Green tea polyphenols prevent Parkinson’s disease:

in vitro and in vivo studies

by

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CHAPTER 1. GENERAL INTRODUCTION

Parkinson’s disease (PD) is associated with the degeneration of dopaminergic neurons in the substantia nigra (SN). One of the pathological hallmarks of PD is the presence of intracellular Lewy bodies (LBs). The soluble protein α-synuclein is expressed in presynaptic region and its aggregation is highly involved in the formation of LBs. Other factors that may also contribute to the progression of PD include: aging, genetic mutations, oxidative stress, environmental toxins, and inflammation. Among these factors oxidative stress has been considered the main factor in causing PD. Due to the high energy metabolism ratio and the structure of SN in normal brain, there is relatively high levels of basal oxidative stress. Postmortem studies and in vitro neuron studies demonstrate that elevated oxidative stress is linked to PD.

Oxygen-generated free radical is the most important class which contributes to ROS and oxidative stress. The enzyme or metal-catalyzed process can directly or indirectly generate free radical. Current evidence shows the changes in the balance of redox transition metals are involved in free radical generation. Iron is an important trace element which participates in many physiological functions, such as neurotransmission and neuromelination. Many iron transporters and regulators are responsible for maintaining iron homeostasis. Iron can be up-taken via divalent metal transporter (DMT1), transferrin, and transferrin receptors (TfR). Ferritin is responsible for iron chelation and storage; while ferroportin 1 (Fpn1) is used for iron exportation. Other regulators such as hepcidin and iron regulatory proteins (IRP) are also important in regulating the iron-related factors and maintaining the regular iron labile pool. Various factors can regulate iron transporters and cause iron dyshomeostasis including environmental toxins and genetic mutations. If iron dyshomeostasis occurs
it could exaggerate the generation of free radicals. The excess free radicals such as superoxides, can force enzymes to release free iron from iron-containing molecules. The neurotoxins 6-hydroxydopamine (6-OHDA) and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) act as the classic toxins to induce PD, and even though the mechanisms remain unclear they are still widely used in PD research. There is evidence that 6-OHDA releases iron from ferritin and the released iron can generate highly reactive hydroxyl radical via the Fenton reaction, consequently leading to neurodegeneration. Markers of oxidative stress in the SN which are reflected by enhanced lipid peroxidation, protein carbonyls, are confirmed in PD. In addition, the oxidative burst triggers inflammation, which is also involved in neurodegeneration.

Several methods such as pharmacological treatments and surgical treatments can be used to treat PD. However, since the PD symptoms represent more than 50% neuronal cell death in SN, prevention methods are ideal ways for neuroprotection rather than the therapeutic methods. Prevention methods such as, antioxidation, antiinflammation, are considered as effective neuroprotection strategies. Attention is focused on the metal-induced formation of free radicals and the protective role of antioxidants (nuclear factor erythroid 2-related factor 2, Nrf2/antioxidant system), anti-inflammatory system (such as peroxisome proliferator-activated receptor γ, PPARγ) and flavonoids (catechins). The iron chelators, such as desferroxamine have shown to be effective in preventing or delaying PD progression. Epidemiological data shows the positive relationship between green tea consumption and the lowered risk of PD in Asian countries. In vitro and animal studies showed epigallocatechin gallate (EGCG) protected against 6-OHDA induced neurotoxicity. However, limited human
study data shows green tea as well as its main component EGCG directly protected against neurotoxicity.

EGCG may benefit neurological disorders from several aspects: iron chelation, antioxidation, antiinflammation, and other pathway modulators. In this study we assessed the effect on iron homeostasis with 6-OHDA induced neurotoxicity and the preventive effects of EGCG against neurotoxicity in N27 cells by attenuating reactive oxygen species (ROS) derived-oxidation and inflammation. We also show the beneficial effects of green tea consumption in PD patients. Our entire project contains three sets of studies. In the first study, we hypothesized that 6-OHDA and EGCG treatment would regulate iron related elements. We used dopaminergic N27 cells to determine the 6-OHDA induced dyshomeostasis by regulating iron transporter mRNAs and protein expressions, such as DMT1, Fpn1, and hepcidin. Meanwhile, EGCG protected against the adverse effects of 6-OHDA by normalizing and maintaining iron metabolism and homeostasis. In the second study, we showed the direct evidence that EGCG maintained the ROS homeostasis by suppressing 6-OHDA induced oxidative damage on lipids and proteins. We also conducted the preliminary study examining the stabilization of Nrf2 antioxidant system and PPARγ anti-inflammatory system by EGCG in N27 cells. In the third study, our hypothesis was to identify the beneficial effects of green tea consumption on PD patients, including slowing down the PD progression by improving the antioxidant status and reducing oxidative damage. We found out that the intervention might improve the quality of life of PD patients based on PD symptom rating scales and other measures as well as enhance the antioxidant status, reduce oxidative damage on lipids and proteins. We also showed that green tea intervention would not affect the iron status. All together these findings in this study reveal the importance of iron regulation and
oxidative damage in PD progression; the neuroprotection of EGCG by its iron chelation, antioxidant, and antiinflammation capabilities in vitro, as well as beneficial effect of and green tea consumption in PD patients.

Dissertation Organization

This dissertation is organized into six chapters, including a general introduction, a literature review, three chapters on research publications, and a final conclusion. Chapter 1 is a general introduction. Chapter 2 is a literature review of nutritional prevention on oxidative stress-associated PD. Chapter 3 and 4 are the manuscripts prepared to be submitted to Journal of Nutrition. Chapter 5 is manuscript prepared to be submitted to Movement Disorders Journal. Chapter 6 is the general conclusion of the studies which include the general conclusions and future directions. The references of this dissertation are formatted in the Journal of Nutrition requirement at the end of chapter 1 to 4. All the tables, figures, and legends in chapter 3, 4 and 5 are placed as close as possible to the original text references.
CHAPTER 2. LITERATURE REVIEW

I. PARKINSON’S DISEASE

Parkinson’s disease (PD) is the second most common neurodegenerative disease after AD, affecting approximately 1% of the population over 50 years old and causes an approximate $25 billion economic burden annually (1). PD is characterized by progressive loss of control over voluntary movement, which results primarily from the progressive loss of dopaminergic neurons in the SN, located in the mid-brain (2). The symptoms of PD include tremor, rigidity, bradykinesia, hypokinesia and akinesia. The pathological hallmarks of PD are the loss of the nigrostriatal dopaminergic neurons and the presence of intraneuronal proteinaceous cytoplasmic inclusions, LBs. LBs are spherical eosinophilic cytoplasmic protein aggregates composed of numerous proteins, including α-synuclein, parkin, ubiquitin, and neurofilaments (4, 5). LBs are more than 15 μm in diameter and have an organized structure containing a dense hyaline core surrounded by a clear halo. PD-associated loss of dopaminergic neurons is concentrated in ventrolateral and caudal portions of the SN, whereas during normal aging the dorsomedial aspect is affected. The striatal dopaminergic nerve terminals are the primary target of the degenerative process. Neurodegeneration and LB formations are found in noradrenergic, serotonergic, and cholinergic systems, as well as in the cerebral cortex, olfactory bulb, and autonomic nervous system. Degeneration of hippocampal structures and cholinergic cortical inputs contribute to the high rate of dementia related to PD, particularly in older patients. The disease is also considered as a tyrosine hydroxylase (TH) deficiency syndrome, since TH catalyzes the formation of L-3,4-dihydroxyphenylalanine, the rate-limiting step in the biosynthesis of DA in the striatum (6). In general, the appearance of disease symptoms is observed
after loss of 80% of striatal DA and 50% of the TH-immunoreactive dopaminergic neurons in the SN (7).

