In addition, 6-OHDA can also initiate cell damage

through mitochondrial complex I inhibition, cytochrome c release, activation of caspase cascades, and inducing kinase signaling modulation accompanied with inhibition of antioxidant systems (Tobon-Velasco *et al.*, 2013).

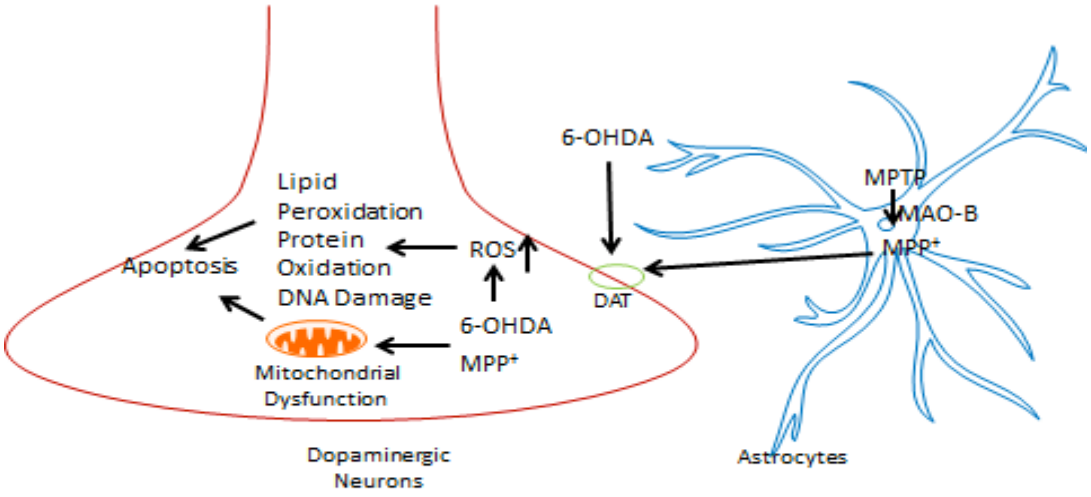

Figure 2-5 Mechanisms involved in the neurotoxicity of 6-OHDA and MPTP. 6-OHDA: 6-hydroxydopamine; MPTP: 1-methyl-4-phenyl-1,2,3,6- tetrahydropyridine; MPP^+: 1-methyl-4-phenylpyridinium; MAO-B: Monoamine oxidase B; DAT: Dopamine transporter; ROS: Reactive oxygen species. Part of the figure made using the template from Motifolio drawing toolkits (www.motifolio.com).

Unlike MPTP, 6-OHDA can't cross blood brain barrier and administration is carried out by the direct injection (frequently as a unilateral injection) in the SN, medial forebrain bundle consisting of efferent fibers from nigral cell bodies to the striatum, or striatum (Jagmag *et al.*, 2015). Degeneration of dopaminergic neurons starts within 12 h after injection of 6-OHDA into the SN or the medial forebrain bundle, with dopamine depletion 2-3 days later (Schober, 2004). Intra-striatal injection of 6-OHDA can cause striatum terminal death first and then result in the progressive retrograde neuronal degeneration in the SN, replicating the pathological process of PD in humans. The model using 6-OHDA does not mimic all of the clinical features of PD. It induces dopamine depletion, nigral dopamine cell loss, and

behavior deficits, but not able to affect other brain regions such as olfactory structures (Blesa *et al.*, 2012). In addition, 6-OHDA does not produce lewy body like inclusions. Recent research suggests that iron overload may play an important role in 6-OHDA mediated neuronal degeneration through the stimulation of dopamine oxidation (Hare and Double, 2016).

Pathogenesis in PD

In the past 20 years, there have been significant advances in understanding the mechanisms of PD and different pathological factors including oxidative stress, mitochondrial dysfunction, altered proteolysis, neuroinflammation, excitotoxicity have been thought to contribute to the cell death in PD (Dexter and Jenner, 2013). These factors may interact with each other and result in a snowball effect triggering or exacerbating the neurodegenerative process.

1. Oxidative stress in PD

A free radical is defined as molecule with unpaired electrons in their outer orbit. The free radicals are often referred as ROS since most biological significant free radicals are oxygen centered (Aprioku, 2013). The most common ROS human body generated includes H_2O_2, superoxide, and hydroxyl radicals. Although these free radicals have deleterious effects on the normal human body, they also act as cellular messengers and play an important role in maintaining homeostasis (Gemma *et al.*, 2007). At the same time, human body has an antioxidant defense system that regulates ROS, including antioxidant enzymes such as superoxide dismutase, catalase, glutathione peroxidase, and non-enzyme molecules such as selenium, zinc, vitamin E and vitamin C (Uttara *et al.*, 2009).

Oxidative stress is described as a condition in which cellular antioxidant defense is insufficient to inactivate the ROS. The major consequence of oxidative stress includes damage to nuclei, lipids and proteins, which severely compromises cell functions, induces a variety of cell responses and leads to cell apoptosis (Dalle-Donne *et al.*, 2006) (**Figure 2-6**).

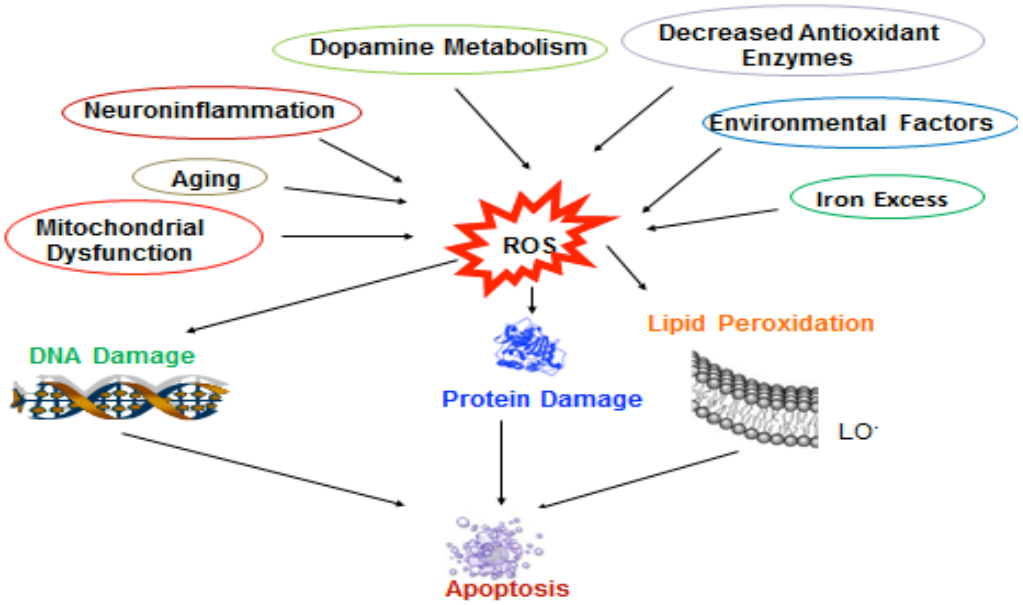

Figure 2-6 The role of oxidative stress in PD.

The role of oxidative stress in PD is indicated by the evidence that brain is vulnerable to oxidative damage due to high oxygen utilization to produce energy and unsaturated fatty acids. Brain is the busiest organ to keep other organs active and under control, and needs large amount of energy to maintain active transport of the ions required for neuronal excitation and neurotransmission (Nakabeppu *et al.*, 2007). It is estimated that brain uses about 20% of body's total oxygen consumption and 10 moles of ATP per day (Halliwell, 2006). The brain is rich in polyunsaturated fatty acids especially arachidonic acid and docosahexaenoic acid, which are primary lipid peroxidation targets (Gandhi and Abramov,

2012). In addition, high iron content and relative low antioxidant enzymes such as catalase, superoxide dismutase and glutathione peroxidase, make brain even more susceptible to oxidative stress (Bharath *et al.*, 2002). Dopaminergic neurons are particularly susceptible to oxidative damage because of the presence of ROS generating enzymes such as tyrosine hydroxylase and monoamine oxidase (Hwang, 2013). Tyrosine hydroxylase is a rate limiting enzyme in the dopamine synthesis and produces H_2O_2 as a side product in their activities. In addition, dopamine is unstable and produces ROS and reactive quinones through the auto-oxidation or monoamine oxidase catalyzed oxidation (Khan *et al.*, 2005).

Mitochondrial dysfunction is another source of oxidative stress in PD. The main function of mitochondria is to provide cellular energy source ATP through the process of respiration and oxidative phosphorylation (Hauser and Hastings, 2013). Free radicals such as superoxide are normally produced as by-products as electrons are transferred to oxygen in the respiration chain. Inhibition of mitochondrial complexes can overthrow the cellular antioxidant capacity and dramatically increase ROS production ultimately leading to cell death (Van Laar and Berman, 2009). The direct evidence showing mitochondrial dysfunction in PD came from the observed defect of mitochondrial complex I activity in SN of PD patients (Schapira *et al.*, 1989). Later studies also found reduced activity in complex I, II and IV in skeletal muscle cells from PD patients (Bindoff *et al.*, 1991). Research also shows high levels of mitochondrial DNA deletions in SN in postmortem tissues of both aging and PD patients, supporting the role of mitochondrial dysfunction in PD pathogenesis (Bender *et al.*, 2006).

The occurrence of oxidative stress in PD is also supported by postmortem brain analyses showing elevated levels of lipid peroxidation, protein carbonyls and nucleic acid oxidation

(Dias *et al.*, 2013). Increased lipid peroxidation products such as malondialdehyde and 4-hydroxynonenal have been found in the SN of PD brains (Gandhi and Abramov, 2012). Increased concentrations of both protein carbonyls and 3-nitrotyorsine were also observed in aging brains and neurodegenerative disorders including PD (Beal, 2002). Moreover, oxidative DNA lesions such as 8-oxoguanine was demonstrated accumulated in nuclear and mitochondrial genomes during aging, and increased dramatically in patients with PD (Nakabeppu *et al.*, 2007). Oxidant production and oxidative damage were also found in neurotoxin-induced PD model. For example, the neurotoxin MPTP can induce neurodegeneration by entering astrocytes and converting to the active metabolite MPP^+, which inhibits complex I of the mitochondrial respiratory chain, and increases ROS production. The study has shown that the ROS produced by MPTP is triggered not only by complex I inhibition but also by the auto-oxidation of dopamine resulting from MPP^+-induced massive release of vesicular dopamine (Bove and Perier, 2012). Neurotoxin 6-OHDA-induced PD model also supports the link between oxidative stress and neurodegeneration in PD. 6-hydroxydopamine is a naturally occurring endogenous product of dopamine synthesis that has been extensively used to model dopaminergic degeneration in both in vitro and in vivo studies (Bove *et al.*, 2005). The deleterious effect of 6-OHDA is due to the oxidative stress triggered by the production of ROS after the auto-oxidation of 6-OHDA. 6-hydroxydopamine-induced oxidative damage includes DNA damage, increased lipid peroxidation and protein carbonyls.

2. Neuroinflammation in PD

Neuroinflammation is also considered as a major component in the pathogenesis of PD. Inflammation is a beneficial process to protect against pathogens and repair tissue damage.

However, it can also be detrimental when the helpful response is not controlled leading to the destruction of normal tissue and chronic inflammation (Hsieh and Yang, 2013).

Recent epidemiological studies have suggested the neuroinflammatory processes in the development of PD. A prospective study was conducted on 136197 participants to investigate the association between use of nonsteroidal anti-inflammatory drugs and the risk of PD (Gao *et al.*, 2011). The result showed that users of ibuprofen had a significantly lower risk of PD than non-users after the data was adjusted for age, smoking and caffeine and other covariates. Another nested case control study including 84 incident cases and 165 matched controls examined whether plasma concentrations of inflammatory biomarkers assessed before PD diagnosis were predictive for future risk of PD (Chen *et al.*, 2008). Results show that higher levels of interleukin-6 (IL-6) but not C-reactive protein, fibrinogen and TNFα receptor were associated with a greater risk of PD, although the small sample size in the study limited the statistical power in the analysis. Later studies also confirm the elevated circulating levels of IL- 6 in PD patients (Scalzo *et al.*, 2010; Koziorowski *et al.*, 2012), indicating the possible involvement of inflammation in the PD pathogenesis. Other studies have observed the increased levels of cytokines such as TNFα, IL-1B, IL-2, IL-4, IL-10, interferon-ɣ in the serum or plasma of PD patients (Katsarou *et al.*, 2007; Brodacki *et al.*, 2008; Rocha *et al.*, 2015).

Both the innate and adaptive immune responses have been suggested to play important roles in the pathophysiology of PD (Hunot and Hirsch, 2003; Stone *et al.*, 2009). The innate immunity plays an important role in initiating inflammation whereas adaptive immunity is induced by the innate immunity, which is specific, targeted and highly potent against the antigens (Kannarkat *et al.*, 2013). The involvement of the innate immunity in the

development of PD is demonstrated by the presence of activated microglia in the SN of patients with PD or neurotoxin-induced animal models (Hirsch *et al.*, 2012). A postmortem study shows the number of activated microglia were significantly higher in SN, putamen, hippocampus, transentorhinal cortex, cingulate cortex and temporal cortex of PD patients compared to normal control (Imamura *et al.*, 2003). Positron emission tomography (PET) studies demonstrate the widespread microglia activation in early PD patients, supporting the involvement of intrinsic microglia in the progressive degeneration process of PD (Ouchi *et al.*, 2005; Gerhard *et al.*, 2006). Studies with animal models also demonstrate the presence of microglia activation in neurotoxin-induced PD. 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine was found to induce microglial activation by increasing cell number, changing cell morphology in both SN and striatum of mice (Lull and Block, 2010). 6-hdyroxydopamine was found to activate microglia through a process called reactive microgliosis, which leads to exacerbation of dopamine neuron neurotoxicity (Peterson and Flood, 2012).

Microglia are the resident macrophages of the central nervous system playing an important role in sustaining brain homeostasis and performing immune surveillance (More *et al.*, 2013). In normal brain, resting microglia continuously examine their environment in the surrounding tissues by extending and contracting their cellular protrusion (Peterson and Flood, 2012).

However, various environmental challenges such as aggregated α-synuclein or neuromelanin released from damaged dopaminergic neurons can cause microglia activation, leading to a series of changes such as shape and increased proliferation. In addition, activated microglia can migrate to the lesion region and secrete cytotoxic substances such as pro-

inflammatory mediators, nitric oxide and superoxide radicals, creating an environment that can initiate and amplify the neuron damage (**Figure 2-7**). In addition, Astrocytes can also undergo a state of gliosis in response to neuronal injury or toxic insults and release cytokines and chemokines that lead to neurodegeneration. (Rappold and Tieu, 2010).

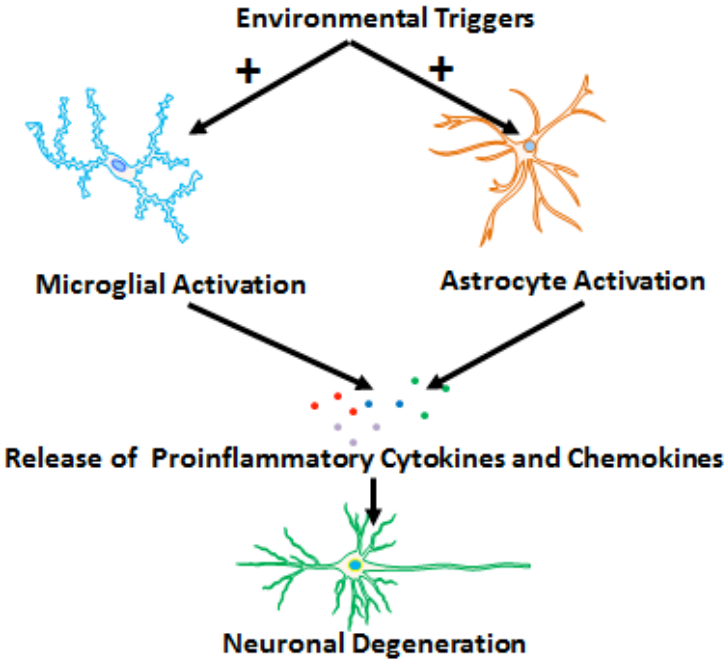

Figure 2-7 The involvement of the innate immune responses in PD. Part of the figure made using the template from Motifolio drawing toolkits (www.motifolio.com).

Innate immune activation may also affect the normal barrier function of cerebral endothelial cells and lead to the infiltration of peripheral leukocytes and adaptive immune cells (Kannarkat *et al.*, 2013). Both CD4+ and CD8+ T cells were discovered within the SN of patients with PD and MPTP intoxicated mice, indicating the role of the adaptive immunity in the pathogenesis of PD (Brochard *et al.*, 2009; Stone *et al.*, 2009). Among those T cells, CD8+ T cells can induce direct neurotoxicity by lysis of targeted cells through the release of granzymes or perforins, or kill cells by engagement of cell death receptors via cytokines such

as TNFα. CD4+ T cells can promote the activation and phagocytic function of microphages or induce B cells to produce high affinity antibodies leading to antibody dependent cytotoxicity (German *et al.*, 2011; Kannarkat *et al.*, 2013; More *et al.*, 2013).

Recent studies demonstrate the interaction between neuroinflammation and oxidative stress in the pathogenesis of PD (Mosley *et al.*, 2006; Tufekci *et al.*, 2012). For example, glial cells can release diverse inflammatory mediators in response to oxidative stress (Chiurchiu and Maccarrone, 2011; Hsieh and Yang, 2013). On the other hand, enzymes like NADPH or cytokines like IL-6, TNFα produced by activated glial cells have the potential to initiate or exacerbate the oxidative damage (Stone *et al.*, 2009; Dias *et al.*, 2013; Blesa *et al.*, 2015). Reactive oxygen species can also act as a key signaling molecule to trigger inflammatory responses in the central nervous system through the activation of the redox sensitive transcription factors such as nuclear factor kB (Hsieh and Yang, 2013).

3. Iron overload in PD

I. Systemic iron homeostasis

Iron is an essential micronutrient for all organisms including human. A healthy male individual contains about 3.5g total body iron, approximate 65% of which is distributed within red blood cell hemoglobin, about 10% of which is in muscle fibers myoglobin and other tissues as enzyme and cytochromes, the remaining of which is stored in the liver, macrophages and bone marrow (Munoz *et al.*, 2009). Iron possesses vital physiological functions. It is an important part of hemoglobin found in circulating red blood cells and serves as oxygen transporter. It is also a component of other heme containing proteins such as cytochrome P450 and the cytochromes a b and c in the electron transport and involved in mitochondrial respiration and ATP synthesis (Levenson and Tassabehji, 2004). In addition,

iron is also required for the activity of a variety of critical enzymes such as tyrosine hydroxylase, tryptophan hydroxylase, ribonucleotide reductase. Since iron plays an essential physiological and biochemical role, iron deficiency can impede behavior and cognitive development. Iron deficiency anemia is a common nutrient deficiency disease in the world (Zhang *et al.*, 2014). On the other hand, excess iron participates in Fenton reaction to produce ROS and induce cytotoxicity affecting multiple organ systems.

Thus, human body has evolved a tight regulation system to control iron uptake, distribution and export. An adult needs 25-30mg of iron daily, about 90% of which is acquired from the recycle iron through the degradation of red blood cells, and the remaining 10% of which is absorbed from the diet to compensate iron loss caused by bleeding, urinary excretion, and sloughing of epithelial and mucous cells (Zhang *et al.*, 2014). The normal diet contains 15-20 mg of iron, from which the body absorbs 1-2mg/d (Munoz *et al.*, 2009). Dietary iron is found in heme (10%) and non-heme (90%) and their absorption occurs in duodenum. Dietary ferric iron (Fe^{3+}) is reduced to ferrous iron (Fe^{2+}) by cytochrome b and subsequently transported across the duodenum epithelium by divalent metal transporter-1 (DMT-1). Enterocytes can also absorb heme iron by heme carrier protein-1, and degrade heme through the reaction of heme oxygenase to release ferrous iron. Ferrous iron in the enterocytes can either be oxidized to its ferric state and stored in ferritin, or released to the periphery through the transmembrane protein ferroportin (Fpn). Ferroportin is the current only known iron exporter in mammals (Le Gac *et al.*, 2013). It cooperates with a ferroxidase such as ceruloplasmin catalyzing the oxidation of ferrous iron to ferric iron, removing iron from cells into bloodstream (Musci *et al.*, 2014). Once in the bloodstream, circulating ferric iron is bound to transferrin to form holo-transferrin which is imported to cells via receptor

mediated endocytosis after binding to the transferrin receptors (TfRs) (MacKenzie *et al.*, 2008). In healthy adults, transferrin is about 30% saturated with iron and increased transferrin saturation levels (>45%) always indicates iron overload disorder such as hereditary hemochromatosis (Hower *et al.*, 2009). Mammals have two forms of TfRs including TfR1 and TfR2. TfR1 is widely expressed in all types of cells and TfR2 is predominantly expressed in hepatocytes (Chen and Paw, 2012). TfR2 doesn't contain iron responsible elements (IREs) on the 3' untranslated regions and is not regulated by iron regulatory proteins (IRPs) (Silvestri *et al.*, 2014). In addition, DMT-1 can also directly transport non-transferrin bound iron (NTBI) into cells under conditions such as hemochromatosis when serum transferrin is saturated with iron and NTBI accumulates (Zhang *et al.*, 2014).

II. Cellular iron regulation

Cytosolic iron can either be stored in ferritin, or utilized by mitochondria for heme and iron sulfur cluster synthesis, or exported out of cells by Fpn. Ferritin is the major iron storage protein found in cytoplasm, nucleus and mitochondria and responsible for sequestration of reactive iron to participate in Fenton reaction (MacKenzie *et al.*, 2008). Two subunits of ferritin have been identified including heavy (H) and light (L) chains. The major function of H subunits is converting ferrous to ferric iron through ferroxidase activity, whereas the L subunits stabilize the ferritin structure and are involved in iron nucleation to induce iron core nucleation (Levi *et al.*, 1992; Takaesu *et al.*, 2008).

Intracellular iron level is regulated at posttranscriptional level by IRE / IRP regulatory system (Munoz *et al.*, 2009). There are two forms of IPRs, including IRP1 and IRP2. Although they have similar IRE binding affinities and both are regulated by intracellular iron

level, their expression levels are different in various cell types. IPR1 are highly expressed in the kidneys, livers and brown fat, while IPR2 are highly expressed in the central nervous system (Zhang *et al.*, 2014). In addition, they have different regulatory mechanisms with IRP1 functioning as a bifunctional protein. In iron rich condition, IPR1 binds an iron sulfur cluster to function as a cytosolic aconitase that catalyzes the conversion of citrate to isocitrate in the cytosol. However, IRP2 has no aconitase function and is degraded by the proteasome system (Zhang *et al.*, 2014). Both IPR1 and IPR2 bind to IRE located on either 3' or 5' untranslated regions of mRNA and control cellular iron homeostasis. In cellular iron deficient condition, IRPs bind to IRE on the 3' untranslated region mRNAs of TfR1, DMT-1, and stabilize the transcription and facilitate the protein synthesis. IRPs can also bind to IRE located on the 5' untranslated region mRNA of ferritin and Fpn, and block the translation of the targeted mRNA. Conversely, in cellular iron overload condition, IRPs are unavailable for IRE binding, allowing the degradation of TfR1, DMT-1, and translation of ferritin and Fpn.

III. Hepcidin-Fpn axis

Besides iron regulatory proteins, intracellular iron balance might also be accomplished by hepcidin, which was discovered in 2000 by Krause et al. and Park et al. (Krause *et al.*, 2000; Park *et al.*, 2001). Hepcidin is a small peptide that is mainly secreted by hepatocytes as a precursor pro-peptide. It undergoes proteolytic processing to convert to a bioactive peptide with 25 amino acids (Wang and Pantopoulos, 2011). Hepcidin binds to Fpn and causes the internalization and lysosomal degradation of the transporter leading to decreased iron efflux (Myhre *et al.*, 2013; Schmidt, 2015). Ferroportin is both the hepcidin receptor and the only known cellular iron exporter in vertebrates (Nemeth and Ganz, 2009). By this mechanism, hepcidin controls both systemic and intracellular iron levels through regulating the dietary

iron absorption from the duodenum, the recycled iron release from macrophages, and the stored iron movement from hepatocyte (Nemeth *et al.*, 2004). Chronic overexpression of hepcidin leads to iron restricted anemia accompanied with elevated iron storage in macrophages and hepatocytes, and limited uptake from dietary sources (**Figure 2-8**). Conversely, hepcidin deficiency can cause increased iron transfer to plasma, causing a severe systemic iron overload (Nemeth and Ganz, 2009; Ganz and Nemeth, 2011).

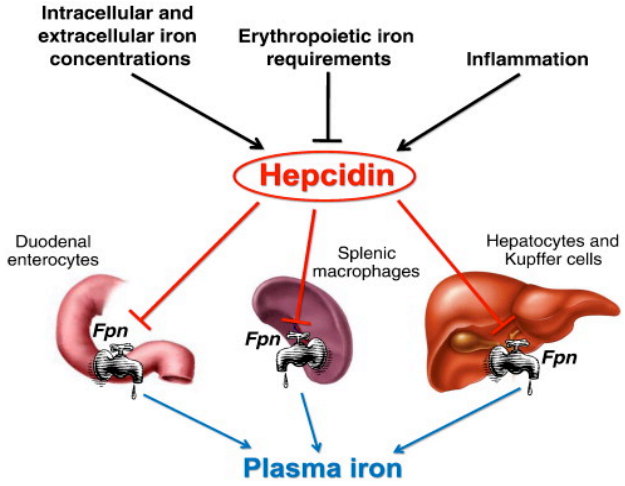

Figure 2-8 The role of hepcidin in systemic iron homeostasis. Figure reproduced from (Ganz and Nemeth, 2012).

Recent research also suggests that Fpn can be regulated independently of hepcidin (Ward and Kaplan, 2012). For example, it can be regulated by cellular iron content at the posttranscriptional level through IRE/IRP system or degraded at the posttranslational level due to the absence of ceruloplasmin. Hepcidin is modulated at transcriptional level by different stimuli including both negative and positive regulators. It is stimulated by iron status and inflammation, and inhibited by iron anemia, hypoxia (Schmidt, 2015). Research demonstrates that hepcidin expression is response to both systemic and intracellular iron

status through Bone Morphogenetic Protein (BMP)/Small Mothers Against Decapentaplegic (SMAD) pathway. Briefly, elevated circulating iron overload or intracellular iron can activate BMP or enhance BMP expression, which can bind with BMP receptors and result in phosphorylation of cytoplasmic SMAD1/SMAD5/SMAD8. SMAD1/SMAD5/SMAD8 proteins form complex with common mediator SMAD4 and translocate into the nucleus and activate the transcription of hepcidin (Nemeth and Ganz, 2009). Hepcidin expression is also induced by inflammatory stimuli such as cytokine IL-6 through the signal transducer and activator transcription 3 (STAT-3) pathway. IL6 binds to its receptor and causes phosphorylation of STAT-3, which can translocate to the nucleus and interact with hepcidin promotor to induce its transcription (Schmidt, 2015). Although hepcidin is predominantly expressed in the liver, recent research also demonstrates the wide distribution of hepcidin in the brain. One study shows the upregulation of hepcidin mRNA in aging mouse brain, especially in cerebral cortex, hippocampus and striatum, resulting in decreased level of Fpn associated with iron overload (Wang *et al.*, 2010). Another study shows peripheral iron overload can induce increased level of hepcidin and decreased level of Fpn in the SN of rats, indicating the significant role of hepcidin in brain iron homeostasis (Sun *et al.*, 2012).

IV. Brain iron homeostasis in PD

Brain particularly needs iron for high metabolic requirements and normal functions since it is a cofactor for a group of enzymes involved in neurotransmitter synthesis such as tyrosine hydroxylase for dopamine and norepinephrine synthesis, tryptophan hydroxylase for serotonin synthesis, monoamine oxidase A and B for dopamine catabolism (Urrutia *et al.*, 2014). Iron is also essential for normal myelin production and maintenance, and iron accumulation is an early event for the development of oligodendrocytes (Todorich *et al.*,

2009). It was found that human infants with iron deficiency anemia tested lower in cognitive, motor, social-emotional and neurophysiological development than comparison group of infants (Lozoff and Georgieff, 2006). On the other hand, excess iron can participate in Fenton reaction to generate highly toxic hydroxyl radicals and enhance lipid peroxidation, DNA damage and glutathione consumption leading to deleterious effects on brain. Various studies have demonstrated an association between iron accumulation and both aging and neurodegenerative disorders (Hagemeier *et al.*, 2012).

Since excessive iron is toxic to the nervous system, brain has a complex system that coordinates iron uptake, release, storage and utilization. Once in the blood stream, ferric iron binds to the major serum iron carrier protein transferrin, which will cross the blood brain barrier via TfR on the brain capillary endothelial cells (BCEC) (Salvador, 2010). The subsequent iron release into the brain interstitium is highly controversial but two major hypotheses have been proposed based on the presence of DMT-1 or not : (1) receptor mediated endocytosis followed by iron release from endosome via DMT-1 and export into the brain interstitium through the Fpn; (2) receptor mediated transcytosis followed by direct holo-transferrin release into brain interstitium (Yang *et al.*, 2013; Belaidi and Bush, 2015; Skjorringe *et al.*, 2015). Except transferrin-TfR dependent iron uptake system, brain iron uptake can also be facilitated by transferrin homologues such as lactoferrin (Mills *et al.*, 2010). Lactoferrin is an iron binding protein involved in host defense against infection and inflammation. It accumulates in the brain with aging and neurodegenerative disorders such as PD. Both in vitro and in vivo studies have demonstrated it can cross blood brain barrier through the receptor mediated transcytosis (Fillebeen *et al.*, 1999; Ji *et al.*, 2006). Under physiological conditions, lactoferrin can function as an iron scavenger and chelate free iron

from participating in Fenton reaction. However, under pathological conditions, brain iron uptake may be increased through lactoferrin mediated transcytosis.

After entering ventricles or cerebrospinal fluid, iron moves between neurons, astrocytes, microglia and oligodendrocytes depending on the need (Ward *et al.*, 2014) (**Figure 2-9**). Neurons can take up iron through transferrin-TfR pathways or DMT-1, store iron mainly as neuromelanin and export iron through Fpn mediated efflux (Lee and Andersen, 2010; Mills *et al.*, 2010; Rouault, 2013). Neuromelanin is a dark/brown granular pigment produced in some dopaminergic neurons in SN. Its exact function is not known but may be involved in neuroprotection against oxidative stress through chelating redox active metals including iron, copper, and zinc (Gerlach *et al.*, 2003). It is estimated about 50% of neuromelanin are saturated with iron with the ferric form in non-neurodegenerative human SN (Zecca *et al.*, 2001). Astrocytes might take significant amount of iron through DMT-1, store iron in ferritin, and export iron through Fpn and ceruloplasmin (Mills *et al.*, 2010; Ward *et al.*, 2014). Microglial cells express DMT-1, ferritin and other iron related proteins like amyloid precursor protein (APP) in order to help neurons maintain iron homeostasis in the brain environment (Oshiro *et al.*, 2011). Oligodendrocytes, which has highest iron concentration for myelin synthesis, is reported to import iron through the ferritin heavy chain or via DMT-1, and store iron mainly as ferritin and transferrin (Hare *et al.*, 2013; Rouault, 2013; Ward *et al.*, 2014). In addition, recent research shows oligodendrocytes also regulate iron efflux via Fpn and require a ferroxidase hephaestin for oxidation before proper iron release in normal central nervous system (Schulz *et al.*, 2011).

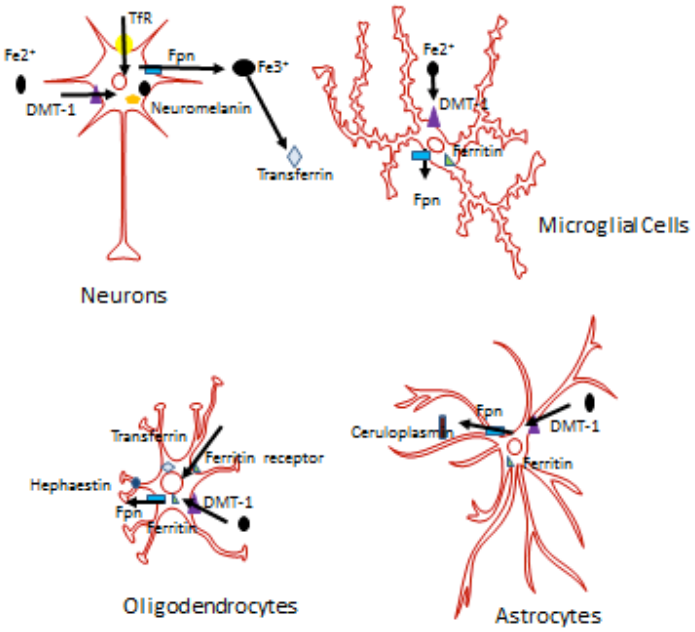

Figure 2-9 Brain iron homeostasis. Fpn: ferroportin; TfR: transferrin receptor; DMT-1: divalent metal transporter. Part of the figure made using the template from Motifolio drawing toolkits (www.motifolio.com).

V. Iron overload in PD

Accumulated evidence has suggested the disruption of iron homeostasis leading to iron accumulation in PD. Postmortem studies have demonstrated the increased levels of iron deposits in the SN and globus pallidus in parkinsonian brains (Gotz *et al.*, 2004; Rhodes and Ritz, 2008). The presence of increased level of total iron in SN of PD patients has also been confirmed by magnetic resonance imaging (MRI) and ultrasound studies, and the extent of deposits is linked to the severity and the duration of disease (Kaur and Andersen, 2004; Weinreb *et al.*, 2013; Ayton and Lei, 2014). In a 3-year-followup study, iron accumulation measured by R2* MRI was observed in SN and caudal putamen in PD subjects but not controls, suggesting the role of iron in the neuron death (Ulla *et al.*, 2013). The process of iron accumulation in PD may involve in various factors (**Figure 2-10**) including increased

iron uptake by DMT-1, TfR and lactoferrin receptor, decreased iron export by Fpn and ceruloplasmin, or altered iron storage regulation by ferritin or neuromelanin (Weinreb *et al.*, 2013; Le, 2014). Decreased ferritin levels was found in SN, caudate putamen, globus pallidus, cerebral cortex and cerebellum in postmortem PD brain (Dexter *et al.*, 1990). Decreased ferritin with increased iron contents suggests that reactive iron amount may increase in SN of PD patients. Neuromelanin was also observed decreased in SNpc of PD patients. A postmortem study found that neuromelanin levels were 1.2-1.5 µg/mg in SNpc of PD patients, which was less than 50% with respect to the age-matched controls (Zecca *et al.*, 2002). In addition, reduced ferroxidase ceruloplasmin activity was observed in PD cerebrospinal fluid and serum, which can in turn promote intracellular iron accumulation and affect brain iron levels (Boll *et al.*, 1999; Bharucha *et al.*, 2008; Olivieri *et al.*, 2011; Ayton and Lei, 2014). The increased iron concentration in SNpc was also observed in neurotoxin MPTP or 6-OHDA-induced PD models, and elevated brain iron level was suggested to result from either increased influx or decreased efflux (Song *et al.*, 2007; Wang *et al.*, 2007; Salazar *et al.*, 2008; Lv *et al.*, 2011). Recent studies report that the upregulation of DMT-1 and downregulation of Fpn expression might be associated with MPTP-induced iron accumulation in mice (Salazar *et al.*, 2008; Lv *et al.*, 2011). Decreased Fpn expression and increased DMT-1 expression were also reported in 6-OHDA-induced neurotoxicity (Song *et al.*, 2007; Wang *et al.*, 2007). Moreover, the effectiveness of a number of iron chelators such as deferoxamine (DFO), clioquinol to attenuate neurotoxins-induced animal models of PD further confirms the role of iron in the progression of neurodegeneration (Lan and Jiang, 1997; Kaur *et al.*, 2003).

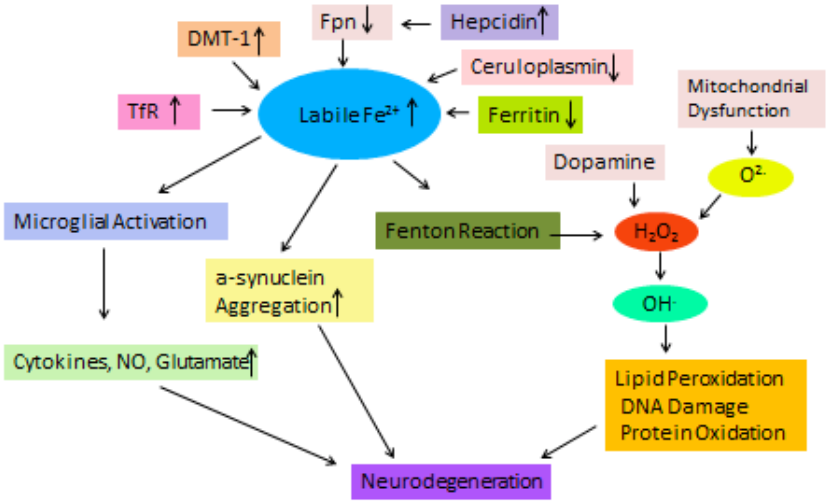

Figure 2-10 Schematic illustration of the role of iron dysregulation in the pathogenesis of PD. DMT-1: divalent metal transporter-1; Fpn: ferroportin; NO: nitric oxide; TfR: transferrin receptor.