A. Pathogenesis and Risk Factors of PD

Major hypotheses related to the progressive loss of dopaminergic neurons are misfolding, aggregation of proteins, mitochondrial dysfunction, and the consequent oxidative stress, including toxic oxidized DA species. LBs consist of aggregates of the presynaptic soluble protein, α-synuclein. α-synuclein decreases mitochondrial complex I activity and increases ROS production (8). Oxidative damage can also interact with α-synuclein and enhance its misfolding. Moreover, multiple cell death-related molecular pathways are activated during PD progression, such as apoptosis and necrosis.

1. Age

Age has been considered a potential risk factor for PD because most patients develop PD after 50 years of age. The incidence of PD increases markedly over age 55, with a 20/100,000 incidence at age 55 to a 120/100,000 incidence at age 70 (3). Pathologically, aging is associated with a decline of pigmented neurons in the SN. It is also suggested that the aging nigrostriatal system may be more vulnerable to damage by exogenous and endogenous toxins since the neurotoxin MPTP causes more severe pathological and neurochemical injury in older mice (9).

2. Genetic Susceptibility

Common sporadic PD shares similar phenotype with genetic forms of PD, and the same pathogenic and genetic mechanisms help to clarify the key biochemical pathway
of PD progression. These PD genes that have been identified and studied in some detail: α-synuclein, parkin, and ubiquitin c-terminal hydrolase L1 (UCHL1) appear to participate in the ubiquitin-proteasome system (UPS), affecting protein clearance and cause LBs protein aggregation, which is the characteristic of PD neuropathology. In addition, familial PD genes, such as PTEN-induced putative kinase 1 (PINK-1), DJ-1 or dominant Leucine-Rich Repeat Kinase 2 (LRRK2) are closely related to the impairment of mitochondrial complex I.

3. Environment

Several epidemiological studies have suggested that exposure to different environmental agents including pesticides, insecticides, heavy metals, and microbial toxins may increase the risk of PD (10, 11). A byproduct of an illicit narcotic drug, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) as well as its metabolite MPP+, has been shown to cause the same signs and symptoms as PD. As mitochondrial poisons, rotenone, and paraquat also activate the progression of PD. Human epidemiological studies have implicated a higher incidence of PD in residents of rural environments with exposure to herbicides and pesticides (12). However, cigarette smoking and coffee drinking are inversely associated with the risk for development of PD (13), reinforcing the concept that some environmental factors do modify PD susceptibility.

4. Inflammation

Inflammation has also been implicated in the pathogenesis of PD. Pro-inflammatory cytokines such as interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) may induce microglia activation and cause microglia related to
neuronal injury (14). The negative association between the low risk of PD and high anti-inflammatory activities indicates the role of inflammation in PD progression, and suggests anti-inflammatory therapy as a potential neuroprotective strategy.

5. Oxidative Stress

Although environmental, genetic, and inflammatory factors contributed to the etiology of PD, oxidative stress has been confirmed to play the main role in PD progression (14). Brain tissue is susceptible to oxidative stress since it is rich in polyunsaturated fatty acids, which can easily undergo lipid peroxidation. In addition, high levels of ATP consumption in the brain could cause high oxidative metabolism as the electrons might leak to form oxygen free radicals. The features of enhanced oxidative stress linked with PD are supported by both postmortem studies and by studies showing oxidative stress inducing nigral cell degeneration (15, 16). The factors, including an impairment of antioxidant status, such as reduced glutathione (GSH), increased reactive species, augmented DA oxidation, and high iron levels, which are associated with oxidative stress, have also been found in the SN in PD (17).

B. Animal Models of PD

6-OHDA administration is widely used to induce PD in animal models, especially in rats (19-21). 6-OHDA impairs sympathetic neuron nerve terminals in the peripheral nervous system when administered systemically. Due to its chemical similarity with catecholamine, it has a high affinity for the catecholamine transporter and is able to damage the catecholaminergic neurons (22). Different from systemic administration of MPTP, 6-OHDA only partly crosses the blood brain barrier (BBB). Brain injection into the SN is one main way to kill TH-positive neurons, causing a dose-dependent
loss in DA in the central nerve system (CNS) (23). Mice with 6-OHDA lesions in the medial forebrain bundle (MFB) indicate the dose-dependent manner of damage on midbrain DA neurons (30). Lundblad (2004) reported the first 6-OHDA lesioned mice developing dyskinesia, which was similar to those previously characterized in the rat and in other animal models of PD (31) and PD patients (32, 33). Compared with the corresponding rat model, MFB-lesioned mice show a much larger number of TH-positive cell bodies in the striatum. However, the functional importance of these neurons is yet to be clarified.

C. Cell Models of PD

Cell cultures, such as mesencephalic slice cultures, primary immature dopaminergic neurons, and immortalized cell lines, either in a proliferating state or in a differentiated state, are used for the screening of pathogenesis and drug candidates in PD research (39). The loss of TH+ immunoreactivity is used as a marker in primary neuron model cells, and neurotoxins, including 6-OHDA and MPP+, can cause significant TH+ cell death (40-42). The difficulty in producing the primary mesencephalic neuron cells in large quantities limits their usefulness even if they have the physiological characteristic of neurons of the SN. The catecholaminergic neuroblastoma cell lines, such as SH-SY5Y, have been used to clarify the proposed mechanisms of 6-OHDA and MPTP neurodegeneration in PD, including oxidative stress and inflammation (43-45). The SH-SY5Y cells are less sensitive to neurotoxins since they are not neuronal cells. N27 cells are from an immortalized line derived from the rat mesencephalon, and may be more physiologically similar to dopaminergic neurons. N27 cells can express both tyrosine hydroxylase and the DA transporter, and produce measurable amounts of DA (46). N27 cells are sensitive to
6-OHDA and can help to clarify the 6-OHDA induced neurotoxicity. The MN9D cell line, a fusion of embryonic ventral mesencephalic and neuroblastoma cells, is extensively used as a model of DA neurons because they express tyrosine hydroxylase and can synthesize and release DA. These cells are also used to understand the mechanisms and to develop potential therapeutics to prevent DA neuronal loss in PD research (47).

Non-dopaminergic cell lines, such as CHO-K1 and HEK293 are also used for studying PD (48, 49). The PC12 cell line, a rat pheochromocytoma cell line, has been extensively used as a tool for studying the function of neurotrophic factors and neuronal differentiation, and is widely used as a model cell to study the environmental effects, oxidative stress, and genetic manipulation on PD progression (50). Several studies showed that 6-OHDA induced PC12 cell death by oxidative stress, cell apoptosis, and necrosis in a pro-inflammatory manner (50, 51).

There are still several other cell lines, such as peripheral T-lymphocytes, that can be used to study the inflammatory and cell apoptotic pathways during PD progression (52).

D. Mechanism of 6-OHDA-induced Neuron Cell Damage

Neurotoxins are shown to depolarize the mitochondrial membrane potential, disturb the mitochondrial function, inhibit the ubiquitin proteasome system (UPS), and decrease proteasomal function (53-55). Consequently, they cause ubiquitinated protein accumulation in the mitochondria, such as α-synuclein, resulting into a reduced activity of mitochondrial complex I, and increase influx of Ca\textsuperscript{2+} into the cell, causing an excitotoxic condition (56). 6-OHDA has been shown to reversibly inhibit both mitochondrial complexes I and IV activities in rat brain with inhibition
concentration (IC$_{50}$) of 10 and 34 µM (57-59). 6-OHDA is mechanistically similar to DA, binding to DA receptors on neuropeptide substance P (SP)-containing striatal projection neurons, releasing SP. SP can then potentiate both DA and glutamate release to cause oxidative stress and glutamate excitotoxicity, contributing to dopaminergic cell death (60). In addition, 6-OHDA is also subjected to auto-oxidation to generate ROS, including quinines, hydrogen peroxide, superoxide radicals, and hydroxyl radicals (61). It has been shown in vitro that DA reacts with Fe (II) in the presence of hydrogen peroxide to generate 6-OHDA (62). 6-OHDA can then react with ferritin to cause iron release and subsequent cellular damage (63). 6-OHDA also induces NO and iNOS over-expression, cell apoptotic and necrotic cell death, and pro-inflammatory gene (IL-1B, TNF-a, COX-2) activation (50, 51, 64).