VI. The interaction between iron overload, oxidative stress and neuroinflammation in the pathogenesis of PD

Accumulated evidence has suggested that iron overload, oxidative stress and neuroinflammation may interact with each other and result in self-propagating effects leading to or exacerbating the neurodegenerative process (**Figure 2-11**) Iron overload is thought to be related to oxidative stress since the highly toxic hydroxyl radicals are produced (**Figure 2-12**) from superoxide and H_2O_2 through iron dependent Haber-Weiss and Fenton reactions (Puntarulo, 2005). On the other hand, intracellular free iron levels can also be elevated by oxidative stress through several pathways such as superoxide-induced iron release from ferritin, peroxidase-induced iron release from hemeprotein, or nitric oxide and peroxynitrite-induced iron release from iron sulfur clusters (Soum and Drapier, 2003; Dias *et al.*, 2013). It is also suggested that ferrous iron in the dopaminergic neurons can enhance the auto-oxidation and monoamine oxidase mediated dopamine metabolism contributing to the continuous production of ROS (Hermida-Ameijeiras *et al.*, 2004).

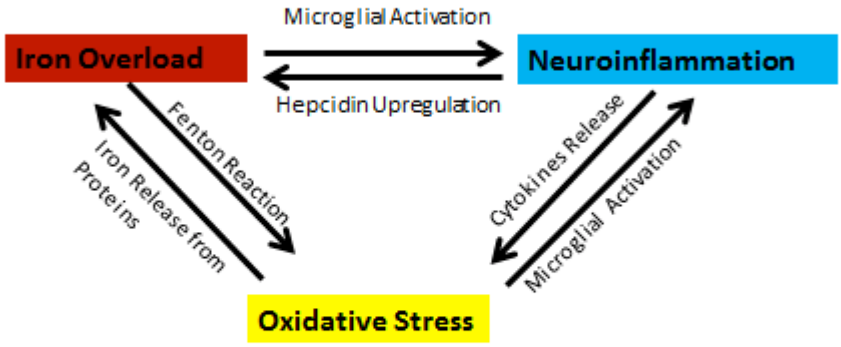

Figure 2-11 The interaction between iron overload, oxidative stress and neuroinflammation.

Fenton Reaction

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\bullet}OH + OH^-$$

Haber-Weiss Reaction

$$O_2^- + H_2O_2 \rightarrow O_2 + {}^{\bullet}OH + OH^-$$

Figure 2-12 Fenton reaction and Haber-Weiss reaction.

Iron accumulation is also involved in mitochondrial dysfunction, which is an important source of oxidative stress in PD. Mitochondrion is the place where free radicals are normally produced during oxidative phosphorylation as by-products. Inhibition of mitochondrial complexes can overthrow the cellular antioxidant capacity and dramatically increase ROS production (Van Laar and Berman, 2009). Studies have shown that mitochondrial complexes activity and expression are reduced in brain and muscle cells of PD patients (Schapira *et al.*, 1989; Bindoff *et al.*, 1991; Van Laar and Berman, 2009). Iron overload can decrease mitochondrial activity through decreasing antioxidant enzyme glutathione (Harley *et al.*, 1993; Pardo Andreu *et al.*, 2009; Urrutia *et al.*, 2014). Moreover, superoxide produced by

electron leak from respiratory chain can further oxidize iron sulfur clusters and cause release of iron contributing to mitochondrial dysfunction and oxidative stress (Liochev and Fridovich, 1999; Isaya, 2014; Bresgen and Eckl, 2015).

The discovery of hepcidin provides a novel insight to explain the interconnected relationship between iron accumulation and neuroinflammation. A recent study shows that oxidative stress can downregulate hepcidin expression via transcriptional factors CCAAT/enhancer-binding protein α and result in iron overload in alcohol fed mice (Harrison-Findik *et al.*, 2006). Another study found that H_2O_2 with low concentrations (0.3 μM-6 μM) is able to induce hepcidin expression through STAT3 signaling pathway (Millonig *et al.*, 2012). Hepcidin is also induced by inflammatory signals such as IL-6 through JAK-STAT 3 pathway leading to iron accumulation in neurons and glial cells (Myhre *et al.*, 2013; Urrutia *et al.*, 2013; Qian *et al.*, 2014). Moreover, Iron accumulation may in turn increase the activation of nuclear factor kB and the secretion of pro-inflammatory cytokines and induce neuroinflammation (Lin *et al.*, 1997; Wessling-Resnick, 2010). Recent research shows iron accumulation in microglial cells can stimulate microglial activation and enhance the release of pro-inflammatory cytokines and free radicals (Rathnasamy *et al.*, 2013), creating the snowball effects among oxidative stress, iron accumulation and neuroinflammation, subsequently causing neurodegeneration.

Treatment for PD

1. Conventional treatment

Conventional treatment for PD can significantly improve the motor symptoms but unfortunately is not able to slow down the progression of the disease. The most common

drugs include dopamine precursor L-dopa, dopamine agonists, catechol-o-methyl-transferase inhibitors and other non-dopaminergic agents (Jankovic and Aguilar, 2008). L-dopa is an effective dopamine replacement agent and has been widely used in clinics for more than 40 years (Salat and Tolosa, 2013). It is the most potent drug that improves patients' life quality, and considered as gold standard to relieve PD symptoms. L-dopa is always administered with a dopa-decarboxylase inhibitor such as carbidopa, which blocks its peripheral conversion to dopamine, thereby minimizing the side effects of circulating dopamine and increasing its availability to the brain (Salat and Tolosa, 2013). Although L-dopa is highly effective during the early stages of treatment, prolonged treatment is associated with significant complications such as motor fluctuations (periods on and off), dyskinesia (involuntary movement), and psychiatric problems (Nagatsua and Sawadab, 2009). Dopamine agonists exert their functions by directly activating dopamine receptors. They can significantly attenuate patients' symptoms and are considered as either first therapy in early stage of the disease or as an adjunct to L-dopa. Unlike L-dopa, they do not require enzymatic conversion to an active metabolite, do not depend on the functional capacities of the nigrostriatal neurons (Goldenberg, 2008). However, it also has adverse effects including nausea, neuropsychiatric effects including hallucinations, impulse control disorders (Fernandez and Chen, 2007). Monoamine oxidase B inhibitors are also widely used in treating motor symptoms as both monotherapy and an adjunct to L-dopa (Riederer and Laux, 2011). The basis for using it in PD is that it enhances striatal dopaminergic activity by inhibiting the metabolism of dopamine (Fernandez and Chen, 2007). Research shows that monoamine oxidase B inhibitor has weaker symptomatic effects than L-dopa and dopamine agonist, and cannot delay the progression of the disease (Macleod *et al.*, 2005; Caslake *et al.*, 2009).

2. Nutritional approaches to prevent PD

The role of nutrition has gained increasing attention in PD and some components in certain food groups have been identified as neuroprotective agents. Phytochemicals are the bioactive plant compounds in fruits, vegetables, and grain. A growing body of evidence suggests that the high intake of food rich in phytochemicals such as vegetables and fruits was inversely associated with PD risk (Gao *et al.*, 2007; Okubo *et al.*, 2012). Evidence from the experimental studies suggest phytochemicals can directly scavenge ROS, enhance mitochondrial complex I activity, inhibit the production of pro-inflammatory cytokines (Shah and Duda, 2015). For example, research shows blueberry or strawberry extracts showed favorable neuroprotective effects including increasing dopamine release, relieving oxidative stress and suppressing neuroinflammation (Gao *et al.*, 2012).

B vitamins, especially folate, vitamin B12, and vitamin B6 may correlate with PD through regulating homocysteine level (Shen, 2015). Homocysteine is a sulfur containing metabolite in methionine cycle and might increase PD risk by exacerbating oxidative stress, mitochondrial dysfunction, damaging DNA and depleting energy reserves, and eventually inducing apoptosis in dopaminergic neurons(Agim and Cannon, 2015). Since B vitamins are cofactors for homocysteine metabolism, high intake B vitamins may exert neuroprotection by reducing plasma homocysteine. An experimental study shows mice with folate deficient diet exhibit elevated levels of plasma homocysteine, which exacerbates MPTP-induced dopamine depletion, neuronal degeneration and motor dysfunction (Duan *et al.*, 2002). In addition, a recent meta-analysis study based on 10 eligible studies shows association between higher dietary intake of vitamin B6 and a decreased risk of PD (Shen, 2015).

Vitamin E is a fat soluble vitamin found in vegetable oils, seeds, nuts and wheat germ. It is an efficient scavenger of free radicals by hydrogen atom transfer reaction (Niki, 2014). It is the major lipid soluble antioxidant in the body that protects membranes from lipid peroxidation by trapping peroxy radicals. Research has suggested the a protective or preventative role of vitamin E in PD (Agim and Cannon, 2015). An animal study shows repeated intramuscular administration of vitamin E protected against 6-OHDA-induced nigrostriatal dopaminergic neurons degeneration (Roghani and Behzadi, 2001). Another study shows vitamin E could partially prevent intra-nigral injection of MPP^+-induced inhibition of dopamine uptake (Barc *et al.*, 2002). In addition, mice with vitamin E deficiency were found more sensitive to the dopaminergic neurotoxicity MPTP in SN (Adams *et al.*, 1990). A meta-analysis also found that both moderate and high intake of vitamin E protected against PD (Etminan *et al.*, 2005).

3. The therapeutic role of iron chelator in PD

Based on the involvement of iron dysregulation in the pathogenesis of PD, iron chelators aimed to reduce excess brain iron currently shows great promise and might provide a new insight into therapies directed towards prevention or slowing down the disease progression of PD (**Table. 1**). Iron chelators refer to a group of chemicals typically containing oxygen, nitrogen or sulfur donor atoms that can chelate with iron (Hatcher *et al.*, 2009). The hexadentate DFO, the bidentate deferiprone, and the tridentate chelator deferasirox are three most used iron chelators in the clinical studies to treat iron overloading diseases such as thalassemia (Flora and Pachauri, 2010). A recent animal study shows that systemic administration of these three iron chelators significantly attenuated 6-OHDA- induced loss of dopaminergic neurons and striatal dopamine contents in rats (Dexter *et al.*, 2011). Moreover,

Clinical trials demonstrate that deferiprone therapy significantly decreased iron content in specific brain regions and slightly improved patients' motor signs with no apparent side effects or resolved neutropenia(Kwiatkowski *et al.*, 2012; Devos *et al.*, 2014; Ward *et al.*, 2015), indicating the potential usage of iron chelation therapy for PD treatment. Clioquinol (CQ) is an 8-hydroxyquinoline derivative that chelates copper, iron and zinc, and has been investigated in neurodegenerative disorders because of its iron chelating properties (Mounsey and Teismann, 2012). Research shows that oral administration of CQ significantly attenuates MPTP-induced neurotoxicity by reducing SN iron level and inhibiting oxidative stress (Kaur *et al.*, 2003). A recent study shows that CQ rescued Parkinsonism and dementia phenotypes of the tau knockout mouse by increasing tyrosine hydroxylase activity, reducing iron level in the brain, and increasing brain derived neurotrophic factor levels in the hippocampus (Lei *et al.*, 2015).

Although treatment with iron chelators might slow down the disease process, disadvantages associated with iron chelation therapy including their low bioavailability, poor blood brain barrier permeability and toxic side effects limited their further investigation in clinical settings (**Table 2-1**). For example, research shows the absorption of DFO in the gastrointestinal tract is really low (Flora and Pachauri, 2010). Moreover, blood brain barrier was relatively impermeable to DFO (Ward *et al.*, 2012). Large dose of DFO has to be given to overcome its low bioavailability and low availably to brain, which may lead to side effects including ophthalmic, auditory toxicity, bacterial and fungal infections, alterations in blood histology, allergic and skin reaction (Flora and Pachauri, 2010). Toxic side effects associated with CQ such as neurological symptoms, spinal cord abnormalities, Vitamin B 12 deficiency also hinders its further investigation in clinical studies (Bareggi and Cornelli, 2012). Future

studies are needed to develop more potent iron chelators and evaluate the therapeutic efficacy, toxicity, bioavailability of iron chelators in PD treatment.

Table 2-1Neuroprotection of iron chelators

	Neuroprotection in animal models or clinical trials	Blood brain barrier permeability	Toxicity
Deferoxamine	Protect against 6-OHDA- or MPTP-induced neurotoxicity in animals (Ben-Shachar *et al.*, 1991; Lan and Jiang, 1997; Dexter *et al.*, 2011).	Limited ability due to hydrophilic nature (Hanson *et al.*, 2009; Liu *et al.*, 2010; Ward *et al.*, 2012).	Ophthalmic and auditory toxicity; bacterial and fungal infections; alterations in blood histology; allergic and skin reaction (Flora and Pachauri, 2010).

Table 2-1 continued

Deferiprone	Protect against 6-OHDA- or MPTP-induced neurotoxicity in animals (Dexter *et al.*, 2011; Devos *et al.*, 2014); 30 mg/kg/day in two doses for 6 months was found to reduce iron deposits in certain brain regions in patients and improve patients' motor signs in clinical trials (Abbruzzese *et al.*, 2011; Kwiatkowski *et al.*, 2012; Devos *et al.*, 2014).	Good ability due to physicochemical characteristics including low molecular weight, favorable octanol: water partition coefficient, neutral charge, and lipophilicity (Habgood *et al.*, 1999; Abbruzzese *et al.*, 2011).	Arthropathy, gastrointestinal symptoms, headache, and moderate zinc deficiency; neutropenia (Flora and Pachauri, 2010).
Deferasirox	Protect against 6-OHDA-induced neurotoxicity in animals (Dexter *et al.*, 2011).	Limited ability due to water insolubility and low bioavailability (Finkenstedt *et al.*, 2010; Goswami *et al.*, 2015)	Renal disease; acute renal failure; Fanconi syndrome (Grange *et al.*, 2010)

Table 2-1 continued

Clioquinol	Protect against MPTP-induced neurotoxicity in animals and rescue Parkinsonism and dementia phenotypes of the tau knockout mouse (Kaur *et al.*, 2003; Lei *et al.*, 2015).	Cross blood brain barrier due to lipophilic characteristics (Kaur *et al.*, 2003); Brain plasma ratio was 20%; 7% was found in cerebrospinal fluid (Bareggi and Cornelli, 2012).	Neurotoxicity; neurological symptoms; spinal cord abnormalities; Vitamin B 12 deficiency (Bareggi and Cornelli, 2012).
EGCG	Protect against MPTP- or 6-OHDA-induced neurotoxicity in animals (Choi *et al.*, 2002; Li *et al.*, 2006; Bitu Pinto *et al.*, 2015); Green tea consumption (3 cups/day) for 3 months significantly increased antioxidant enzymes and decreased oxidative damage in PD patients (Chen *et al.*, 2015).	May accumulate in the brain after given repeatedly (Suganuma *et al.*, 1998; Mahler *et al.*, 2013)	Maybe hepatotoxic at higher dose (Mereles and Hunstein, 2011).

Recently, natural iron chelators derived from food and plant has attracted increasing interest because of their safety, low toxicity and general acceptance. Phytic acid is a natural antioxidant by its ability to chelate metal ions such as iron, copper and scavenge hydroxyl radicals (Rao *et al.*, 1991). Our previous research shows that phytic acid can protect both MPP^+- and 6-OHDA-induced apoptosis by attenuating caspase 3 activity and decreasing DNA fragmentation in vitro models of PD (Xu *et al.*, 2008; Xu *et al.*, 2011). Although phytic acid is considered as a safer alternative to synthetic iron chelators, its inability to cross blood brain barrier hinders its further investigation in the animal models of PD and clinical settings.

4. The neuroprotective effect of EGCG in PD

Green tea is a popular beverage in the world endowed with biological and pharmacological properties. Green tea consumption has been shown to be useful for prevention of many diseases including different types of cancer, cardiovascular disease and liver disease (Chacko *et al.*, 2010). Research also suggests green tea consumption is inversely correlated with the incidence of neurodegenerative disorders such as PD and Alzheimer's disease, which may explain the lower incidence of neurodegenerative disorders in Asians than in people in western countries (Mandel *et al.*, 2008). The major bioactive constituent of tea is catechin, which includes epicatechin (EC), epigollocatechin (EGC), epicatechin gallate (ECG) and epigallocatechin gallate (EGCG) (**Figure 2-13**).

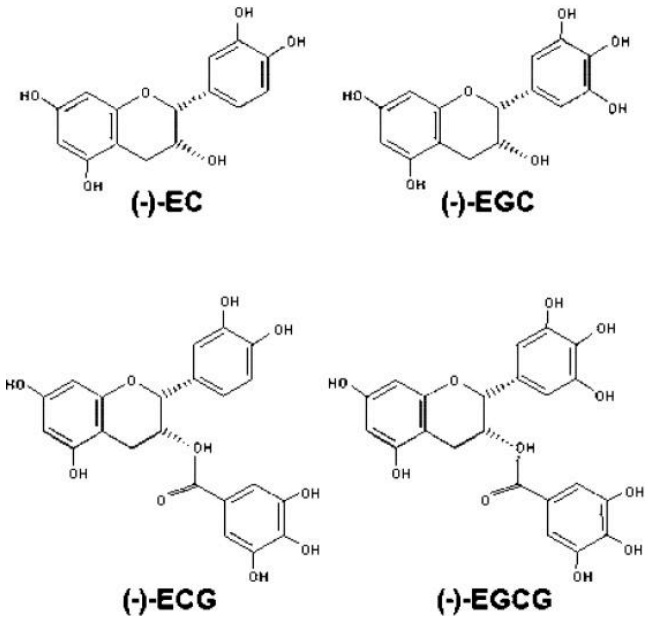

Figure 2-13 Structure of epicatechin (EC), epigollocatechin (EGC), epicatechin gallate (ECG) and epigallocatechin gallate (EGCG). Figure reproduced from (Ravindranath *et al.*, 2006).

Among those catechins, EGCG is the most abundant, best studied and possibly most potent polyphenol (Bode and Dong, 2009). Research has suggested that beneficial effects of EGCG include its antioxidant effects, antiangiogenic and antitumor effect, enhancing weight loss, protecting skin from ionizing radiation damage, and neuroprotective effects (Nagle *et al.*, 2006). Epigallocatechin gallate is by far the most studied natural iron chelator for the treatment of PD. Several epidemiology studies have demonstrated the reduced risk of PD in population with tea consumption (Chan *et al.*, 1998; Checkoway *et al.*, 2002; Tan *et al.*, 2008). Substantial evidence suggests that neuroprotective effect of tea is partially due to its most abundant polyphenol EGCG (Levites *et al.*, 2002; Higdon and Frei, 2003). In vitro studies have shown that EGCG prevented neuronal cell death caused by neurotoxins such as 6-OHDA, MPP^+ (Levites *et al.*, 2002; Ye *et al.*, 2012). In agreement with in vitro findings, in vivo studies have shown that EGCG protected MPTP-induced striatal dopamine depletion

and loss of TH positive neurons by inhibiting microglial cell activation and nitric oxide synthase (NOS) activity (Choi *et al.*, 2002; Li *et al.*, 2006). A recent study shows EGCG also protected 6-OHDA-induced neurotoxicity by reversing striatal oxidative stress and inhibiting pro-inflammatory enzymes cyclooxygenase-2 (COX-2) and inducible NOS (Bitu Pinto *et al.*, 2015). The mechanisms underlying the neuroprotective effect of EGCG includes its chelating ability due to the 3,4-dihydroxyl groups and the gallate group in the structure, its regulation of antioxidant enzymes superoxide dismutase and catalase and its inhibition of microglia activation and TFN α release (Mandel *et al.*, 2005; Mandel *et al.*, 2008). In addition, EGCG was shown to prevent apoptosis through regulating pro-survival phosphatidylinositol 3-kinase (PI3K)/AKT and protein kinase C, inhibiting pro-apoptotic kinase pathways, upregulating the expression of anti-apoptotic proteins like Bcl-2 and downregulating pro-apoptotic molecules such as Bax and Bad (Levites *et al.*, 2002). Epigallocatechin gallate was also found to mediate the expression of nuclear factor erythroid 2 p45 (NF-E2) related factor (Nrf2), which can further induce detoxifying and antioxidant enzymes such as glutathione peroxidase, glutathione S transferase (Na and Surh, 2008). Recent studies also suggest EGCG could modulate mitochondrial functions such as impacting mitochondrial biogenesis, bioenergetics control (ATP production and anabolism) (Oliveira *et al.*, 2016). EGCG was found to protect mitochondrial function by promoting fusion and suppressing fission and autophagy. Moreover, EGCG was also found to upregulate the activities of mitochondrial enzymes involved in the maintenance of the tricarboxylic acid cycle (TCA cycle) and electron transport chain complexes in aged brain mitochondria (Srividhya *et al.*, 2009).

The natural origin of EGCG and its ability to cross the blood brain barrier makes it more attractive for PD treatment. It is reported that EGCG could be easily absorbed from the

digestive tract and widely distributed into various organs, including the brain, which had a similar concentration to the level found in the liver, kidney, lung, heart, spleen and pancreas (Levites *et al.*, 2001). Although the protective effect of EGCG in PD is extensively investigated in preclinical studies, data regarding the beneficial effects in clinical trials are rare or non-conclusive (Mahler *et al.*, 2013). Recent study shows that green tea consumption (3 cups/day) for 3 months significantly increased antioxidant enzymes including catalase and SOD, decreased oxidative damage including lipid peroxidation and protein carbonyls (Chen *et al.*, 2015).

General summary

In summary, research has demonstrated the central role of iron in the pathogenesis of PD. Its potential interaction with other factors such as oxidative stress and neuroinflammation can result in a snow ball effect leading to the subsequent neurodegeneration. Based on the involvement of iron dysregulation in the pathogenesis of PD, use of iron chelators might be a promising therapy for prevention or slowing down the progress of the disease. Epigallocatechin gallate is the major polyphenol in green tea and it has gained attention due to its antioxidant, iron chelating and anti-inflammatory properties. Research has demonstrated the neuroprotection of EGCG in both in vitro and in vivo models of PD. However, future studies are needed to understand the mechanisms underlying the neuroprotection of EGCG and its therapeutic values in clinical settings.

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CHAPTER 3 HEPCIDIN PLAYS A KEY ROLE IN 6-OHDA-INDUCED IRON OVERLOAD AND APOPTOTIC CELL DEATH IN A CELL CULTURE MODEL OF PARKINSON'S DISEASE[1,2]

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Abstract

Background: Elevated brain iron levels have been implicated in the pathogenesis of Parkinson's disease (PD). However, the precise mechanism underlying abnormal iron accumulation in PD is not clear. Hepcidin, a hormone primarily produced by hepatocytes, acts as a key regulator in both systemic and cellular iron homeostasis. **Objective:** We investigated the role of hepcidin in 6-hydroxydopamine (6-OHDA)-induced apoptosis in a cell culture model of PD. **Methods:** We downregulated hepcidin using siRNA interference in N27 dopaminergic neuronal cells and compared with control siRNA transfected cells to investigate the role of hepcidin in 6-OHDA-induced neurodegeneration. **Results:** Hepcidin knockdown (32.3% $P<0.0001$) upregulated ferroportin expression and significantly ($P<0.05$) decreased intracellular iron by 25%. Hepcidin knockdown also reduced 6-OHDA-induced caspase-3 activity by 42% ($p<0.05$) and DNA fragmentation by 29% ($p=0.086$) and increased cell viability by 22% ($P<0.05$). In addition, hepcidin knockdown significantly attenuated 6-

OHDA-induced protein carbonyls by 52% ($p<0.05$) and intracellular iron by 28% ($p<0.01$), indicating the role of hepcidin in oxidative stress. **Conclusions:** Our results demonstrate that hepcidin knockdown protected N27 cells from 6-OHDA-induced apoptosis, and that hepcidin plays a major role in reducing cellular iron burden and oxidative damage by possibly regulating cellular iron export mediated by ferroportin.

Key words: Parkinson's disease, hepcidin, ferroportin iron, 6-OHDA.

Introduction

Parkinson's disease (PD) is an incurable neurodegenerative disease that affects more than 1% of people over 65 years old and approximately 4% of the population aged over 80 years [1]. The prevalence is expected to rise sharply within the next two decades because of progressive aging population [2]. Parkinson's disease is characterized by the progressive loss of dopaminergic neurons in the substantia nigra (SN), degeneration of projecting nerve fibers in the striatum, and accumulation of intracytoplasmic inclusions, known as Lewy bodies [3]. Although the etiology of PD is not clear, both genetic and environmental risk factors including exposure to metals and pesticides are considered to be involved in PD [4].

Iron, the most abundant trace metal in the brain, is thought to play an important role in the pathogenesis of PD. Studies have demonstrated the association between iron dysregulation and PD. Increased levels of iron deposits in the SN are observed in postmortem studies, as well as in 6-hydroxydopamine (6-OHDA)- and 1-methyl-4 phenyl-1,2,3,6,-tetrahydropyridine (MPTP)-induced PD animal models [5]. The imaging studies of living PD patients also confirmed the presence of accumulation of iron in the SN and linked the extent of iron deposits to the severity of disease [6]. Although iron is important in various

physiological functions, such as DNA synthesis, mitochondrial respiration and oxygen transport [7], free iron is potentially toxic as it is involved in the generation of hydroxyl radicals, which can react with lipid, protein and DNA, leading to subsequent neuronal damage and death. Moreover, free iron in dopaminergic neurons can accelerate toxic alpha-synuclein fibril formation, leading to neuronal dysfunction [8].

Because of potential toxicity of iron, iron homeostasis is tightly regulated by a complex system that coordinates iron uptake, release, storage, and utilization. For example, iron is delivered to tissues by circulating transferrin, and excess iron in the cell is stored in the cytosolic ferritin [9]. Hepcidin is a small peptide that mainly secreted by hepatocytes in response to inflammation, iron overload and oxidative stress [10, 11]. It controls systemic iron levels by regulating iron absorption from the intestine, the release of iron from degraded hemoglobin from macrophages, and stored iron from hepatocyte [12]. Hepcidin is also considered as a master regulator in the management of cellular iron homeostasis by binding to iron exporter protein ferroportin in cell membranes and causing its subsequent internalization and lysosomal degradation [3]. Although hepcidin is predominantly expressed in the liver, recent research demonstrates that hepcidin is also widely distributed in the central nervous system. One study showed that hepcidin mRNA level is increased with aging in mouse brain, particularly in the cerebral cortex, hippocampus, and striatum, which leads to decreased level of ferroportin (Fpn) and the associated iron accumulation in aging brain [13]. Another study showed that peripheral iron overload induces hepcidin and decreased level of Fpn in the SN of rats, suggesting the critical role of hepcidin plays in brain iron disturbance [14]. The objective of this study was to determine the role of hepcidin in 6-OHDA-induced cell death by knocking down hepcidin expression in N27 dopaminergic cell model of PD.

Materials and methods

Chemicals

The immortalized rat mesencephalic dopaminergic neuronal cell line (1RB3AN27, generally referred to as N27) was a kind gift from Dr. Kedar N. Prasad, University of Colorado Health Sciences Center (Denver, CO). RPMI-1640 medium, fetal bovine serum, L-glutamine, penicillin, and streptomycin were obtained from Invitrogen (Carlsbad, CA). Calcein-AM, ascorbic acid, mouse β-actin antibody, 6-OHDA, ferrous sulfate, ascorbic acid were purchased from Sigma Aldrich (St. Louis, MO). The Amaxa Nucleofector kit was purchased from Lonza (Allendale, NJ). The Absolutely RNA Miniprep kit and High Capacity cDNA Archive kit were purchased from Stratagene (La Jolla, CA) and Life Technologies (Grand Island, NY), respectively. The hepcidin specific siRNA and scrambled siRNA were purchased from Integrated DNA Technologies (Coralville, IA). Substrate for caspase-3, Acetyl-Asp-Glu-Val-Asp-AFC was obtained from MP Biomedicals (Solon, OH). The Cell Death Detection ELISA Plus kit was purchased from Roche Diagnostics (Indianapolis, IN). Protein Carbonyls Colorimetric Assay kit was purchased from Cayman Chemical (Ann Arbor, MI). The rabbit polyclonal antibody for Fpn was purchased from Alpha Diagnostic (San Antonio, TX). Alexa Fluor 680 conjugated anti-mouse IgG and IRdye 800 conjugated anti-rabbit IgG were purchased from Invitrogen (Carlsbad, CA) and Rockland Inc. (Gilbertsville, PA), respectively. All solutions were prepared fresh prior to each assay.

Cell culture

N27 cells were grown in RPMI-1640 medium containing 10% fetal bovine serum, 2 mmol/l L-glutamine, 50 units penicillin, and 50 µg/ml streptomycin and maintained at 37^{o}C

in a humidified atmosphere containing 5% CO_2, as described in our previous publication [15].

Transient transfections and treatment paradigm

N27 cells were transfected with hepcidin specific siRNA (hepcidin siRNA) or scrambled siRNA (control siRNA) using the Amaxa Nucelofector kit, following the manufacturer's instructions. Briefly, $3x10^6$ cells were resuspended in 100 µL of the Nucleofector solution, along with 1.5 µg of hepcidin siRNA or control siRNA, and subsequently subjected to electroporation using the nucleofector program no. A23. After 72 h of initial transfection, cells were harvested and hepcidin mRNA was analyzed using quantitative real-time RT-PCR to confirm the knockdown efficiency. To evaluate the effect of hepcidin knockdown on 6-OHDA-induced neurotoxicity, both control siRNA and hepcidin siRNA transfected cells were plated for 48 h and treated with or without 100 µM 6-OHDA for 6 h. Cells were collected at the end of each treatment for the following experiments.

Quantitative real-time RT-PCR

Total RNA was isolated and converted to cDNA using the Absolutely RNA Miniprep kit and High Capacity cDNA Archive kit, respectively. Real-time PCR was performed using a Brilliant SYBR Green QPCR Master Mix kit and the Mx3000P QPCR system, as described in our previous publication [16]. The 18s rRNA was used as an internal control for quantifying RNA with the primer set purchased from SABiosciences (Valencia, CA). The reaction mixture included 2 µl of cDNA, 12.5 µl of 2X master mix, and 0.2 µmol/L each primer. Cycling conditions contained an initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s and annealing at 60°C for 10 min. Fluorescence was detected during the annealing/extension step of each cycle. Dissociation curves were run

to verify the singularity of the PCR products. The data were analyzed using the comparative threshold cycle method as described in our previous publication [16].

Western blot assays for ferroportin

Cell lysates were prepared using a modified radio immunoprecipitation assay (RIPA) buffer as described previously [17]. Equal amounts of protein were loaded for each sample and separated on 12% SDS-PAGE gels. After separation, the proteins were transferred onto a nitrocellulose membrane and were incubated with the rabbit polyclonal antibody directed against Fpn (1:1000) and developed with IR-dye 800 anti-rabbit secondary antibody (1:5000). β-actin was used as the loading control. Membranes were visualized on an Odyssey Infrared Imaging system (LICOR, Lincoln, NE).

Calcein quenching to measure intracellular iron levels

The intracellular iron levels were determined by a calcein fluorescence quenching method modified from a previous study [18]. Calcein-AM is a membrane permeable, non-fluorescent molecule that becomes fluorescent by intracellular esterases. It is quenched rapidly by Fe^{2+} or Fe^{3+} and is a good indicator of the 'labile iron pool' [18]. After the treatment, cells were incubated with calcein-AM in HEPES-buffered saline (HBS) for 30 min at 37^{o}C. The excess calcein on the cell surface was washed off three times with HBS, and fluorescence was recorded using a Synergy II microplate reader (BioTek Instruments, Winooski, VT) at 485 nm excitation and 530 nm emission. Change in fluorescence intensity (with and without treatment after normalizing to protein concentrations) reflected the intracellular iron levels. Calcein fluorescence pictures were obtained with FLoid ® Cell Imaging Station (Life technologies).

Cell viability assays

Cell viability was measured using MTT assay as described earlier [15]. After each treatment, cells were incubated with serum-free RPMI medium containing 0.25 mg/mL MTT solution for 3 h at 37°C, followed by adding isopropanol–HCl (200 µL) solution to dissolve intracellular purple formazan. The absorbance was read at 570 nm with a reference wavelength of 630 nm using a microplate reader (Molecular Devices, Sunnyvale, CA).

Caspase-3 activity assays

Caspase-3 activity was measured as described previously [19]. After treatment, the cell pellet after centrifugation was lysed with Tris buffer (50 mol/L Tris-HCL, 1 mmol/L EDTA, and 10 mmol/L EGTA at pH=7.4) containing 10 µmol/L digitonin for 20 min at 37^{o}C. Lysates were subjected to a quick centrifugation at 14,000 x g and then incubated with a specific fluorescent substrate (Ac-DEVD-AFC, 50 µmol/L) for 1 h at 37^{o}C. The caspase-3 activity was measured with excitation at 400 nm and emission at 505 nm using a fluorescence microplate reader. The caspase-3 activity was expressed as fluorescent units/mg protein.

DNA fragmentation assays

DNA fragmentation assays were performed using the Cell Death Detection ELISA Plus kit as described previously [17]. After treatment, cell pellet was incubated with lysis buffer provided in the kit. The lysates were then centrifuged and the supernatant was incubated for 2 h with the mixture of HRP-conjugated antibody cocktail that recognizes histones, single- and double-stranded DNA. After washing away the unbound components, measurements were made at 490 nm and 405 nm using a fluorescence microplate reader. DNA fragmentation was expressed as absorbance units/mg protein.

Protein carbonyl assays

The oxidative damage to proteins was determined by measuring the protein carbonyl residues using DNPH (2,4-dinitrophenylhydrazine) according to manufacturer's protocol. DNPH reacts with protein carbonyls to produce the corresponding hydrazones, which was measured spectrophotometrically at the wavelength of 360 nm. The carbonyl content was determined from the differences in absorbance between DNPH-reacted samples and non-reacted HCL samples, and then standardized against the protein concentrations in the samples.

Statistical analysis

Data were analysed using the GraphPad Prism 5.0 (GraphPad Software, Inc. La Jolla, CA). All values were expressed as mean ± SEM. Student t test was used to compare the differences between groups. The values for two (control and hepcidin) siRNA transfected cells with 6-OHDA treatments were normalized to their respective controls (without 6-OHDA treatment) before statistical analysis. All the mean differences were considered significant at $p \leq 0.05$.

Results

Downregulation of hepcidin

To address the role of hepcidin in regulation of 6-OHDA-induced neurotoxicity, we first utilized RNA interference (RNAi) technique to downregulate hepcidin levels in N27 dopaminergic cells. As shown in **Figure 3-1A**, hepcidin mRNA levels were significantly downregulated (32.3% $P<0.0001$) in hepcidin siRNA transfected cells compared with control siRNA transfected cells. Since hepcidin regulates Fpn by triggering its degradation, we further determined whether downregulation of hepcidin leads to an increased Fpn protein

expression (**Figure 3-1B**). Compared to the control siRNA transfected cells, the Fpn protein levels were elevated in hepcidin siRNA transfected cells, which confirmed a negative relationship between hepcidin and Fpn expression in dopaminergic cells. We also measured intracellular iron, which was indirectly measured by calcein fluorescence quenching, to ascertain whether decreased expression of hepcidin and increased expression of Fpn reduced intracellular iron levels. To confirm calcein fluorescence quenching method by intracellular iron, we incubated the cells with or without 1 mM exogenous iron (ferrous sulfate in ascorbic acid solution, 1:44 molar ratio, pH 6.0) for 30 min and then examined the calcein quenching by fluorescence microscopy. As shown in **Figure 3-1D**, supplementation of 1 mM ferrous sulfate effectively decreased calcein fluorescence. As shown in **Figure 3-1C**, hepcidin knockdown significantly decreased intracellular iron by 25% ($P<0.05$).

Hepcidin knockdown protects N27 cells from 6-OHDA-induced cytotoxicity

All the values presented in **Figure 3-2** were presented as percentage of respective controls, control and hepcidin siRNAs not treated with 6-OHDA.We evaluated the effect of hepcidin knockdown on 6-OHDA mediated cell death using MTT assay (**Figure 3-1A**). Hepcidin knockdown significantly lessened the toxic effect of 6-OHDA by increasing cell viability by 22% ($P<0.05$). When apoptosis was measured, hepcidin knockdown reduced 6-OHDA-induced caspase-3 activity significantly (**Figure 3-1B**; 42%; $P<0.05$). DNA fragmentation was also reduced, but it was only marginally significant (**Figure 3-1C**; $p= 0.086$). Together, these results demonstrate that hepcidin knockdown protects against 6-OHDA-induced cell apoptosis.

Hepcidin downregulation reduces 6-OHDA-induced protein oxidative damage and intracellular iron

As shown in **Table** 3-1, hepcidin knockdown decreased 6-OHDA-induced protein carbonyls by 52% ($P<0.05$) and intracellular iron by 28% ($p<0.01$). These results show that hepcidin knockdown might protect against 6-OHDA-induced neurotoxicity through attenuating oxidative stress by mediating intracellular free iron.