The molecular pathways involved in 6-OHDA-induced cell death have also been studied in vivo, however less is known about the mechanism of progressive dopaminergic neuronal death by the striatal injection of 6-OHDA. 6-OHDA activates NF-κB, p53 and the c-jun N-terminal kinase (JNK) pathway, which has been identified in dopaminergic cell death in both the MFB and the striatal model (65, 66). In addition, the anti-inflammatory PPARγ was shown to attenuate 6-OHDA toxicity in a rat model, indicating the involvement of an inflammatory reaction. More recently, 6-OHDA has been shown to elevate the activation of an apoptosis signal regulating kinase 1 (ASK1), indicating the oxidative stress and apoptotic pathways in PD progression (47).

II. OXIDATIVE STRESS AND NEURODEGENERATION

Oxidative stress can result from a variety of conditions that represent either increased ROS production or a decreased antioxidant defense. The superoxide radical
is considered as the “primary” ROS, which further generates “secondary” ROS by interacting with other molecules. For example, hydrogen peroxide can be generated by a dismutase reaction. The superoxide dismutase (SOD) enzyme also works in conjunction with other H$_2$O$_2$–removing enzymes, such as catalase and glutathione peroxidase (GPx). The production of H$_2$O$_2$ and O$_2$ is tightly linked with the participation of redox-active trace metals.

\[
2\text{O}_2^- + 2\text{H}^+ + \text{SOD} \rightarrow \text{H}_2\text{O}_2 + \text{O}_2
\]

The redox states should be maintained within strict physiological limits. In vivo, under stressed conditions, an excess of superoxide acts as an oxidant of [4Fe-4S] cluster-containing enzymes, releasing “free iron” from iron-containing molecules. The released Fe$^{2+}$ can participate in the Fenton reaction, generating highly reactive hydroxyl radicals (67). Cells of the immune system also produce both the superoxide anion and nitric oxide during the oxidative burst triggered during inflammatory processes. Under these conditions, nitric oxide and the superoxide anion may react together to produce significant amounts of a much more oxidatively active molecule, such as peroxynitrite anion (ONOO$^-$) (68, 69).

The oxidation of cellular components by oxidative stress results in modifications of DNA, proteins, lipids, and carbohydrates, which are associated with cell death either by necrosis or by apoptosis. 4-hydroxy-2-nonenal (HNE) is the main aldehyde by-product formed during lipid peroxidation, and considered to be one of the most reliable markers of oxidative stress. Protein carbonylation is the ROS-induced post-translational modification of amino acid side chains. The protein carbonyl is widely used as marker of oxidative protein damage (70, 71). In addition, lipid peroxidation can also exacerbate the oxidation of protein. Some protein carbonylation from lipid-derived aldehydes is more prevalent than that formed via direct amino acid
side chain oxidation (72). ROS can also attack DNA, generating DNA lesions and resulting in mutagenesis and cell death, especially in the progression of DNA/RNA polymerases.

Nigral dopaminergic neurons are particularly exposed to oxidative stress due to the high monoamine oxidase (MAO) enzyme activity which yields H$_2$O$_2$ and other ROS. H$_2$O$_2$ could also react with iron in the SN to produce highly toxic hydroxyl radicals. The auto-oxidation of DA can also acerbate the ROS level (73). Due to the significantly lower concentration of antioxidants in the brain compared with other organs (74, 75), the brain is much more sensitive to oxidative stress. Previous work has shown that neurodegeneration is closely related to increased ROS levels in brain. Postmortem brains of PD patients have shown high levels of oxidation of lipids, proteins, and nucleic acids in the SN. The loss of functional nicotinic acetylcholine receptors (nAChRs) in neuron cells due to the intracellular ROS is like to have significant implications for the early progression of PD (76-78), therefore oxidative stress is directly involved in the loss of dopaminergic nigral neurons.

A. Oxidative Stress and Mitochondrial Dysfunction

There is considerable evidence for mitochondrial dysfunction in the brain of PD patients (79, 80). The dramatic loss of function of mitochondrial complex I activity may be the central tenet of sporadic PD, consequently promoting oxidative stress. Damage to mitochondrial complex I by oxidative stress can be acerbated by trace metals, environmental toxins, and familial Parkinson related genes. Epidemiological studies showed MPTP is related to acute and irreversible Parkinsonism in human and non-human primates (81). Different from 6-OHDA, MPTP is a lipophilic molecule that can easily cross the BBB and be transferred to
1-methyl-4-phenyl-2,3-dihydropyridinium (MPDP) in glial cells and metabolized to the active toxic compound (MPP+, 1-methyl-4-phenylpyridinium) (82). MPP+ can be taken up by DA neurons and concentrated in mitochondria, causing complex I dysfunction and oxidative stress (83). MPTP is responsible for several downstream apoptotic events in SN neurons, including up-regulation of inflammatory factors, release of cytochrome c, and activation of caspase-3 activity. In addition to MPTP, pesticides with related properties, such as rotenone, paraquat, dieldrin, and maneb exhibit common features to inhibit the mitochondrial respiratory chain and produce oxidative stress (84, 85).

Significant evidence suggests that accumulation of α-synuclein in mitochondria elevates oxidative stress, which is relevant to PD pathogenesis. Complex I defects cause α-synuclein aggregation and this aggregation would lead to further mitochondrial dysfunction, such as decreased mitochondrial membrane potential, and oxidation of mitochondrial proteins (86, 87). Further studies are needed to elucidate the precise functions of α-synuclein in the regulation of mitochondrial functions. Additionally, other genes linked to familial PD have been implicated in the mitochondrial function and oxidative stress response.

B. Metal Related Neurodegeneration, With Particular Consideration of Iron

Over the last decade, the redox ability of metal ions, such as copper, has been shown to lead to radical formation, and reactive nitrogen and oxygen species, contributing to various diseases. These metals can be coupled with proteins, accelerating the electronic reactions. A number of recent studies showed that α-synuclein interacts with zinc, copper, and iron, consequently in the state of aggregation (88). Therefore, it has become widely accepted that dyshomeostasis of
these redox metal ions (manganese, copper, iron, lead, and aluminum) is accompanied by oxidative stress, which is a key factor in a large number of neurodegenerative diseases, such as PD, Alzheimer’s disease, Huntington’s disease, multiple sclerosis and Friedreich’s ataxia (89). It has been shown that the concentration of zinc and iron are increased and copper is decreased in the SN in PD. However, the mechanisms of these changes remain unclear. Iron accumulation is closely related to neurodegenerative diseases. The elevation of brain iron such as in the putamen, motor cortex, sensory cortex and thalamus, are localized within H- and L-ferritin and neuromelanin with no adverse effects (90). However, excessive iron in cellular constituents, like the mitochondria, or specific regions, such as SN and lateral globus pallidus, can lead to the generation of toxic free radicals which can damage cells (89). Numerous studies have confirmed that there is an elevated iron level in the SN of PD patients compared to age-matched controls (91, 92). The oxidative stress in brain regions can cause peroxidation of polyunsaturated fatty acids in membrane phospholipids, generating protein carbonyls and damaging neurons.