Discussion

Iron is an essential nutrient and involves in many functions, such as acting as a cofactor for key enzymes involved in neurotransmitter biosynthesis [20] . On the other hand, excess free iron can cause significant oxidative stress by involving in the production of hydroxyl radical formation, glutathione consumption, protein aggregation, lipid peroxidation, and nucleic acid modification [21, 22]. Human body has differential expression of iron regulatory proteins to tightly regulate free iron levels to minimize its amount iron available to participate in free radical formation. Among those proteins, hepcidin is considered as a principal regulator because of its function to inhibit cellular efflux of iron by binding to Fpn at the cell surface and inducing its subsequent degradation [23].

Recent studies have suggested a critical role for hepcidin in a variety of disorders, including anemia of inflammation, chronic kidney disease, and familial hemochromatosis [23-25]. However, the participation of hepcidin in neurodegenerative disorders is very limited. In our earlier study in cell culture [26], 6-OHDA increased the expression hepcidin and decreased the expression of Fpn, which made us to design this current study to investigate the effect hepcidin with knockdown experiments. We used a N27 dopaminergic neuronal cell model to detect hepcidin and Fpn expression, since N27 cell line possesses all physiological and biochemical properties of dopaminergic neurons [27]. Our results show

that both hepcidin and Fpn are expressed in N27 cells, and that knockdown of hepcidin remarkably increased Fpn expression and reduced intracellular iron levels as measured by calcein quenching. These results are consistent with previous studies, which demonstrate that hepcidin is widely expressed in murine brain and might play a key role in regulating iron levels in the brain by down-regulating Fpn expression [13, 28, 29].

Our study also shows that regulation of brain iron efflux by hepcidin may play a protective role in 6-OHDA-induced neurotoxicity. Hepcidin knockdown and subsequent upregulation of Fpn protein significantly attenuated the protein oxidative damage induced by 6-OHDA, ultimately leading to a reduction in cell apoptosis, as evidenced by decreased caspase-3 activation and marginally decreasing DNA fragmentation. Increasing caspase-3 activity and DNA fragmentation respectively by more than 2- and 1.5-fold in the control siRNA cells but only showing a small increase in hepcidin siRNA cells with 6-OHDA treatment clearly shows the protection with hepcidin downregulation. 6-hydroxydopamine is a hydroxylated analogue of the neurotransmitter dopamine and represents a classic neurotoxin used for the initiation of the PD neurodegeneration both in vitro and in vivo [30]. Studies have shown abnormal iron accumulation in 6-OHDA-induced PD models, and 6-OHDA-induced neurotoxicity may result from free iron and the ensuing production of free radical species [31]. However, the precise mechanism underlying abnormal iron accumulation in 6-OHDA-induced neurotoxicity is not very clear. Song et al. [32] demonstrated that upregulation of iron regulatory protein 1 (IRP1) might be responsible for a decreased expression of Fpn and increased cellular iron accumulation. Another study reported that divalent metal transporter-1(DMT-1)+IRE upregulation is involved in 6-OHDA-induced iron accumulation and aggravated oxidative injury [33]. Our study provides

direct evidence for the first time that hepcidin-ferroportin axis at least partially accounts for iron accumulation in 6-OHDA-induced neurodegeneration. Hepcidin knockdown resulted in upregulation of Fpn, which may enhance iron release and alleviate iron accumulation in dopaminergic neurons, and eventually protected neurons from 6-OHDA-induced apoptosis. Our data with calcein quenching support this relationship. In addition to its role in iron homeostasis, hepcidin is also recognized as a principal mediator in inflammation [34, 35], which is also directly linked to the pathogenesis of PD [36, 37]. Thus, further study is needed to study the linkage between hepcidin expression, iron status and neuroinflammation in PD.

Conclusions

In conclusion, our study demonstrates that hepcidin plays an important role in iron accumulation, thus causing oxidative stress and associated neurotoxicity. Hence, the approaches that can reduce hepcidin and increase Fpn expression might be effective strategies in preventing the progression of PD.

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Conflict of interest

Author disclosure: Qi Xu, Anumantha G. Kanthasamy, Huajun Jin and Manju B. Reddy have no conflicts of interest.

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Tables and figures

Table 3-1 The role of hepcidin knockdown on 6-OHDA-induced oxidative damage measured by protein carbonyls (n=4) and intracellular iron measured by calcein quenching method (n=6).

	Protein Carbonyls (nmol/mg protein)	Calcein Quenching (fluorescent units/mg protein)
Control siRNA	18.5±2.9	112.4±4.8
Hepcidin siRNA	8.9±1.1*	80.8±6.2**

Values are mean ± SEM; *P<0.05; **P<0.01. Differences between two groups were based on student's t-test; control siRNA: scrambled small interfering RNA; hepcidin siRNA: hepcidin small interfering RNA.

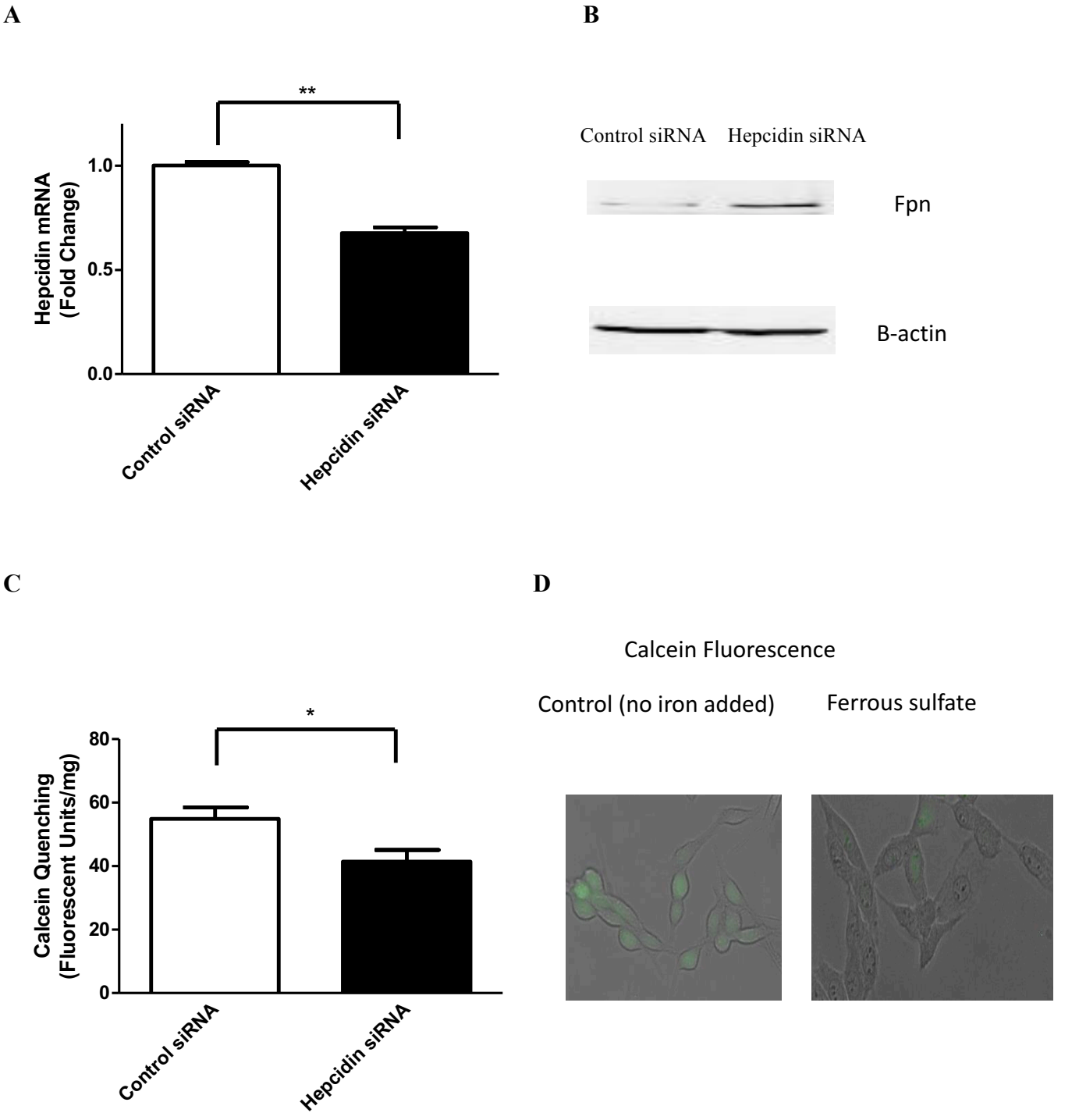

Figure 3-1 Effect of hepcidin knockdown on hepcidin mRNA levels measured by quantitative real-time RT-PCR (A, n=7-8), ferroportin protein levels (normalized to β-actin) measured by Western blot (B) and intracellular iron measured by a calcein quenching method (C, n=6) in N27 cells. Representative calcein fluorescence images with and without incubation of 1 mM ferrous sulfate for 30 min are shown (D). Values

are mean ± SEM. Differences between two groups was based on student's t-test; *P<0.05, **P<0.0001. Control siRNA: scrambled small interfering RNA; Hepcidin siRNA: hepcidin small interfering RNA; Fpn: ferroportin.

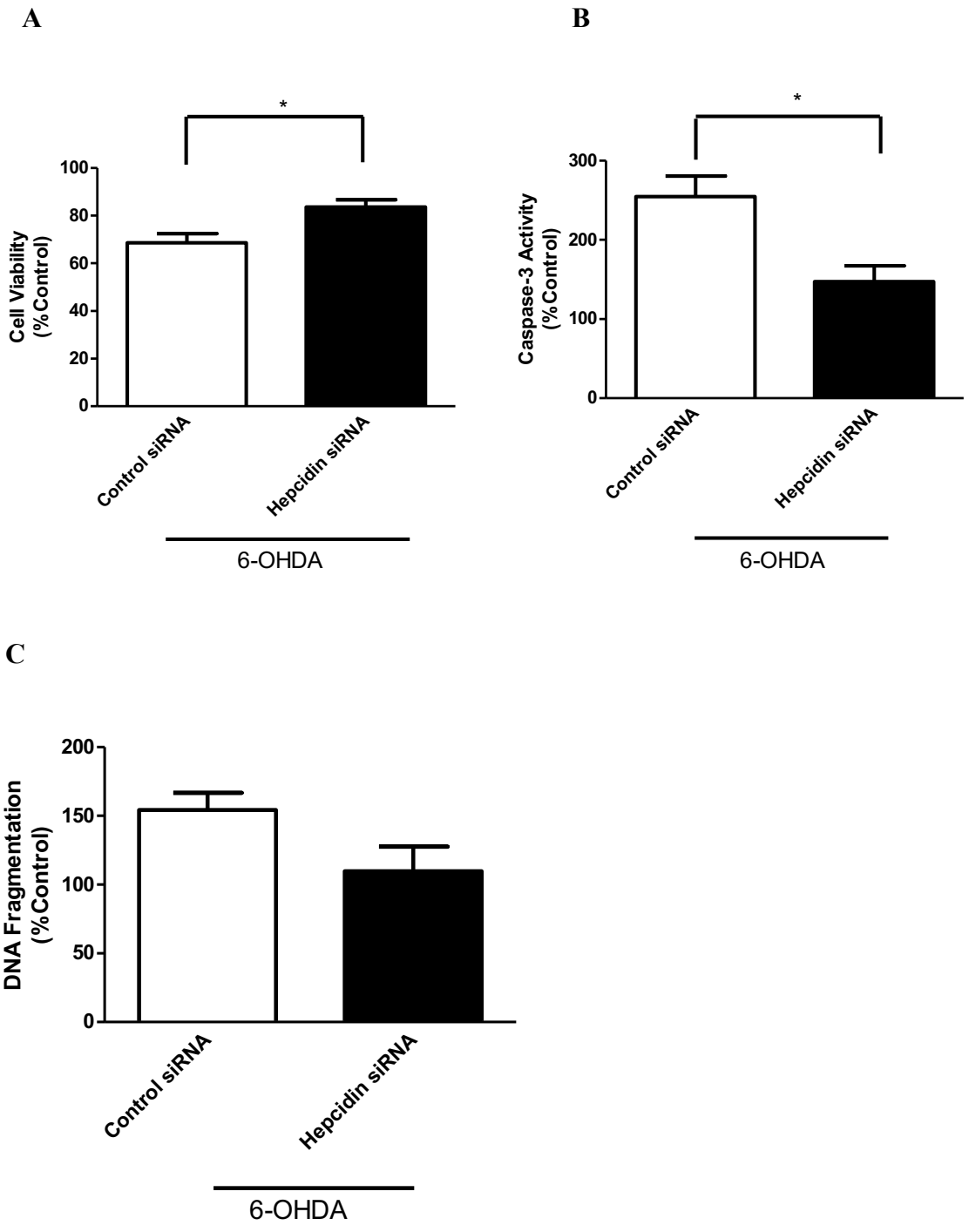

Figure 3-2 The role of hepcidin knockdown on 6-OHDA-induced cytotoxicity measured by MTT (A, n=6), caspase-3 activity (B, n=4-5) and DNA fragmentation (C, n=4) in N27 cells; Cells were treated with 100 µM 6-OHDA for 6 h and the values (mean ± SEM) are normalized to their respective controls without 6-OHDA treatment; *P<0.05, difference

between two groups was based on student's t-test; control siRNA: scrambled small interfering RNA; hepcidin siRNA: hepcidin small interfering RNA.

CHAPTER 4 EPIGALLOCATECHIN GALLATE PROTECTS AGAINST TUMOR NECROSIS FACTOR ALPHA- AND HYDROGEN PEROXIDE-INDUCED APOPTOSIS IN A CELL CULTURE MODEL OF PARKINSON'S DISEASE

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Abstract

Several factors including oxidative stress, iron dysregulation, and inflammation have been implicated in the pathogenesis of Parkinson's disease (PD). Considering the entwined relationship among these factors, Epigallocatechin gallate (EGCG) may be a good candidate due to its antioxidant, iron chelating and anti-inflammatory properties. The objective of this study is to determine whether EGCG protects immortalized rat mesencephalic cells from hydrogen peroxide (H_2O_2)- and tumor necrosis factor alpha (TNFα)-induced neurotoxicity. The neuroprotective effects of EGCG were assessed by cell viability assay, caspase-3 activity, intracellular reactive oxygen species (ROS) generation, and iron related protein expressions. Our results show that caspase-3 activity was increased to 2.8 fold ($P<0.001$) and 1.5 fold ($P<0.01$) with H_2O_2 and TNFα treatment; However, EGCG pretreatment significantly decreased the caspase activity by 50.2% ($P<0.001$) and 30.1% ($P<0.05$). Similarly, cell viability was reduced to 69.2% ($P<0.01$) and 89% ($P<0.01$) by H_2O_2 and TNFα, which is

partially blocked by EGCG pretreatment. Epigallocatechin gallate pretreatment also significantly ($P<0.001$) protected against H_2O_2-induced ROS in a time dependent manner. In addition, both H_2O_2 and TNFα significantly upregulate hepcidin expression and marginally reduce ferroportin (Fpn) expression. However, iron alone treatment had a more significant effect on Fpn than hepcidin. All these effects were partially reversed by EGCG, indicating its ability of altering iron efflux. Collectively, our results show that EGCG protects against both TNFα- and H_2O_2-induced neuronal apoptosis. The observed neuroprotection may be through the inhibition of oxidative stress and inflammation which is possibly mediated by iron regulated proteins hepcidin and Fpn.

Key words: Parkinson's disease EGCG MPTP

Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disorder affecting about 1.5% global population over 65 years old [1]. It is characterized by the progressive degeneration of dopaminergic neurons in the substantia nigra pars compacta (SNpc), which manifests as motor dysfunction including tremor, bradykinesia, postural instability and rigidity [2]. Currently, there are no available therapies that can effectively slow down or reverse the disease progression and there is a great need to understand the pathogenesis and develop the new neuroprotective agents for the treatment or prevention of PD.

Although the etiology of PD has to be established, it is widely accepted that many factors including oxidative stress, inflammation, iron overloading may be the underlying mechanisms that lead to neurodegeneration and development of PD. Oxidative stress is a

condition caused by the imbalance in the production of reactive oxygen species (ROS) and the biological system's antioxidant capacity to detoxify those species and repair the resulting damage [3]. The major consequence of oxidative stress includes damage to nuclei, lipids and proteins, which severely affects cellular function and may induce cell death [4]. Oxidative stress has been thought to be involved in both idiopathic and genetic cases of PD, and oxidative damage such as increased levels of oxidized lipids, proteins, and DNA, and decreased levels of reduced glutathione have been observed in the substantia nigra (SN) of PD patients [5]. A number of sources and mechanisms responsible for the generation of ROS including the metabolism of dopamine, mitochondrion dysfunction and aging have also been implicated in PD pathogenesis [6]. Neuroinflammation is considered as another major component in the pathogenesis of PD, which is demonstrated by the presence of activated microglia in the SN of PD patients or neurotoxins such as 1-methyl-4-phenyl-1,2,3,6-tertrahydropyridine (MPTP)-induced animal models [7, 8]. Microglia are the resident macrophages of the central nervous system playing an essential role in the immune response [9]. However, over-activated or chronically activated microglia are a significant source of oxidative stress and damage the neighboring neurons through the secretion of cytotoxic substances such as nitric oxide or superoxide radicals [10]. In addition, microglia produced pro-inflammatory cytokines such as tumor necrosis factor alpha (TNFα), can mediate direct apoptosis in neurons through the activation of caspase 8 [11].

The role of iron has gained increasing attention in PD due to its complicated interplay with other pathological factors including oxidative stress and neuroinflammation. Although iron possesses essential physiological roles in all organisms, excess iron can participate in Fenton reaction to generate highly reactive hydroxyl radicals leading to lipid peroxidation,

DNA and protein damage. Moreover, iron accumulation might stimulate the activation of glia cells leading to the release of neurotoxic substances such as TNFα, IL-6, nitric oxide contributing to the progression of PD [12]. Accumulated evidence has demonstrated that iron accumulation is a hallmark of several neurodegenerative disorders including PD [12]. It is demonstrated that that iron concentration is significantly elevated in SN in PD patients as well as neurotoxins, such as 6-hydroxydopamine (6-OHDA)-, 1-methyl-4-phenyl-1,2,3,6-tertahydropyridine (MPTP)-, and rotenone-induced PD model in animals [13, 14]. The iron accumulation in PD may be due to altered expressions of iron related proteins such as increased iron importer, divalent metal transporter-1 (DMT-1), transferrin receptor (TFR), decreased iron exporter ferroportin (Fpn) and ceruloplasmin, or altered iron storage protein ferritin or neuromelanin [15]. Hepcidin is a small peptide that controls intracellular iron balance by binding to the sole cellular iron exporter Fpn and inducing its degradation [16]. Recent studies have shown that hepcidin and Fpn are widely expressed in the central nervous system and dysregulated hepcidin-Fpn axis might account for iron accumulation in neurodegenerative disorders [17, 18]

Based on the multifactorial pathogenesis of PD, natural compounds targeted to affect multiple functions are ideal candidates for the prevention or treatment of the disease. Epigallocatechin gallate (EGCG) is the major polyphenol in green tea and it gained attention due to its antioxidant, iron chelating and anti-inflammatory properties [19]. Both in vitro and in vivo studies have shown that EGCG prevented neurotoxin 1-methyl-4-phenylpyridinium (MPP^+)-induced neuronal cell death and MPTP-induced striatal dopamine depletion and loss TH positive neurons, respectively [20-22]. In agreement with in these findings, our recent in vitro study also show that EGCG protected against 6-OHDA-induced neurotoxicity by

regulating genes and proteins involved in brain iron homeostasis [23]. The objective of this study is to further investigate the antioxidant and anti-inflammatory effects of EGCG in a cell culture model of PD. Our hypothesis was that EGCG exerts neuroprotective action against hydrogen peroxide (H_2O_2)- and TNFα-induced neurotoxicity through regulating iron related proteins, hepcidin and Fpn.