C. Brain Iron and Neurodegeneration

Iron is a necessary cofactor in many metabolic processes in the central nervous system, including oxidative phosphorylation, myelin synthesis, nitric oxide metabolism, and oxygen transport. It plays an important role in electron transfer and affects numerous enzymes, including a number of key enzymes for neurotransmitter biosynthesis in the brain, such as tyrosine hydroxylase, which is involved in the metabolism of DA (93).

Serum iron is delivered to cells via transferrin (Tf). Endothelial cells can express transferrin receptor (TfR) on the luminal side of the capillaries in the BBB, and serum
iron can be delivered into the brain via the transferrin (Tf)-TfR-cell model, which is regulated by astrocytes. The iron which was in a diferric form in transferrin (Fe$^{2+}$-Tf) binds to TfR, followed by receptor-mediated endocytosis, therefore iron can be transported from the luminal to the abluminal sides of the blood capillary endothelial cells (BCECs). Later, iron is released from transferrin within the endosome under the acidic pH environment and the apotransferrin is recycled back to the plasma membrane to bind to more iron. DMT1 is responsible for transporting iron in and out of the endosome into the cytoplasm, and then metalloreductases can reduce Fe$^{3+}$ to Fe$^{2+}$. Therefore, cytoplasmic iron can be transferred to the mitochondria for biological usage, while the extra iron is stored in cytosolic ferritin (94, 95). Astrocyte cell membranes contain ceruloplasmin (Cp) which is linked to glycoposphoinostitide (GPI) (96). The ferroxidase activity of GPI-Cp is required for the stability of Fpn1 in cells, facilitating the iron efflux from the cells (97). Fpn1 is present in neurons and could therefore represent the mechanism by which iron is exported from such cells (98). Additionally, another iron regulator, hepcidin, is responsible for regulation of iron homeostasis. Hepcidin can bind to Fpn1 and down-regulate Fpn1 mRNA and protein expression by inducing its internalization and degradation, promoting iron retention (99). Hepcidin is expressed in several organs, such as liver and brain, and is closely related to the increased iron. It is also considered as an oxidation indicator, associated with oxidative stress.

Overall, iron metabolism and iron homeostasis at both the cellular and systemic levels is mainly regulated by the iron-associated proteins, such as ferric reductase, DMT1, the iron exporter Fpn1, and the membrane-bound ferroxidase. The translation of these proteins is modulated by the IRP system, together with the other iron homeostasis regulators, including hepcidin.
Fig. 1 The iron uptake into cell. Holotransferrin (HOLO-TF) binds to transferrin receptors (TFR) at the cell surface. The complexes localize to clathrin-coated pits, which initiate endocytosis. Specialized endosomes form and are acidified by a proton pump. At acidic pH, iron is released from transferrin and is co-transported with protons out of the endosomes by the divalent metal ion transporter DCT1. Apotransferrin (APO-TF) bound to TFR is returned to the cell membrane, where, at neutral pH, they dissociate to participate in further rounds of iron delivery. In non-erythroid cells, iron is stored as ferritin and hemosiderin (89).

IRPs are the key factors to post-transcriptionally regulate the iron-associated proteins involved in cellular iron metabolism. IRP1 and IRP2 also function as cytosolic iron sensors. In conditions of iron deficiency, IRPs bind with IRE, which is in the 5’-untranslated regions of the mRNA, preventing initiation of translation of ferritin and Fpn1. In contrast, in the case of transferrin receptor and DMT1 where the IREs are in the 3’-untranslated region, binding of the IRPs to the mRNAs protects them against degradation by nucleases. Therefore, this leads to increased iron uptake and reduced iron storage and export. In conditions of iron abundance, the IRPs are no longer active in binding, allowing ferritin and Fpn1 mRNA to be translated and inhibiting iron uptake by reducing transferrin receptor and DMT1 synthesis.

Disruption of iron transport proteins could cause excess iron in the brain. The cause might be genetic or by neurotoxins. The interruption of iron homeostasis is involved in a number of neurodegenerative disorders, including PD. The main adverse effect of
excess iron is the production of highly reactive hydroxyl radicals via the Haber-Weiss and Fenton reactions (100):

\[
\begin{align*}
\text{Fe}^{3+} + \text{O}_2^{•−} & \rightarrow \text{Fe}^{2+} + \text{O}_2 \\
\text{Fe}^{2+} + \text{H}_2\text{O}_2 & \rightarrow \text{Fe}^{3+} + \text{OH}^- + •\text{OH} \\
\text{O}_2^{•−} + \text{H}_2\text{O}_2 & \rightarrow \text{OH}^- + •\text{OH} + \text{O}_2 \\
\end{align*}
\]

If the increased ROS levels exceed the capacity of the self-antioxidant defense system, the cells will experience oxidative stress, membrane oxidation and protein damage. Increased oxidative stress is one of the contributors to PD progression. In addition, oxidative stress also exacerbates the intracellular free iron level, further promoting the α-synuclein aggregation to form LBs in PD brains (101).

Even if the iron dyshomeostasis is related to neurological diseases, it is still under debate whether impaired iron homeostasis is the primary cause. Genetic hemochromatosis and thalassemic patients with high circulating iron, transferrin saturation, and high levels of low-molecular weight iron, do not exhibit increased brain iron. Evidence shows that higher iron distribution in different brain regions was associated with pathologic status.

D. Oxidative Stress and Neuroinflammation in PD

The immune responses within the brain are regulated by resident immune cells. Microglia are the major resident immune cells in the brain, providing innate immunity via the production of neurotoxic factors including cytokines, chemokines, and prostaglandins. Those factors also contribute to the immune activities of surrounding astrocytes and oligodendrocytes (102). Pro-inflammatory cytokines such as TNF-α and IL-1β act on astrocytes, inducing the adaptive immune response, while chemokines such as monocyte chemotactic protein-1 (MCP-1)/Chemokine (C-C motif)
ligand 2 (CCL2) recruit additional immune cells. These inflammatory responses may potentiate neuronal cell damage through oxidative stress. Neuroinflammation and oxidative stress share common linkages and influence each other. One of the functions of proinflammatory cytokines is to produce oxidative stress, which can subsequently shape the profile of cytokine release. Microglia are a major source of ROS in the CNS. Under the pathologic condition where there is reduced antioxidant level, oxidative stress causes more free radicals to be released into the cytosol, inactivating proteins or interrupting intracellular signaling pathways. In addition, ROS can initiate pro-inflammatory pathways in neuronal cells. Compared to cortical or hippocampal neurons, midbrain DA neurons are more susceptible to pro-inflammatory cytokines such as TNFα (103). Therefore, future therapeutic interventions to limit the early pro-inflammatory response would be beneficial in reducing microglia activation and elevated oxidative stress (104).