Material and Methods

Chemicals

The immortalized rat mesencephalic dopaminergic neuronal cell line (1RB3AN27, generally referred to as N27) was a gift from Dr. Kedar N. Prasad, University of Colorado Health Sciences Center (Denver, CO). RPMI-1640 medium, fetal bovine serum, L-glutamine, penicillin, and streptomycin, 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA) were purchased from Invitrogen (Carlsbad, CA). EGCG, ferrous sulfate, ascorbic acid, mouse β-actin antibody, H_2O_2 were purchased from Sigma Aldrich (St. Louis, MO). Substrate for caspase-3, Acetyl-Asp-Glu-Val-Asp-AFC was obtained from Calbiochem (San Diego, CA). Rat TNFα recombinant was purchased from peprotech (Rocky Hill, NJ). The Cell Titer 96® AQueous Non-Radioactive Cell Proliferation assay kit was bought from Promega (Madison, WI). The rabbit polyclonal antibody for Fpn or hepcidin was purchased from Abcam (Cambridge, MA). Alexa Fluor 680 conjugated anti-mouse IgG and IRdye 800 conjugated anti-rabbit IgG were purchased from Invitrogen (Carlsbad, CA) and Rockland Inc (Gilbertsville, PA), respectively. All solutions were prepared fresh prior to each assay.

Cell culture and treatment paradigm

N27 cells were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum, 2 mmol/l L-glutamine, 50 units penicillin, and 50 µg/ml streptomycin and maintained at 37°C in a humidified atmosphere containing 5% CO_2 as described in our previous studies [24]. Cells were treated with different concentrations of EGCG, H_2O_2, TNFα to determine the optimal doses for the experiments. To investigate the protective effect of EGCG against H_2O_2- or TNFα-induced cytotoxicity, cells were pretreated with 10 µM EGCG for 3 h, followed by 50 µM H_2O_2 or 30 ng/ml TNFα for another 24 h. Cells were collected at the end of each treatment for the future assay.

Cell viability assay

Cell viability was measured using the Cell Titer 96 Aqueous Non-Radioactive Cell Proliferation kit as described earlier [25]. Briefly, cells were incubated with 10 µL tetrazolium compound MTS solution reagent mix at 37°C for 45 min, followed by adding 25 µL DMSO to dissolve the formazan crystals. The absorbance was read at 490 nm with a reference wavelength of 670 nm using a microplate reader (Molecular Devices, Sunnyvale, CA).

Caspase-3 activity assay

Caspase-3 activity was measured as described previously [26]. The cell pellet was lysed with Tris buffer (50mM Tris-HCL, 1 mM EDTA, and 10 mM EGTA at pH=7.4) containing 10 µmol/L digitonin for 20 min at 37°C. Lysates were quickly centrifuged and cell free supernatants were incubated with 50 µM Ac-DEVD-AFC as the fluorometric caspase-3 substrate for 1 h at 37°C. The caspase-3 activity was measured using a fluorescence microplate reader with the excitation at 400 nm and emission at 505 nm. The caspase-3 activity was expressed as fluorescent units (FU)/mg protein.

Intracellular ROS measurement

The formation of intracellular ROS was measured using the CM-H_2DCFDA fluorescent probe as described in our early publication with minor modifications [25]. In brief, cells were incubated with 10 μM CM-H_2DCFDA along with the treatment. Fluorescence intensity was continuously measured using a fluorescence microplate reader with the excitation 488 nm and emission 515 nm with 30 min interval for 2 h.

Western blot

Cell pellets were lysed using a modified radioimmunoprecipitation assay (RIPA) buffer as described previously [27]. Cell lysates containing equal amount of protein were loaded and separated on 12% SDS-PAGE (for Fpn) gels or 16% Tricine-SDS-PAGE gels (for hepcidin). After separation, the proteins were transferred onto a nitrocellulose or polyvinylidene difluoride (PVDF) membrane and probed with proper antibody directed against hepcidin rabbit polyclonal (1:500) or Fpn rabbit polyclonal (1:1000), followed by IR-dye 800 anti-rabbit secondary antibody (1:5000). Membranes were visualized on Odyssey Infrared Imaging system (LICOR, Lincoln, NE) and β-actin was used as an internal control.

Statistics

Data were analyzed with Prism 5.0 software (Graph Software, San Diego, CA). The measurements were normalized to the respective controls in each experiment. The differences among the treatments were compared with ANOVA with Dunnett's or Tukey's Multiple Comparison and considered significant $P<0.05$.

Results

Cytotoxic effect of EGCG, H_2O_2 and TNFα

To determine the optimal dose of EGCG, H_2O_2 and TNFα for the future experiments, we first measured the dose response cytotoxic effects of EGCG, H_2O_2 and TNFα using MTS assay. As shown in **Table 4-1**, no cytotoxic effect was found when cells were treated with 5 or 10 μM EGCG for 24 h. However, N27 cell viability was reduced to 61.6% ($P<0.001$), 31.2% ($P<0.001$) and 31.6% ($P<0.001$) after 24 h incubation of 25 μM, 50 μM, and 100 μM EGCG, respectively. The cytotoxic effects of different concentration of H_2O_2 and TNFα were also shown in Table 1. Cytotoxicity was not found with 10 μM H_2O_2 after 24 h incubation, but a reduction of 12% ($P<0.01$) and 48.3% ($P<0.001$) of cell viability was found with 30 μM and 100 μM. Similarly, 24 h treatment of TNFα 10 ng/ml didn't affect cell viability but TNFα 30 ng/ml, 60 ng/ml, 100 ng/ml significantly decreased cell viability by 24.4% ($P<0.05$), 38.6% ($P<0.001$), 29.9% ($P<0.01$). Based on these results, 50 μM H_2O_2 and 30 ng/ml TNFα were chosen to induce cytotoxicity, and 10 μM EGCG was selected as the highest safe dose for the evaluation of neuroprotective effect in the subsequent experiments.

Epigallocatechin gallate protects N27 cells from both TNFα and H_2O_2 induced-cytotoxicity

Protective effects of EGCG against H_2O_2- and TNFα-induced cytotoxicity was evaluated by MTS (**Figure 4-1A** and **Figure 4-1B**), caspase-3 activity (**Figure 4-1C** and **Figure 4-1D**), and intracellular ROS measurement (**Figure 4-1E**). Cell viability was decreased to 69.2% ($P<0.01$) and 89% ($P<0.01$) after treating with H_2O_2 or TNFα. However, EGCG shows marginal protection and increased cell viability to 88.5% and 94.8% respectively. Similarly, caspase-3 activity was increased to 283.9% ($P<0.001$) and 154% ($P<0.01$) after treatment

with H_2O_2 or TNFα, but EGCG significantly protected against H_2O_2- or TNFα-induced apoptosis by reducing caspase-3 activity by 50.2% ($P<0.001$) and 30.1% ($P<0.05$), respectively. In addition, intracellular ROS was increased by 4.5% ($P<0.001$), 5.5% ($P<0.001$), 6.8% ($P<0.001$) after incubating with H_2O_2 for 60 min, 90 min, 120 min, and EGCG pretreatment significantly counteracted the effect ($P<0.001$) and protected against H_2O_2-induced ROS in a time dependent manner.

Epigallocatechin gallate protects N27 cells from both TNFα- and H_2O_2-induced cytotoxicity through downregulation of hepcidin and upregulation of Fpn

To further explore the mechanisms involved in the protective effect of EGCG against H_2O_2- and TNFα-induced cytotoxicity, we assessed the hepcidin and Fpn protein expressions. As shown in (

Figure 4-2**A** and

Figure 4-2**B)**. As expected, 24 h of EGCG alone treatment didn't affect either hepcidin or Fpn expressions. However, H_2O_2 and TNFα significantly upregulated hepcidin expression by 66.3% ($P<0.05$) and 64.1% ($P<0.05$), and 3 h pretreatment of EGCG down regulated H_2O_2-induced hepcidin expression by 34.1% ($P<0.05$) and TNFα-induced hepcidin expression by 32.1% ($P>0.05$) respectively (

Figure 4-2**A** and

Figure 4-2**C**). Moreover, H_2O_2 and TNFα lowered Fpn expression by 25% ($p>0.05$) and 55.1% ($p<0.05$) and EGCG pretreatment marginally counteracted this effect, and increased Fpn expression by 42.5% ($P>0.05$) and 44.5% ($P>0.05$), respectively (

Figure 4-2**B** and

Figure 4-2**D**). As a positive control, we also tested the effect of 50 μM iron on hepcidin and Fpn expressions (

Figure 4-2**E** and

Figure 4-2**F**). Iron partially elevated hepcidin expression by 79.9% and significantly reduced Fpn by 47.6% ($P<0.05$), and EGCG pretreatment reversed these effects and decreased hepcidin expression by 19.7% and increased Fpn to the control level ($P<0.01$).

Discussion

The goal of this current study was to investigate the antioxidant and anti-inflammatory effects of EGCG in a cell culture model of PD. We used H_2O_2 and TNFα to induce oxidative stress or inflammation mediated damage in dopaminergic neurons. Hydrogen peroxide is produced from the enzymatic or spontaneous dismutation of superoxide and further converted to highly toxic hydroxyl radicals via Fenton reaction [28]. Hydrogen peroxide can be generated during dopamine turnover and auto-oxidation of dopamine, and H_2O_2 derived radicals is implicated in neurotoxins MPTP or rotenone mediated neuronal death [29]. Therefore, H_2O_2 is extensively used in vitro studies to elicit the mechanisms of which oxidative damage-induced neuronal apoptosis, as well as to screen neuroprotective agents in neurodegenerative diseases [30-32]. A previous study has shown that 250 μM H_2O_2 increased intracellular ROS by 50 % after 24 h treatment and caspase 3 activity by 210 % after 8 h treatment [33]. Similarly, our current study showed a lower dose of 50 μM H_2O_2 started to increase intracellular ROS after 60 min, and significantly elevate caspase 3 activity after 15 h and induce cell death after 24 h treatment. Our study also found that TNFα had similar effects on dopaminergic N27 cells as H_2O_2. TNFα is a pro-inflammatory cytokine that is secreted by microglia in response to various stimuli, and has been considered to play a key role in the neuroinflammation mediated cell death in neurodegenerative disorders including PD [34]. TNFα not only activates and recruits immune cells to propagate inflammation, but also directly induces oxidative stress by the activation of ROS generation [11]. This might explain why TNFα and H_2O_2 have similar toxic effects and induce caspase activity and apoptosis in dopaminergic neurons. Moreover, the protection of EGCG against both TNFα- and H_2O_2-induced apoptosis suggests both antioxidant and anti-inflammatory properties of

EGCG. Since ROS and inflammation can have synergistic effect and eventually result in a feed-forward loop of neurodegeneration in PD, EGCG might be the promising candidate for prevention or halting the progression of the disease.

Hepcidin-Fpn axis is a master regulation of cellular iron metabolism and controls cellular iron export in response to iron stores, oxidative stress, inflammation [35]. Although the role of hepcidin in neurodegenerative disorders is very limited, recent research has shown that hepcidin and Fpn were widely expressed in the central nervous system and might be involved in neuroinflammation and brain iron dysregulation [17, 18, 36]. One recent in vivo study shows intracerebroventricular injection of lipopolysaccharides (LPS) in the rat brain upregulated hepcidin and downregulated Fpn in cortex and SN [36]. Another in vitro study shows that inflammatory cytokines such as TNFα upregulated the expressions of iron importer divalent metal transporter 1 (DMT-1) and suppressed Fpn expression, resulting in iron accumulation in neurons or astrocytes [17]. Our previous study found that neurotoxin 6-OHDA increased the expression hepcidin and decreased the expression of Fpn, leading to iron accumulation in dopaminergic neurons [23]. In agreement with these studies, this study shows that H_2O_2 and TNFα can significantly upregulate hepcidin expression and marginally reduce Fpn expression. These results further implicate the role of iron related proteins in both oxidative stress and inflammation mediated cell damage and demonstrated the link among iron dysregulation, oxidative stress and neuroinflammation. We also found that iron alone treatment on dopaminergic neurons had more significant effects on Fpn expression than hepcidin. Since Fpn is regulated not only by hepcidin at the post-translation level, but also by iron regulatory protein/iron responsive element at the posttranscriptional level [37], our

results suggest that iron alone treatment might reduce Fpn expression via hepcidin independent mechanism.

Epigallocatechin gallate is the most abundant polyphenol in green tea and has been shown to prevent neurotoxin MPTP- and 6-OHDA-induced neurodegeneration in both in vitro and in vivo studies [21, 38-40]. The underlying protective mechanisms include its antioxidant, anti-inflammatory, iron chelating properties, its ability to interfere with protein aggregation and intracellular signaling pathways [41]. Our current study demonstrates that EGCG can prevent both oxidative stress and inflammation mediated neurodegeneration through the mediation of iron related proteins, hepcidin and Fpn.

Overall, our study suggests the cross talk between iron accumulation, oxidative stress and inflammation, and that EGCG protects against H_2O_2- and TNFα-induced neurotoxicity through the mediation of iron regulated proteins hepcidin and Fpn. Our study shed light on some of the mechanisms by which EGCG provides protection in PD and further study is needed to confirm the role of hepcidin-Fpn axis in EGCG mediated protection in an in vivo model of PD.

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Tables and figures

Table 4-1 Dose response effects of EGCG, H_2O_2 and TNFα on cell viability.

	Cell Viability (%)		Cell Viability (%)		Cell Viability (%)
EGCG (μM)		H_2O_2 (μM)		TNFα (ng/ml)	
0	100.0	0	100.0	0	100.0
5	92.0	10	93.1	10	81.4
10	99.9	30	88.0**	30	75.6*
25	61.6***	100	51.7***	60	61.4***
50	31.2***			100	70.1**
100	31.6***				

The values (mean ± SEM) represent percentage of the respective controls (no treatment); ANOVA with Dunnett's multiple Comparisons test was used to detect the differences between the treatments and controls; *P<0.05; **P<0.01; ***P<0.001.

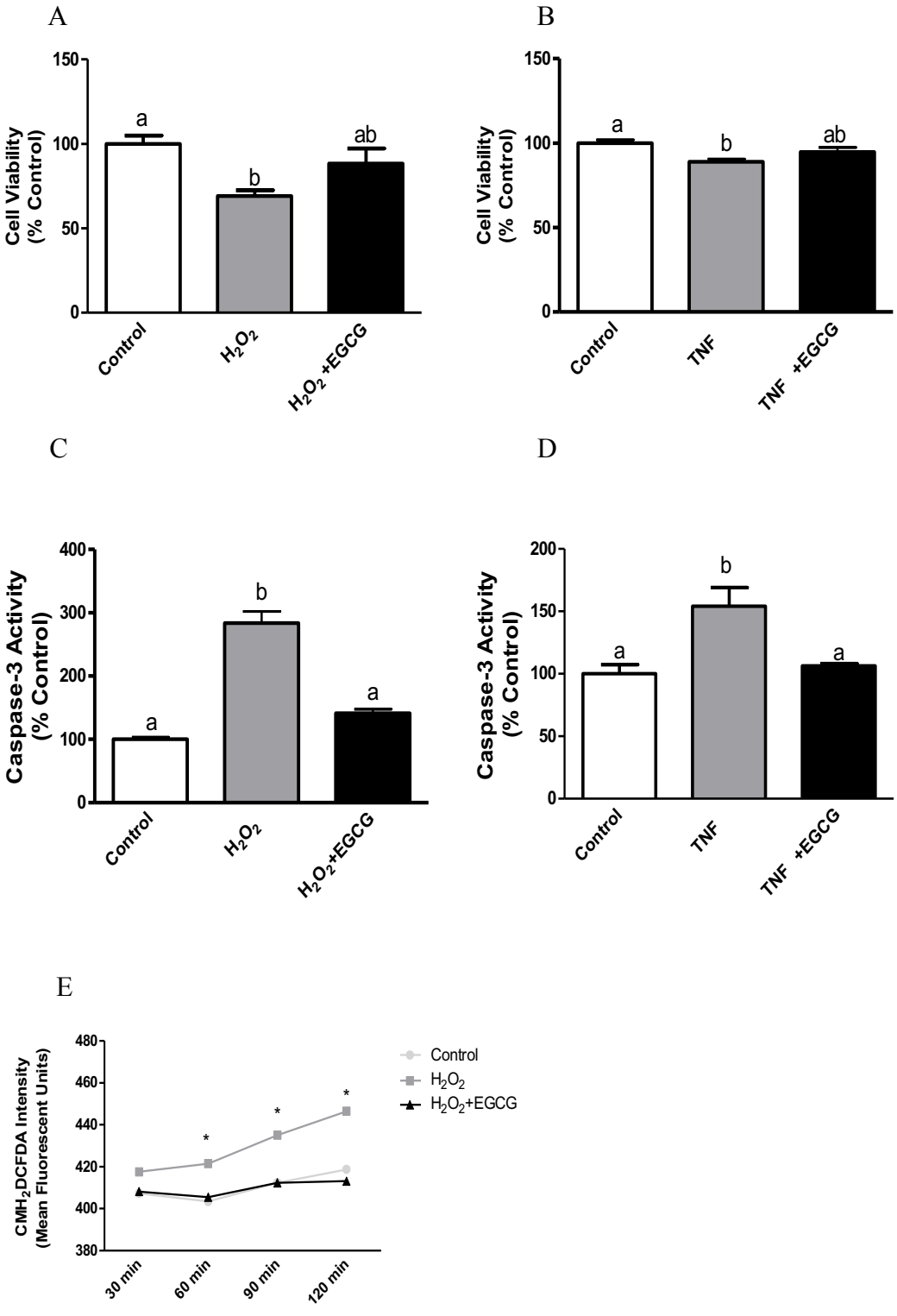
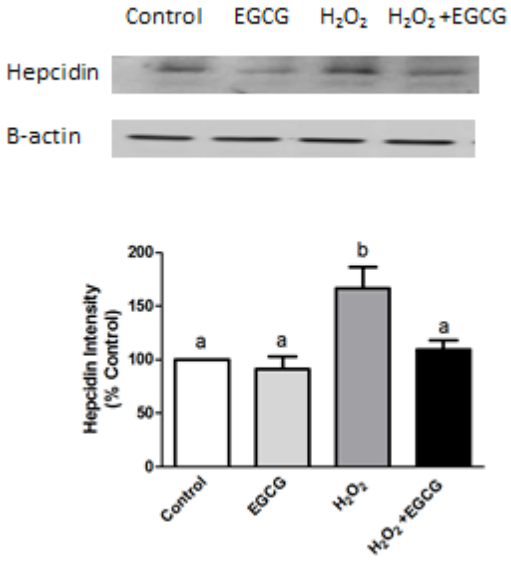


Figure 4-1 The protective effect of EGCG against H_2O_2- or TNFα-induced neurotoxicity measured by MTS (A, n=8; B, n=8), caspase-3 activity (C, n=4; D, n=5) and intracellular ROS (E, n=8) in N27 cells; Cells were treated with 10 μM EGCG, followed by the treatment of 50 μM H_2O_2 or 30 ng/ml TNFα for another 15 or 24 h. The values (mean ± SEM) are normalized to their respective controls and ANOVA with Tukey's Multiple Comparison was used to detect the differences among the treatments and controls; *P<0.001. Bars sharing same letters are not significantly different.

A



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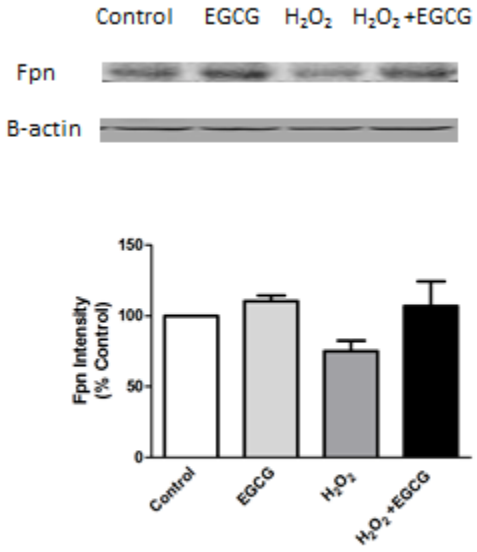
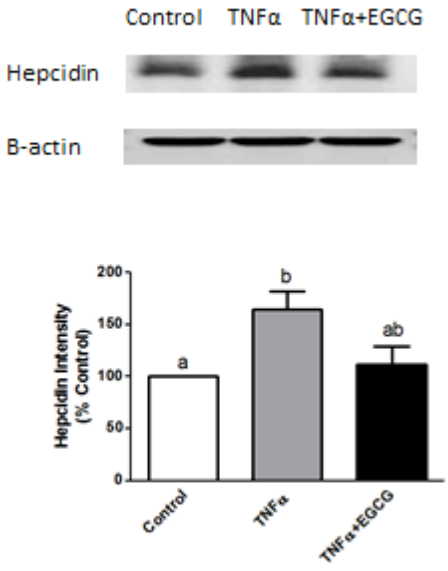
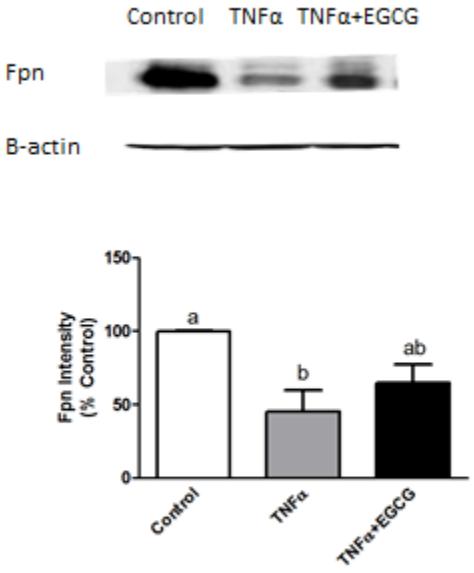


C

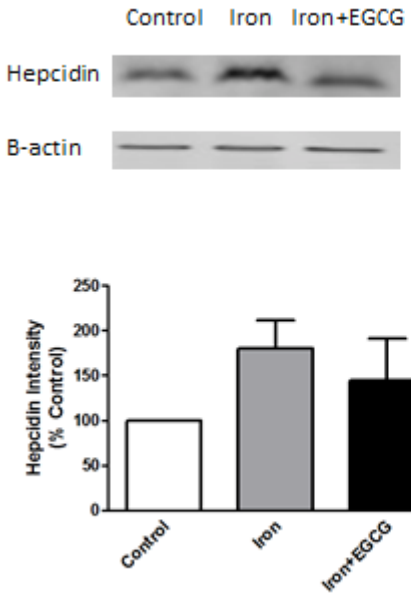


D



continued

E



F

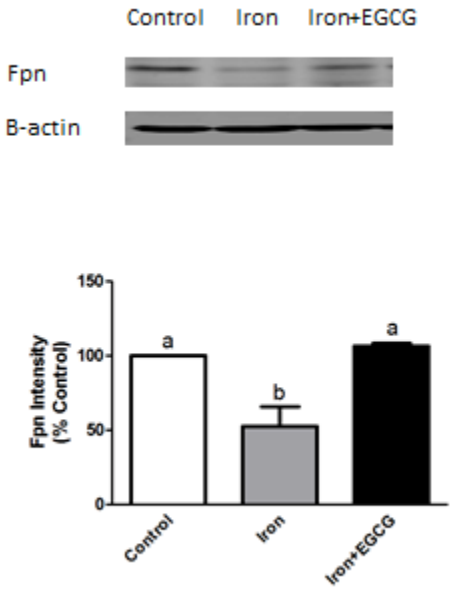


Figure 4-2 The protective effect of EGCG against H_2O_2 (A and B), TNFα (C and D), or ferrous sulfate (E and F)-induced altered expressions of hepcidin or Fpn in N27 cells (n=3); Cells were treated with 10 μM EGCG with 3 h, followed by the treatment of 50 μM H_2O_2 or 30 ng/ml TNFα or ferrous sulfate for another 15 or 24 h. The values (mean ± SEM) are normalized to their respective controls and ANOVA with Tukey's Multiple Comparison was used to detect the differences among the treatments and controls; Bars sharing same letters are not significantly different.

CHAPTER 5 NEURORESCUE EFFECT OF EPIGALLOCATECHIN GALLATE IN AN ANIMAL MODEL OF PARKINSON'S DISEASE

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Abstract

Background: Parkinson's disease (PD) is a neurodegenerative disorder that has been associated with many factors, including oxidative stress, iron accumulation and inflammation. Epigallocatechin gallate (EGCG) is the major polyphenol in green tea with antioxidant, anti-inflammatory and iron chelating properties.

Objective: The objective of the present study is to determine the neurorescue effects of EGCG in 1-methyl-4-phenyl-1,2,3,6- tetrahydropyridine (MPTP)-induced PD and to examine the involvement of iron-related proteins in that protective effect.

Methods: We evaluated the neurorescue effect of EGCG (25 mg/kg, 7 d, oral administration) against MPTP (20 mg/kg, 3 d, IP)-induced neurodegeneration in C57 black mice. The neurorescue effect of EGCG was assessed by motor behavior tests, neurotransmitter analysis, oxidative stress indicators, and iron related protein expressions.

Results: MPTP treatment shortened mice's latency to fall from the rotarod by 16% ($P<0.05$), decreased striatal dopamine (DA) level by 58% ($P<0.001$) and dihydroxyphenylacetic acid

(DOPAC) by 35% ($P<0.05$), and increased serum protein carbonyls by 68% ($P<0.05$). However, EGCG post-treatment significantly rescued MPTP-induced neurotoxicity by increasing the rotational latency by 17% ($P<0.05$), elevating DA (40%, $P<0.05$) and DOPAC (32%, $P>0.05$), and reducing serum protein carbonyls by 29% ($P>0.05$). EGCG significantly ($P<0.05$) increased ferroportin (Fpn) by 44%, and reduced hepcidin expression by 36% ($P>0.05$), suggesting the protection of EGCG might be associated with increasing iron exporter expression to relieve nigral iron burden but not by reducing iron import by divalent metal transporter-1 (DMT-1).

Conclusion: Overall, our study demonstrated that EGCG not only can restore MPTP-induced functional and neurochemical deficits but also offer a neurorescue effect by regulating iron export protein Fpn in substantia nigra (SN) and reducing oxidative stress.

Key words: Parkinson's disease EGCG MPTP Neurorescue

Introduction

Parkinson's disease (PD) is a chronic neurodegenerative disorder characterized primarily by the progressive degeneration of dopaminergic neurons in the substantia nigra (SN), resulting in irreversible motor dysfunction such as resting tremor, bradykinesia, and postural instability (1). The exact causes and mechanisms of pathogenesis of PD remain unknown, however, the involvement of oxidative stress, chronic inflammation, and iron accumulation have been the focus of attention in recent years (2,3).

The role of oxidative stress in initiating or promoting neurodegeneration is demonstrated by the postmortem brain analyses showing increased levels of lipid peroxidation, carbonyl modifications of proteins, and DNA and RNA oxidation (4). Iron accumulation is also

thought to be involved in PD pathogenesis since free iron can enhance oxidative stress by generating highly toxic hydroxyl radicals through Fenton reactions. Abnormal iron accumulation in the SN of PD patients has been substantiated by MRI as well as in postmortem brains, and is considered an invariable pathological feature of PD (5-7). It is suggested that brain iron accumulation may be caused by a number of factors including a disturbed blood-brain barrier, occupational exposure, or misregulation of iron related proteins (8,9). Hepcidin-Fpn axis is a key regulator for cellular iron metabolism. Hepcidin is a peptide primarily secreted by the liver that regulates cellular iron efflux by binding to iron exporter Fpn on the cell surface and inducing its internalization and degradation (10). Recent studies suggest the hepcidin-Fpn axis is widely expressed in the brain and might play an important role in brain iron homeostasis (11,12).

For the past several decades, several animal models of PD have been developed to study the pathophysiology and to assess the potential of neuroprotective therapies. 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is a widely used neurotoxin that crosses the blood brain barrier, converts to its metabolite 1-methyl-4-phenylpyridinium (MPP^+), and induces neurodegeneration by inhibiting mitochondrial complex I activity and generating reactive oxygen species (ROS) (13). Recent studies have also demonstrated nigral iron accumulation in MPTP-induced animal models (14,15), which might be associated with altered expression of iron related proteins such as increased expression of iron importer divalent metal transporter-1 (DMT-1), or decreased expression of iron exporter, Fpn (16,17). Moreover, the effectiveness of an iron restricted diet or pharmacological and genetic iron chelation further supports the participation of iron in MPTP-induced neurodegeneration (18,19).

Green tea has been widely consumed in Asian countries and the inverse relationship of tea consumption and the incidence of dementia, PD and Alzheimer's has been observed (20). Epigallocatechin gallate (EGCG) is the most abundant tea polyphenol and shows diverse biological effects such as radical scavenging, iron chelating, and anti-inflammatory properties (21-23). A number of studies have demonstrated the neuroprotective effects of EGCG against MPP^+- or MPTP-induced neurodegeneration in both cell culture and animal models of PD (24-26). However, its neurorescue potential in post MPTP-induced Parkinsonism is not very well studied. One study suggested that oral EGCG administration resulted in a substantial recovery of tyrosine-hydroxylase-positive neurons post MPTP treatment (20), but studies on the neuroprotective effects of EGCG through iron related proteins are limited. The objective of our study is to determine the neurorescue effects of EGCG in MPTP-induced PD and to examine the involvement of iron-related proteins in that protective effect.

Materials and Methods

Chemicals:

Mouse β-actin antibody, MPTP, EGCG were purchased from Sigma-Aldrich (St. Louis, MO). Perchloric acid and sodium metabisulfite ($Na_2S_2O_5$) were purchased from Fisher Scientific (Pittsburgh, PA). The rabbit polyclonal antibodies for ferroportin (Fpn), divalent metal transporter-1 (DMT-1) with and without iron response element (IRE), and hepcidin were purchased from Abcam (Cambridge, MA). Alexa Fluor 680 conjugated anti-mouse IgG was purchased from Invitrogen (Carlsbad, CA). IRdye 800 conjugated anti-rabbit IgG and western blot blocking buffer were purchased from Rockland Inc (Gilbertsville, PA).

Commercial assay kit for protein carbonyl was purchased from Cayman Chemical Company (Ann Arbor, MI).

Animals and treatment:

Male C57 black mice (~25 g) were purchased from Charles River (Wilmington, MA). The mice were housed individually in a temperature/humidity controlled room with a 12-h light/dark cycle. Food and water were provided *ad libitum*. All the procedures were approved by the Institutional Animal Care and Use Committee at Iowa State University. Mice were divided into 3 groups: control (n=10), MPTP (n=10), MPTP+EGCG (n=10). The mice in the last two groups were given MPTP intraperitoneally at the dose of 20 mg/kg for first 3 d to induce neurodegeneration. On day 4, MPTP+EGCG group was given EGCG (25 mg/kg via oral gavage) for additional 7 d. The control group was given equal volume of PBS. All the animals were sacrificed by decapitation 3 d after the last dose of EGCG.

Accelerated rotarod test:

The motor coordination and balance alterations were measured by the accelerating rotarod test as described previously (27). Briefly, the mice were first trained on a stationary rod for 2 min and the mice that fell during training were placed back on the rod. For the performance test, the mice were assessed on five occasions at an accelerating speed of 4 to 60 rpm for 3 min. The length of time each mouse was able to stay on the rotating rod was recorded with the computer software and averaged for the analysis. The trials were excluded if the mice jumped off the rod.

Protein carbonyl assay:

Blood samples from mice were collected by cardiac puncture and serum was used for the assessment of protein carbonyls following the instructions provided in the commercial kit.

Protein carbonyl content is a general indicator of oxidative stress measured as protein oxidation (28). According to the protocol, protein carbonyls react with 2,4-dinitrophenylhydrazine (DNPH) to produce corresponding hydrazones, which can be quantified spectrophotometrically at the wavelength of 360 nm. The carbonyls were determined from the differences in absorbance between DNPH-reacted samples and non-reacted samples, and normalized to protein concentration.

Striatal dopamine and its metabolite analysis:

The striatal dopamine (DA) and its metabolites were determined by high performance liquid chromatography (HPLC) with electrochemical detection (EC) as described previously (29). The neurotransmitters from striatal tissues were extracted using an antioxidant solution containing 0.1M perchloric acid, 0.05% Na_2EDTA, and 0.1% $Na_2S_2O_5$ and centrifuged at 13200 x g for 25 min. Dopamine (DA) and 3,4-dihydroxyphenylacetic acid (DOPAC) were separated isocratically on a C-18 reversed-phase column at a flow rate of 0.6 ml/min using a Dionex Ultimate 3000 HPLC system (pump ISO-3100SD, Thermo Scientific, Bannockburn, IL) equipped with a refrigerated autosampler (model WPS-3000TSL) and electrochemical detection system (CoulArray model 5600A coupled with microdialysis cell 5014B and a guard cell model 5020). The integration and data analysis was performed in ESA Coularray 3.10 software (ESA Inc., Bedford, MA). DA and DOPAC levels were normalized to wet tissue weight and converted to ng/mg protein. Data were represented as percentage of the control group.

Western blot analysis:

Substantia nigral tissue was lysed with modified RIPA lysis buffer and the lysates were loaded and separated on 12% SDS-PAGE gels or 16% Tricine-SDS-PAGE gels as described

previously (30). After separation, the proteins were transferred onto a nitrocellulose or polyvinylidene difluoride (PVDF) membrane and nonspecific binding sites were blocked with western blot blocking buffer for 1 h. The membranes with transferred proteins were probed with primary antibody directed against DMT-1 $\pm$ IRE rabbit polyclonal (1:1000), hepcidin rabbit polyclonal (1:500) or Fpn rabbit polyclonal (1:1000), followed by incubation with IR-dye 800 anti-rabbit secondary antibody (1:5000). Membranes were visualized on Odyssey Infrared Imaging system (LICOR, Lincoln, NE) and quantified with image J with β-actin as an internal control.

Statistics:

Data were analyzed with Prism 5.0 software (Graph Software, San Diego, CA). The values for each treatment group were normalized to the control group. The differences among the treatments were compared with ANOVA with Tukey's multiple comparison test and considered significant at $P \leq 0.05$.

Results

Epigallocatechin gallate reversed MPTP-induced reduction of rotarod activity:

The differences in weight gain of the animals were not significantly different among the treatments (**Table 5-1**). The average weight gain in three groups ranged from 1.5 g to 2.5 g. The protection of EGCG against MPTP-induced behavioral deficits was evaluated by the accelerated rotarod test as shown in **Figure 5-1A**. The MPTP treated mice showed an impaired ability to remain on the rod, showing a 16% reduction in the average time spent on the rotarod ($P<0.05$) when compared with the control group. However, rotarod activity with

EGCG co-administration was similar to control and significantly improved ($P < 0.05$) when compared with the impaired MPTP treatment group.

Epigallocatechin gallate protected against MPTP-induced oxidative stress:

Epigallocatechin gallate treatment reduced MPTP-induced oxidative stress, as measured by protein carbonyls (**Figure 5-1B**). Serum protein carbonyls were 1.7-fold higher ($P<0.05$) in MPTP treated mice compared to the control, however, they were reduced by 29% ($p>0.05$) with EGCG post-treatment.

Epigallocatechin gallate preserved MPTP-induced striatal DA reduction:

Epigallocatechin gallate showed protection against MPTP-induced depletion of striatal DA (**Figure 5-2A**) and its metabolite DOPAC (**Figure 5-2B**). Dopamine and DOPAC were significantly reduced by 58% ($P<0.001$) and 35% ($P<0.05$), respectively in MPTP treated mice. However, EGCG post-treatment partially reversed the reduction, and increased DA and DOPAC level by 40% ($P<0.05$) and 32%, respectively.

Epigallocatechin gallate alters iron-related protein expression:

To further study the mechanisms involved in the neurorescue effect of EGCG, we assessed iron related proteins DMT-1, hepcidin and Fpn expression in the SN. As shown in **Figure 5-3**, MPTP treatment slightly upregulated DMT-1 and hepcidin by 14% and 11%, and down regulated Fpn by 6% but the differences were not significant. Although EGCG post-treatment had no effect on DMT-1 expression, a decreased trend in hepcidin expression by 36% ($P>0.05$), but increased Fpn expression by 44% ($P<0.05$) compared to MPTP alone group.