Epidemiological, animal and human studies implicated the innate and peripheral neuroinflammatory response contributing to the onset of PD. Increased immune cells, such as CD4+ and CD8+ have been found in post-mortem human PD brains (105, 106). The use of positron emission tomography (PET) imaging has confirmed the activated microglia in human PD brain (107). Elevated levels of pro-inflammatory cytokines have been found in the SN of PD brains. Factors known to promote neuroinflammatory response, including traumatic brain injury, and exposure to viruses and infectious agents, have all been proposed as increased risk factors for developing PD (108). In addition, 6-OHDA and MPTP are also commonly shown to cause PD in animal models, and display features of neuroinflammation.
1. **Antioxidation**

Peroxisome proliferator receptor (PPAR) is a family of ligand-dependent nuclear hormone receptor transcription factors that play a key role in the regulation of inflammatory responses, antioxidation, and brain function. PPARs stimulate gene expression by binding to peroxisome-proliferator response elements (PPREs) of the target genes (109). There are three structurally homologous iso-types: PPARα, PPARβ/δ, and PPARγ. PPARα is expressed in several organs, including liver, kidney, intestine, heart, skeletal muscle, and brain. PPARα is involved in acetylcholine metabolism, excitatory neurotransmission, and oxidative stress defense. PPARα can regulate lipid metabolism and energy homeostasis (110). PPARβ/δ is ubiquitously expressed in all cell types, including immature oligodendrocytes, and promotes differentiation and myelination in the CNS. PPARβ/δ also can regulate lipid metabolism in the brain (111). PPARγ can be detected in adipose tissue, intestinal mucosa, retina, skeletal muscle, heart, liver, and lymphoid organs. PPARγ has been implicated in the pathology of numerous diseases including obesity, diabetes, atherosclerosis, and cancer. PPARγ is not only an anti-inflammatory factor, but also has antioxidant properties, and is used as treatment of oxidative stress related diseases, including PD, AD, and multiple sclerosis (112, 113). In these disorders, PPARγ agonists have the potential to modulate various signaling pathways, including matrix metalloproteinase-9, mitogen-activated protein kinases (MAPK), signal transducer and activator of transcription, mitochondrial uncoupling protein 2, and amyloid precursor protein degradation (114). PPARγ may directly modulate the expression of several antioxidant genes in response to oxidative stress, including catalase, SOD, GPx, the scavenger receptor CD362, and nitric oxide synthase (eNOS) (115-119). PPARγ can regulate heme oxygenase-1 (HO-1) expression, however this effect is still
under debate (120, 121). It is obvious that PPARγ is closely related to antioxidative defense mechanisms. Recent evidence had confirmed that Nrf2 and PPARγ were linked by a positive feedback loop that sustained the expression of antioxidant genes and antioxidant system under oxidative stress (119, 122). Therefore, antioxidation and anti-inflammatory pathways might be simultaneously involved in the progression of neurodegeneration and the clarification of the mechanisms on the interaction will be needed to be researched.

2. Anti-inflammatory System

The neuroinflammatory diseases, including AD, and PD, present major challenges to the health care system and impose substantial economic costs around the world (123). Since evidence shows that PPARγ is expressed in certain areas of the brain, such as neurons and glial cells, it may possibly be involved in neuroinflammation and subsequent neurodegeneration. As shown in a MPTP model, PPARγ exerts both anti-inflammatory and antioxidant effects by inhibiting glial activity, which may account for the attenuation of dopaminergic cell loss in both the SN and striatum. In the LPS-induced inflammatory model of PD, PPARγ showed the ability to restore striatal DA, mitochondria function, and significant dopaminergic neuroprotection by directly inhibiting mitochondrial fatty acid metabolism and altering mitochondrial uncoupling protein expression (124, 125).

PPARγ could reduce the infiltration of immune cells to the BBB and down-regulate pro-inflammatory genes such as COX-2, iNOS, and various cytokines, which were associated with inflammation-induced neurodegeneration (126-128). The neuroprotection of PPARγ activation was also based on antiinflammation through inhibiting IL-6, TNFα expression, and toll-like receptor (129, 130).
Additionally, epidemiological studies showed that 7% of PD patients have type II diabetes or insulin desensitization. They suggest that diabetes accelerated the progression of the motor and cognitive symptoms of PD, and insulin in the nigrostriatal system is related to PD (131). Drugs used to treat PD, such as L-dopa or bromocriptine alter insulin signaling and sensitivity. PPARγ had been associated with insulin sensitivity alteration and inhibition of inflammation. Therefore, PPARγ activation may offer a new clinical treatment approach to neuroinflammation and PD (132).

III. TREATMENT AND PREVENTION STRATEGIES OF PD

Since it is impossible to cure PD, preventing and controlling the symptoms of PD and minimizing side effects are the goals of anti-Parkinsonian therapy. Data is lacking in many areas of PD treatment and prevention, therefore, there is currently wide variation in the management of PD.

A. Treatments

1. Pharmacological Treatment

L-dopa has been used as the main pharmacological treatment of PD, and peripheral decarboxylase inhibitor is used to limit metabolism of L-dopa. Even if L-dopa is the most effective drug in the treatment of PD, long-term use can cause motor fluctuations and dyskinesias (133). There is still debate over how to use L-dopa as a neurological treatment, such as dosage and time. L-dopa has been shown to be toxic to neurons in vitro, but these findings have not been substantiated in humans (134, 135).
DA agonists are frequently used as adjunct therapy with L-dopa in early PD, and they include bromocriptines, pergolide, lisuride, cabergoline, pramipexole and ropinirole. The mechanism of action of DA agonists depends on their DA releasing, inhibiting reuptake and DA receptor and stimulating capabilities. Monoamine oxidase B (MAO-B) inhibitors can be used as dopaminergic therapy in patients with early PD because they can inhibit DA catabolism and increase nigrostriatal DA levels. The inhibition of catechol O-methyl transferase (COMT) can increase the bioavailability and prolong the action of DA and is also considered as a therapy for neuroprotection. In addition, anticholinergic medication methods are also used to treat PD, such as benztropine and procyclidine. However, they are typically used in younger PD patients, instead of patients older than 65.

2. Surgical Treatment

Recognition of the limitations of pharmacological therapy and improvement in surgical technologies has led to a resurgence of surgery on PD patients in recent years. Even though surgical treatment may raise concerns regarding safety, it may reduce drug-induced dyskinesias and dystonias in PD, including ablative procedures (globus pallidus or thalamus), deep brain stimulation and tissue transplant (136). Thalamic stimulation has equal efficacy for tremor suppression, but with fewer adverse effects compared to thalamotomy (137). Transplantation has shown promising benefits, but studies are questioning its efficacy and safety, which has impeded this treatment procedure (138).
B. Prevention Strategies

Due to the efficacy and safety concerns about the treatment therapies of PD, the use of antioxidants, anti-inflammatory drugs, and specific stress signal inhibitors as prevention methods have provided potential beneficial effects for common neurodegeneration.

1. Antioxidants

Selegiline, vitamin E, and rasagiline have been used in clinical trials due to their antioxidant properties (139, 140). Selegiline reduces DA oxidation by inhibiting MAO-B and has been used as an antioxidant drug. Although there exist concerns about the efficacy of vitamin E in the prevention of PD, long-term, high dose vitamin E dietary supplementation (e.g. 1600-2000 IU d-α-tocopheryl succinate) was shown to be a successful therapeutic strategy for the prevention of PD (143, 144). Rasagiline is a newer MAO-B inhibitor and its metabolite has potential antioxidant property. A clinical study showed rasagiline reduced the oxidative effect of symptomatic efficacy. Coenzyme Q10 (CoQ10) is a cofactor in the electron transport chain in mitochondria metabolism and has been shown to reduce dopaminergic neurodegeneration in mouse PD models (145). However, CoQ10 showed less or no significant protection on PD progression based on different study designs, initiating concerns on the stability of its usefulness (146-148). Even if none of these clinical studies have convincingly shown that these antioxidant therapies slow disease progression, several of these trials indicate that selegiline, rasagiline, and CoQ10 may be promising. In addition, compared to current PD treatments, all of the antioxidant approaches are relatively safer in neuroprotection. Epidemiological studies identified uric acid as a potential
25

neuroprotective agent in PD, which acts as an antioxidant by scavenging ROS and NOS.

2. Receptor antagonists

Epidemiological studies have shown that caffeine may reduce the incidence of PD, leading to adenosine receptor antagonists as potential neuroprotective agents (13, 153). Adenosine receptor antagonist A2a can improve symptoms in PD and be neuroprotective, which was shown in several in vivo studies (154, 155). A2a can also exert synergistically effects with L-dopa (156).

DA receptor agonists have been proposed as potentially neuroprotective by acting at D2 autoreceptors found on dopaminergic SN terminals to suppress DA release and thus reduce oxidative stress in vitro and in animal studies (157-160). Certain agonists, such as pramipexole, may also act as direct antioxidants due to their hydroxylated benzyl ring structure. However, it is still difficult to establish long-term systems for neuroprotection of the receptor antagonists’ neuroprotection.

3. Anti-inflammatory agents

The use of anti-inflammatory drugs has been investigated in various diseases. In PD, antiinflammation has been considered as a potential neuroprotective strategy in drug development (161). Anti-inflammatory agents, including non-steroidal anti-inflammatory drug (NSAIDs) and minocycline have been used to treat PD in tissue culture and in animal models. NSAIDs, such as aspirin, have anti-inflammatory effects, including reduction of TNFα, nitric oxide, and superoxide production by microglia (162). Statins may also act to show neuroprotection due to their anti-inflammatory ability beyond scavenging free radicals (163). Epidemiological
studies showed that statin use, particularly simvastatin, was associated with reduced PD incidence (164, 165). Simvastatin had been shown to reduce DA loss in MPTP animal models (162). Minocycline has anti-inflammatory effects independent of its antimicrobial activity and protects against dopaminergic cell loss in both MPTP and 6-OHDA animal models (166, 167). Minocycline blocks microglial activation and may also have anti-apoptotic activity in vitro (168).

4. α-synuclein-directed therapies

Since protein aggregations, like α-synuclein, are related to PD progression, the disruption of α-synuclein aggregation has been the focus of research to develop novel therapies against PD. Augmentation of parkin or ubiquitin carboxy-terminal hydrolase-L1 (UCH-L1) activity could promote the clearance of α-synuclein and other aggregated proteins (169, 170). Activation of lysosomal degradation could also clear α-synuclein. Therefore, the lysosomal enzyme cathepsin D could inhibit α-synuclein-induced neurotoxicity in cell culture and animal models (171). Because oxidative modification and phosphorylation of α-synuclein both promote protein aggregation, antioxidants and kinase inhibitors could reduce α-synuclein aggregation and toxicity (172-174). In addition, direct blockers of α-synuclein aggregation could be a therapeutic strategy for PD. Increasing the expression of chaperone-like proteins, such as 14-3-3, has been demonstrated to reduce α-synuclein aggregation and could serve as a basis for therapeutic intervention (175, 176). The chaperone protein Hsp70 reduced insoluble α-synuclein aggregates in vitro and in vivo (177).

The COMT inhibitors entacapone and tolcapone are able to block fibril formation of α-synuclein. However, a strong limitation for the use of these compounds is that they have a wide range of side effects, such as vomiting, nausea and hypotension, or
in the case of tolcapone, hepatotoxicity (178). In addition, cholesterol reducing agents have been shown to be effective in blocking \( \alpha \)-synuclein aggregation in vitro and in vivo.

5. Anti-apoptotic agents

Several anti-apoptotic agents have been examined in controlled clinical trials. Vasoactive intestinal peptide (VIP) can be considered as a good anti-apoptotic candidate agent for 6-OHDA induced neurotoxicity and the treatment of PD (179). Tauroursodeoxycholic acid (TUDCA) acts as an anti-apoptotic agent in MPTP mouse PD models, used as a modulator of neurodegeneration in PD (180). The propargylamine TCH346 is an anti-apoptotic factor and was shown in both 6-OHDA and MPTP animal models to reduce dopaminergic cell loss (181, 182). Overall, anti-apoptotic agents would be a perspective treatment for PD progression.

6. Kinase inhibitors

The activation of kinase pathways has been shown to be involved in the progression of PD in in vitro and in vivo studies. The most common genetic cause of PD to date is mutation in the gene LRRK2, which is associated with increased kinase activity. Therefore, kinase inhibitors seems to be new neuroprotective strategies against the progression of PD. Kinase inhibitors, such as Z-Asp(Ome)-Ile-Pro-Asp(OMe)-FMK can block PK\( \Delta \) cleavage and reduce caspase-3 related neurotoxicity. It can also rescue TH+ neurons from MPP+ and 6-OHDA-induced toxicity in primary mesencephalic cultures (183).
7. Polyphenols

Polyphenols are a class of highly bioactive compounds, and can be categorized into flavonoids, stilbenes, phenolic acids, phenolic alcohols, and lignans. A number of *in vitro* and *in vivo* studies indicate that these small molecules are able to modulate neurological progression pathways due to their strong antioxidant and anti-inflammatory effects. A natural polyphenol resveratrol can directly scavenge the hydroxyl radical, inhibit H$_2$O$_2$ or lipid peroxide-dependent peroxidation (190, 191), suppress COX-2-induced neuroinflammation (192), and reduce dopamine-induced cell death *in vitro* (193, 194). *In vivo*, resveratrol attenuated 6-OHDA-induced DA depletion and loss of dopaminergic neurons in rats (195).

It is important to note that many *in vitro* studies have focused on the neuroprotective properties of flavonoids that differ from their forms *in vivo*. For example, natural forms of flavonoids are hydrolyzed and conjugated by gut and liver enzymes before entering the circulation (196), resulting in reduction of their antioxidative activity (197, 198). Both intact and derivative forms of flavonoids such as tea catechins and naringenin have been found to pass through the BBB (199). The degree of permeability is governed by several factors, including compound lipophilicity and the action of specific transporters on the BBB (200). Therefore, elevating the bioavailability of flavonoids in CNS would benefit PD drug development.

8. Nutritional strategies

Oxidative stress causes the loss of dopamine neuron within the central nervous system. Therefore, an antioxidative has to potential to impart neuroprotection. A diet containing high levels of antioxidants, such as complex phenols, vitamin E, and
carotenoids, acts as a preventative measure for the disease. Higher intake of vegetables, legumes, fruits, cereals, unsaturated fatty acids, and fish may add more antioxidants, while a diet rich in dairy and animal fat products results in a higher risk of developing PD. Foods which cause high plasma urate may reduce PD risk because of urate’s ability to scavenge peroxynitrite and hydroxyl radicals resulting in decreased oxidative stress.

Oxidative modification can cause mitochondrial decay, resulting in neurodegeneration. Supplementation with mitochondrial nutrients may prevent mitochondrial decay and the possible mechanisms may include: a. preventing mitochondria from oxidative stress, with nutrients such as lipoic acid, vitamin E and CoQ; b. repairing mitochondrial membranes, with nutrients such as carnitine; c. functionally repairing and preventing mitochondrial damage, such as substrates or coenzymes, or precursors of mitochondrial enzymes (quinine reductase and glutathione-S-transferase), with nutrients such as lipoic acid, niacin, pantothenic acid, riboflavinthiamine, biotin, and carnitine; d. inducing phase-2 enzymes to enhance antioxidant defenses in cytosol as an indirect protection to mitochondria, with nutrients such as lipoic acid.

The combination of two mitochondrial nutrients may have more effective antioxidant capability than each has individually. It is reported that the combination of R-alpha-lipoic acid (LA) and acetyl-L-carnitine (ALC) has a synergistic action against rotenone-induced mitochondrial dysfunction, oxidative damage and a-synuclein accumulation at lower concentrations than they have individually (100-1000-fold lower).
Figure 2. Possible protective mechanisms of nutrients in mitochondria. B1, thiamine; B2, riboflavin; B3, niacin; B5, pantothenic acid; B6, pyridoxine; B12, cobalamin; CoQ, coenzyme Q10; Cyt C, cytochrome c; LA, alpha-lipoic acid; PDH, pyruvate dehydrogenase; alpha-KGDH, alpha-ketoglutarate dehydrogenase; BC-KADH, branched chain ketoacid dehydrogenase, CAT, carnitine acyltransferases; Carboxilase includes pyruvate carboxylase, acetyl-CoA carboxylase, and propionyl-CoA carboxylase, and THF-related enzymes includes methionine synthase, methylene tetrahydrofolate reductase.