Discussion

Parkinson's disease is the second most common neurodegenerative disorder and it affects around 5.2 million people worldwide (31). However, to date there is no strategy available for curing PD patients and the traditional therapies such as with levodopa only provide symptomatic relief while presenting significant motor complications (32). Based on the involvement of iron accumulation and oxidative stress in the pathogenesis of PD, the compounds with free radical scavenging and iron chelating properties have been thought of as promising candidates for treating PD. The iron chelator deferoxamine (DFO) is reported to reduce iron accumulation and oxidative stress, and protect against MPTP-induced neurotoxicity in mice (33). The metal chelator, clioquinol is also demonstrated to chelate both ferrous and ferric iron and protect against MPTP-induced loss of striatal DA *in vivo* (19). Our previous studies also found that the natural iron chelator phytic acid could protect both MPP^+- and 6-hydroxydopamine (6-OHDA)-induced dopaminergic neuron apoptosis in normal and iron excess conditions (34,35). Although these iron chelators may be effective in providing neuroprotection in PD, their therapeutic use in PD patients is limited because of their inability to cross the blood-brain barrier and/or causing severe side effects. Deferoxamine has limited ability to cross blood brain barrier due to its hydrophilic nature and is reported to cause neurotoxicity with high doses (36,37). The safety of clioquinol was also questioned since it might cause serum vitamin B12 deficiency (38). Recent clinical studies utilizing deferiprone have shown decreased iron brain iron content and slight improvement in motor symptoms, suggesting the importance of iron chelation in treating PD patients (39,40).

Epigallocatechin gallate is the major green tea polyphenol that has gained attention in PD because of its free radical scavenging, iron chelating and anti-inflammatory properties

(23). *In vitro* studies have shown that EGCG could protect from both MPP^+ and 6-OHDA-induced neurotoxicity (24,41). In agreement, *in vivo* studies in mice also show that EGCG could significantly prevent striatal dopamine depletion and loss of TH positive neurons induced by MPTP (26,42). The natural origin of EGCG and its ability to cross the blood brain barrier also make it an appealing clinical approach for PD treatment (23). It is reported that EGCG could be easily absorbed from the digestive tract and widely distributed into various organs, including the brain, which had a similar concentration to the level found in the liver, kidney, lung, heart, spleen and pancreas (42).

Previous studies focused on the neuroprotective effect of EGCG (24,26), but studies to evaluate the neurorescue effect after inducing neurotoxicity are limited. In addition, the effect of EGCG on iron related proteins in the brain that are perturbed in PD is not well studied. One study showed that oral EGCG (5 mg/kg) administration for two weeks after MPTP treatment (20 mg/kg, 4 d) resulted in a substantial recovery of the nigral dopaminergic neurons (20). Consistent with this study, our results also show that EGCG post-treatment (25 mg/kg, 7 d) not only rescued MPTP-induced dopamine depletion, but also improved motor deficits caused by MPTP as assessed by accelerated rotarod test. Striatal dopamine depletion in MPTP mice was attenuated by EGCG treatment, although the dopamine concentrations with EGCG were not increased compared to the control group. Since dopamine depletion is the major cause of motor dysfunction in PD, it is encouraging to see the improvement with EGCG for its future potential use in humans. The accelerated rotarod test is a behavior test used to measure animals' innate motor skills which resemble akinesia and bradykinesia in human Parkinsonism (41)(43). Administration of MPTP resulted in decreased rotarod duration and EGCG post-treatment completely corrected motor deficits, suggesting its ability

to alleviate PD symptoms. In addition, our results also show that EGCG post-treatment reduced serum protein carbonyls that were elevated by MPTP. This finding is consistent with our previous publication showing 3 cups of green tea consumption for 3 months improved antioxidant enzymes and reduced oxidative damage to lipids and proteins in PD patients (44).

In our study MPTP treatment didn't significantly affect DMT-1, hepcidin or Fpn expression in the SN. These results are inconsistent with a previous study showing nigral iron accumulation with increased DMT-1 expression and decreased Fpn expression in a chronic MPTP-induced PD model (16). However, our study used a subacute MPTP model (20 mg/kg, 3 d) rather than a chronic MPTP model (30 mg/kg, 10 doses on a 5-week schedule). A higher dose with longer treatment of MPTP in the previous publication might account for observed nigral iron accumulation by altering iron transporters. We found that EGCG did not affect hepcidin or DMT-1 expression but significantly affected nigral Fpn expression. The plausible explanation might be that Fpn mRNA might be regulated not only by hepcidin but also via iron regulatory proteins (IRP) since it contains IRE responsive element (IRE) in the 5' region (45). It has been reported that IRP is regulated by oxidative stress and intracellular iron concentrations. Since EGCG has antioxidant and iron chelating abilities, we expect that IRP is up regulated, thus increasing Fpn expression (45) with its treatment. To our knowledge, this is the first *in vivo* study to show significant upregulation of Fpn after EGCG treatment. Although these results do not support our previous study regarding EGCG's effect on hepcidin and DMT-1, they are in agreement with our study showing protection in 6-OHDA-induced neurotoxicity by alleviating intracellular iron level and upregulating Fpn in a cell culture model of PD (46). However, the differences between two studies are the use of different neurotoxins as well as using different models (*in vivo* vs *in vitro*). Since iron

accumulation can exacerbate MPTP-induced dopaminergic neurodegeneration (47), upregulation of Fpn might be one of the underlying neuroprotective mechanisms of EGCG by reducing nigral iron. The dose of 25 mg/kg EGCG in our study is approximately 2 mg/kg in humans using the body surface area (BSA) normalization method (48), or 140 mg EGCG daily consumed by a 70 kg person. Based on a previous study showing a cup of green tea (2.5 g of green tea leaves / 200 ml of water) may contain up to 90 mg of EGCG (49), habitual consumption of green tea (3 cups per day) can reach more than the target amount 140 mg (44).

Overall, our study demonstrated that EGCG not only can restore MPTP-induced functional and neurochemical deficits, but also offers its neurorescue effect by regulating iron export protein Fpn in the SN and reducing oxidative stress. Although future clinical studies are needed to confirm the protective effect of EGCG, our findings suggest its potential therapeutic use after the onset of PD.

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Tables and figures

Table 5-1 Weight gain* of animals during the study period.

	Initial weight (g)	Final weight (g)	Weight gain (g)
Control	23.9 ± 0.2	25.6 ± 0.3	1.5 ± 0.4
MPTP	23.3 ± 0.3	26.0 ± 0.3	2.5 ± 0.3
MPTP + EGCG	23.0 ± 0.3	25.1 ± 0.2	1.9 ± 0.3

*mean ± SEM. The differences in weight gain are not significantly different among three groups.

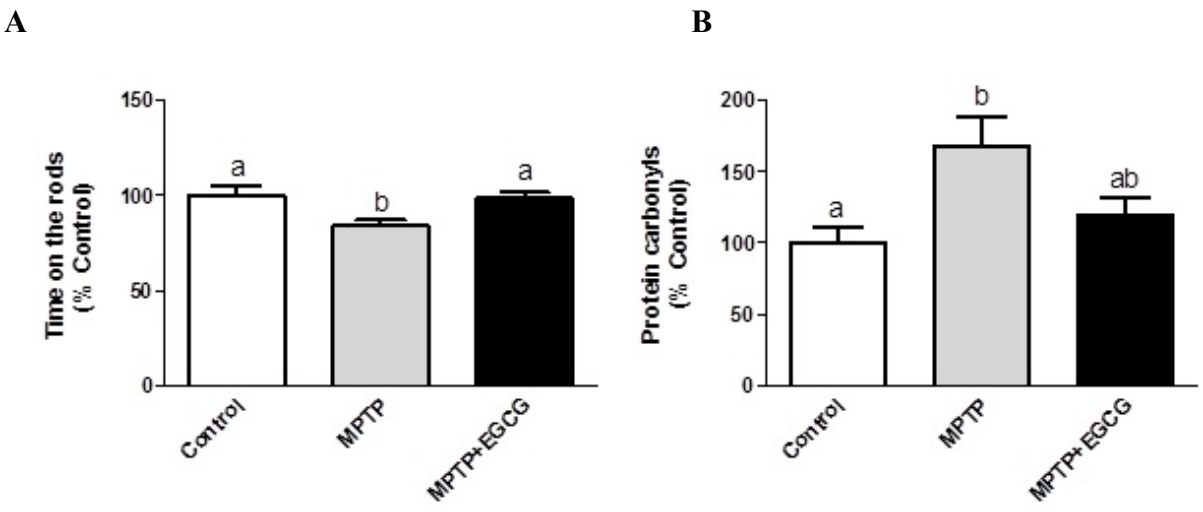


Figure 5-1 The neurorescue effect of EGCG against MPTP -induced motor deficits and oxidative stress. Motor coordination was measured by accelerated rotarod test (A, n=10), and oxidative stress was measured as protein carbonyls in serum (B, n=7-8). The values (mean ± SEM) are normalized to the control group and ANOVA with Tukey's Multiple Comparison was used to detect the differences among the three groups. Bars not sharing the same letters are significantly different (P <0.05).

A **B**

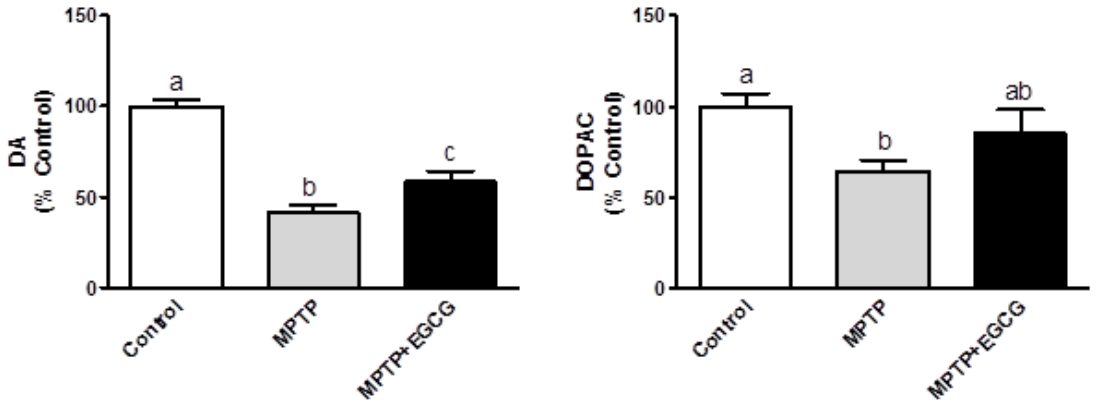
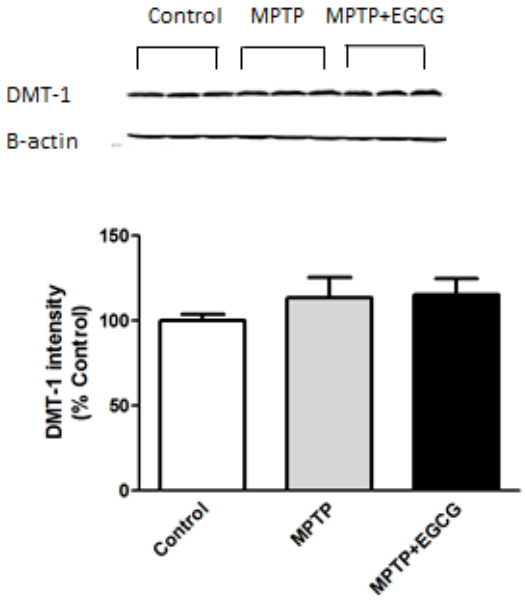


Figure 5-2 The neurorescue effect of EGCG against MPTP-induced neurochemical changes as determined by striatal DA (A, n=10) and DOPAC (B, n=10) concentrations. The values (mean ± SEM) are normalized to control group and ANOVA with Tukey's Multiple Comparison was used to detect the differences among the three groups. Bars not sharing the same letters are significantly different (P <0.05).

A



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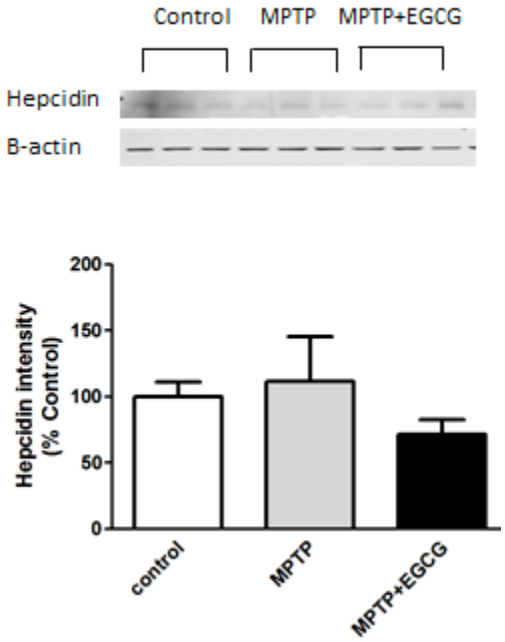

C

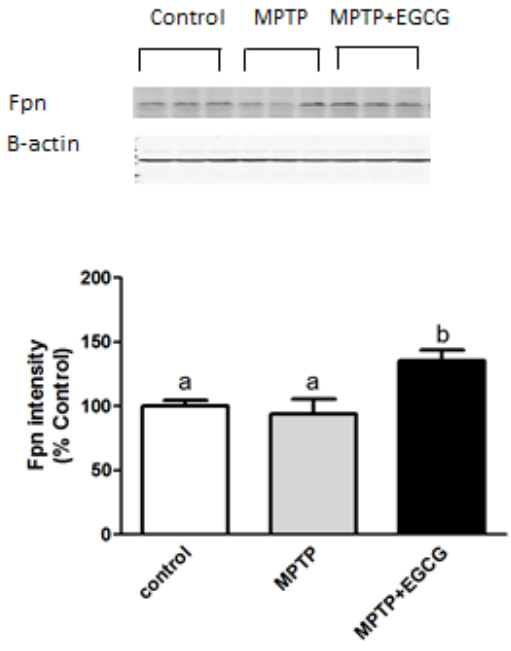

Figure 5-3 The effect of EGCG on MPTP-induced alteration in iron related proteins DMT-1 (A, n=6), hepcidin (B, n=6) and Fpn (C, n=6). The top panel shows the representative western blots (n=3). The values (mean ± SEM) are normalized to the control group and ANOVA with Tukey's Multiple Comparison was used to detect the differences among the three groups. Bars not sharing the same letters are significantly different (P <0.05).

CHAPTER 6 GENERAL CONCLUSION

General Discussion

Parkinson's disease (PD) is the second most common neurodegenerative disorder that is characterized by both motor and non-motor symptoms. Although the exact cause of PD remains elusive, both genetic and environmental factors are suggested to be involved in the development of the disease. Current therapies for PD focus on relieving the symptoms but no treatments are available to attenuate or reverse the disease progression. Therefore, understanding the pathogenesis of the disease and developing the novel therapies to prevent the onset or progression of the disease is important.

Iron is an essential component of the normal cell metabolism and physiology. However, their dysregulation can generate oxidative stress and neuroinflammation and promote protein aggregation leading to neurodegeneration. A growing body of data has demonstrated that iron accumulation in substantia nigra (SN) in PD patients and neurotoxin-induced PD models, suggesting the role of iron dysregulation in the pathogenesis of PD. The design of iron chelation therapy aimed to reduce excess brain iron currently shows great promise and might provide a new insight into therapies directed towards prevention or slowing down the disease progression. However, disadvantages associated with iron chelation therapy including their low bioavailability, poor blood brain barrier permeability and toxic side effects limited their further investigation in clinical settings.

Natural iron chelators derived from food and plants have attracted increasing interest because of their safety and low toxicity. Epigallocatechin gallate (EGCG) is the major tea polyphenol and might be a good candidate for PD treatment due to its antioxidant, anti-

inflammatory and iron chelating properties. Our study shows EGCG protected against both tumor necrosis factor alpha (TNFα)- and hydrogen peroxide (H_2O_2)-induced neuronal apoptosis in in vitro models of PD, and the observed neuroprotection is through the inhibition of oxidative stress and neuroinflammation, which might be mediated by hepcidin and ferroportin (Fpn). In addition, we also found that EGCG rescued dopaminergic neurons after neurotoxin 1-methyl-4-phenyl-1,2,3,6- tetrahydropyridine (MPTP) treatment in an in vivo model of PD and the protective mechanism might be associated with its ability to regulate Fpn expression in the SN and reduce oxidative stress.

Overall, our study demonstrates the preventative or therapeutic role of EGCG in the treatment of PD (**Figure 6-1**). Future studies are needed to confirm the protective role of EGCG in clinical settings.

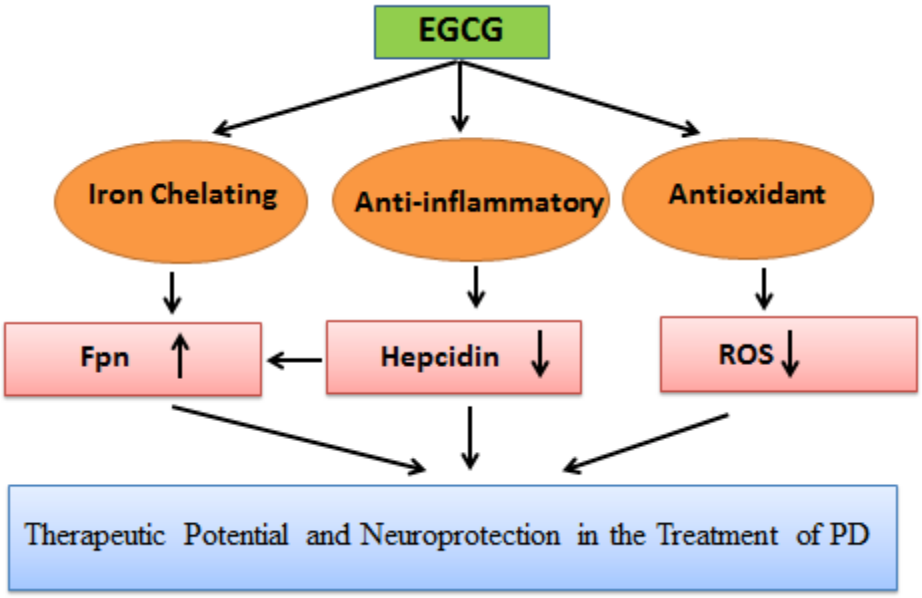

Figure 6-1 The preventive and therapeutic role of EGCG in PD treatment. EGCG: Epigallocatechin gallate; PD: Parkinson's disease; Fpn: Ferroportin.