C. Green Tea Polyphenol Properties & Mechanisms of Protection

Green tea catechines have been shown to have many biological actions, including antioxidant, free radical-scavenging, antiatherosclerotic, cardioprotective, anti-inflammatory, and anticarcinogenic effects. There is still controversy on the cancer-preventive, cardiovascular protective, or neuroprotective roles of green tea intake due to the limitation of data. Most of the results have come from studies using observational designs, case studies, case-control studies or cohort studies that lack the rigor of clinical trials. Detailed information on intake quantities and cumulative exposure to green tea (years of drinking) is lacking. However, human epidemiological studies suggest that tea consumption is inversely correlated with incidence of dementia, AD, and PD. In a cross-sectional study aimed at investigating an association between consumption of green tea and cognitive function in elderly
Japanese subjects, it was found that higher consumption of green tea is associated with lower prevalence of cognitive impairment (201). In a case-controlled study in the US, it was found that people that consumed 2 cups/day or more of tea presented a decreased risk of PD (202). In consensus, a recent prospective 13-year study of nearly 30,000 Finnish adults, aged 25-74 years, demonstrated that drinking 3 or more cups of tea per day is associated with a reduced risk of PD (203). These findings emphasize the importance of well-designed controlled studies to assess a risk reduction of PD and AD in consumers of green tea. The beneficial effects of green tea are attributed to its polyphenolic compounds, particularly the catechins, approximately composing 30% of the dry weight of green tea leaves. There are higher quantities of catechins in green tea than black or oolong tea due to the different processing steps. For green tea, the polyphenol oxidase enzymes are inactivated and maintained in the monomeric forms. Catechins account for 25–35% of the dry green tea extract and consist of eight polyphenolic flavonoid-type compounds, namely, (+)-catechin (C), (−)-epicatechin (EC), (+)-gallocatechin (GC), (−)-epigallocatechin (EGC), (+)-catechingallate (CG), (−)-epicatechin gallate (ECG), (+)-gallocatechin gallate (GCG) and EGCG. The most abundant catechin is EGCG, which accounts for 65% of the total catechin content in green tea. A cup of green tea (2.5 g of green tea leaves/200 ml of water) may contain 90 mg of EGCG and thus is thought to particularly contribute to the beneficial effects attributed to green tea, such as its neuroprotective properties. Green tea and EGCG have been used in many different cells and animal models to investigate mechanisms underlying the various actions of green tea catechins.
EGCG has been shown to improve age-related cognitive decline and protect against cerebral injuries and brain inflammation and neuronal damage in experimental autoimmune encephalomyelitis (204). Long-term administration of EGCG was demonstrated to improve spatial cognition learning ability in rats (206). In line with the in vitro findings, cell culture studies had demonstrated that EGCG prevented neuronal cell death caused by the neurotoxins 6-OHDA and MPP+ in several cell lines, such as human neuroblastoma SH-SY5Y cells and rat pheochromocytoma (PC12) cells. EGCG not only has cancer chemopreventive, anti-angiogenic and anti-mutagenic properties, but also has anti-diabetic properties in an insulin animal model and anti-bacterial, anti-HIV, anti-aging, and anti-inflammatory activity. EGCG has a catechol-like structure, which inhibits DA uptake, blocks DA competitors, exerting neuroprotective effects. EGCG also inhibits the activity of the enzyme COMT to block the metabolism of DA and related catecholamines. In addition, EGCG is able to regulate the proteolytic processing of a-synuclein and reassemble the structure of a-synuclein to convert it into a less toxic form. Beyond this, EGCG can also promote the non-amyloidogenic a-secretase pathways of APP to soluble APP, against AD.
Low concentrations of EGCG are responsible for the antiapoptotic/neuroprotective effects, whereas high doses account for its antiproliferative, antiangiogenic and pro-apoptotic actions (207). For example, low concentrations of EGCG decreased the expression of pro-apoptotic genes bax, bad, caspases1 and 6, cyclin-dependent kinase inhibitor p21, fas-ligand and tumor necrosis factor-related neuronal cells, while high concentrations induced apoptosis. Most of the effects of EGCG in cell culture systems have been observed with relatively higher concentrations than those in the plasma or tissues of animals or in human plasma after administration of green tea or EGCG. The reason may be that EGCG is subjected to form a dimer, and auto-oxidizes under cell culture conditions, reducing its half-life. It is not clear whether the activities observed with high concentrations of EGCG in cell lines can be observed in vivo. However, it is possible that increased consumption of green tea by humans could result in the buildup of higher concentrations of EGCG in the plasma.

Bioavailability is an important factor for considering the beneficial effects of EGCG use in vivo. EGCG has only limited systemic availability. There have been some recent efforts to enhance its bioavailability by delivering EGCG using lipid nanocapsules and liposome encapsulation. Indeed, there has been successful in vitro and in vivo testing of the delivery of EGCG in polylactic acid-polyethylene glycol nanoparticles to inhibit angiogenesis and induce apoptosis. It is shown that nanolipid EGCG particles can significantly improve the neuronal α-secretase and have more than two-fold greater oral bioavailability than normal EGCG treatment on AD. Based on the current findings, additional large, long-term cohort studies and clinical trials should be performed to investigate the efficacy, pharmacology, safety, and side-effects of standardized preparations of green tea and EGCG.
1. EGCG Antioxidant Activity

Exogenous antioxidants also play a major role in maintaining cellular redox balance. These include direct scavenging of HO·, singlet oxygen, etc. Tea catechins are powerful hydrogen-donating antioxidants and free radical scavengers of ROS and NOS in vitro. The anti-oxidant property of catechins is due to the presence of the phenolic hydroxyl groups on the B-ring in ungalloylated catechins (EC and EGC) and in the B- and D-rings of the galloylated catechins (ECG and EGCG) (208, 209). The presence of the 3, 4, 5-trihydroxy B-ring is important for antioxidant and radical-scavenging activity.

The neuroprotective effect of green tea polyphenols in vivo may also involve the regulation of antioxidant protective enzymes through the Nrf2/ARE system. EGCG was found to elevate the activity of two major oxygen-radical species metabolizing enzymes, SOD and catalase, in mice striatum (206). In peripheral tissue it has been shown that EGCG at low concentrations activates the expression of some stress-response genes, such as phase II detoxification enzyme, glutathione-s-transferase and HO-1.

2. EGCG & Iron Chelation

The 3,4,5-trihydroxy B-ring of the catechins (shown in Fig. 3) have been shown to be important for antioxidant and radical scavenging activity. EGCG was shown to be more efficient as a radical scavenger due to the presence of the trihydroxyl group on the B- and D-rings and the gallate moiety at the 3’ position in the C ring. The green tea catechins have been shown to be more effective antioxidants than vitamin C and E, and the radical scavenging capability order is ECG>EGCG>EGC>EC>catechin. There are two points of attachment of transition metal ions to flavonoid molecules:
the o-diphenolic groups in the 3’,4’-dihydroxy positions in the B ring, and the keto structure 4-keto, 3-hydroxy or 4-keto and 5-hydroxy in the C ring of the flavonols. Therefore, the metal-chelating properties of green tea catechins are also important contributors to their antioxidative activity. The chelation capability of EGCG has major significance for the treatment of neurodegenerative diseases.

Desferal/desferrioxamine (DFO) was shown to be neuroprotective against 6-OHDA and MPTP-induced neurotoxicity in rat and mouse models (212, 213). However, the hydrophilic nature and large molecular size of DFO limited its absorption across the gastrointestinal tract and prevents it from penetrating the BBB. EGCG has the capability to cross the BBB and possess relatively potent metal-chelating properties due to the gallate moiety present in the C-ring. EGCG and R-apomorphine prevented iron-dependent α-synuclein aggregation in the SN of MPTP-treated mice and restored the nigro-striatal DA neurons (214). The chelation of iron by EGCG also activates hypoxia signal transduction pathways, such as HIF-1, and regulates compensatory factors, including prolyl-4-hydroxylase, chaperones, and the immunoglobulin-heavy chain-binding protein, HSP90. EGCG can reduce the iron pool and activate IRP expression (215, 216). The regulation of IRP is coordinated with iron-related proteins, maintaining cellular iron hoemeostasis, survival, and proliferation, including ferritin, TfR and Fpn1, etc.

Table 1. Iron chelators and neuroprotection

<table>
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<th>Iron Chelators</th>
<th>PD prevention</th>
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<tr>
<td>Desferrioxamine</td>
<td>Higher binding affinity to Fe^{3+} than Fe^{2+}. Protects dopamine in rat PD model. Also inhibits iron-promoted radical damage in dopaminergic cell line in 6-OHDA. Unable to cross the BBB when orally administrated due to the particle size and hydrophilic feature. It has adverse effects.</td>
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Table 1. Iron chelators and neuroprotection (Continued)

<table>
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<th>Iron chelator</th>
<th>Neuroprotection</th>
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| **Clioquinol** (5-chloro-7-iido-8-hydroxyquinone) | Reduces iron level in substantia nigra of mice.  
Pre-treatment prevents mice against MPTP toxicity.  
Produces unintended effects due to no iron selective, by reducing the S-adenosylmethionine level, resulting into vitamin B12 deficiency.  
Can be used orally. |
| **HLA20**                      | Protects P19 cells (mouse embryonal carcinoma cell line) against 6-OHDA.  
Strong ferric chelator, can also inhibit MAO-B activity. |
| **VK-28** (5-[4-(2-hydroxyethyl)piperazine-1-ylmethyl]-quinoline-8-o1) | Penetrates mitochondrial membrane.  
Protects dopaminergic neuron against 6-OHDA in rat. |
| **M30** [5-[(N-methyl-N-propargyaminomethyl)-8-hydroxyquinoline] | Both iron chelator and MAO-B inhibitor. Similar to HLA20.  
More effective MAO-B inhibitor than HLA20 and VK-28 in vivo. |
| **Aroylhydrazones** PCIH (2-pyridylcarboxaldehyde isonicotinoyl hydrazone) | Charged hydrazone can cross membrane and remove iron more effectively. |
| **H₂O₂-triggered prochelator** SIH-B (2-borobenzaldehyde isocotinoyl hydrazone) | H₂O₂ can trigger for the transformation of prochelator SIH-B into the active chelator SIH, which in turn will form an inert complex with iron or copper and inhibit the Fenton reaction. Decrease the adverse effect of iron chelation. Experiment test in vivo is underway. |
| **Green tea polyphenols** EGCG | Inhibits cytochrome p450, GST, lipid peroxidation in vitro and in vivo studies. Strong antioxidant at lower concentration, while prooxidant at high concentration. |
| **Quercetin**                  | Inhibits Fenton reaction in vitro. Strong iron chelator in vivo. Protects mesencephalic dopamine neurons from apoptosis induced by MPP+. |
| **Curcumin**                   | Decreases the iron level in vitro against neurotoxin, even if not as effectively as DFO. Can protect dopamine level in 6-OHDA injected rat. |
3. EGCG & Protein Kinase Signaling Pathways

The neuroprotection of EGCG is shown to be involved in regulating intracellular signaling pathways, such as protein kinase C (PKC) in the SH-SY5Y cells (207, 217). To some extent, the PKC inhibitor can diminish the protection of EGCG. Therefore, interventions to modulate specific PKC activity or PKC mediated signal transduction pathways may constitute a potential therapeutic tool for neuroprotection. PKCε activation agonist was shown to be essential for neuroprotection against oxygen or glucose deprivation in vitro. EGCG has been shown to phosphorylate and activate PKC and protect against several neurotoxins, such as Aβ and 6-OHDA (207). EGCG can also relocate the isoform PKCα to the membrane compartment in PC12 cells. An animal study showed that two weeks of oral EGCG consumption prevented the depletion of PKCα and against Bax protein in the striatum and SN of mice with MPTP administration (218). In addition to PKC, other cell signaling pathways are implicated in the action of EGCG, such as the MAPK, phosphatidylinostitide 3’-OH kinase (PI3K)/Akt, and PKA signaling cascades and cell calcium influx regulation (219). In general, green tea catechin can activate MAPK in both neuronal and extraneuronal cultures to increase the extracellular signal-regulated kinase (ERK1/2) activity, which was suppressed by oxidative stress (220). Epicatechin was shown to stimulate phosphorylation of cAMP-response element binding protein (CREB) to regulate neuronal viability and synaptic plasticity activity via ERK1/2 and Akt in primary cortical neurons (221). Although EGCG had no effect on ERK1/2 phosphorylative levels, it was able to counteract the decline in ERK1/2 induced by 6-OHDA in neuroblastoma cells (207).
4. EGCG & Inflammation

Since neuroinflammation reactions are closely related to PD, the inhibition of inflammation can be a preventive strategy for neurological disorders. EGCG is shown to be involved in the regulation of anti-inflammatory activity. The administration of EGCG reduced expression of IL1β, TNF, and TGF in C57BL/6 mice (222). EGCG can also indirectly regulate the inflammatory effect through down-regulation of NF-κB and up-regulation of PPARγ (223). Interestingly, it was found that higher dose of dietary supplement of EGCG can promote inflammation reaction in C57BL/6 mice, including TNF-a, IL-6, different from the protection effects of low dose (224). However, the mechanism by which EGCG regulates anti-inflammatory action is still unclear. Further studies are needed to clarify the involvement of EGCG in the inflammatory pathways.

In summary, EGCG may prevent against PD progression by its antioxidant, anti-carcinogenic, and anti-inflammatory properties, and by regulating iron homeostasis. Further studies are required to clarify the mechanism in vivo. In addition, the treatment methods, concentration, and structure modification, which are all related to EGCG bioavailability, need to be investigated in animal models. Since limited data is available to show the protection of EGG in humans, newly designed human clinical trials are required to directly demonstrate the preventive effects of EGCG.

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CHAPTER 3. EGCG PROTECTS AGAINST 6-OHDA INDUCED NEUROTOXICITY IN A CELL CULTURE MODEL

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Abstract: Parkinson’s disease (PD) is a progressive neurodegenerative disease that causes severe DA depletion in the striatum of the brain and induces clinical signs such as tremor, rigidity, akinesia, immobility and frequent falls. Disruption of iron metabolism may be involved in the progression of PD. Our objective was to test whether the protective effect of (-)-epigallocatechin-3-gallate (EGCG) against 6-hydroxydopamine (6-OHDA) induced neurotoxicity was by regulating iron homeostasis in N27 cells. The protection of EGCG was assessed by SYTOX Green assay, MTT and caspase-3 activity. Pretreatment of EGCG with 6-OHDA significantly increased (p<0.0001) TH⁺ cell count (~ 3-fold) and neurite length (~ 12-fold) compared to 6-OHDA alone in primary mesencephalic neurons. 6-OHDA treatment significantly increased divalent metal transporter-1 (DMT1), hepcidin, and decreased ferroportin-1 (Fpn1) expression, while pretreatment of EGCG significantly altered these adverse effects of 6-OHDA. The increased ~2-fold (p<0.001) ⁵⁵Fe cell uptake by 6-OHDA confirmed iron burden associated with neurotoxicity. Pretreatment of EGCG protected against the neurotoxicity by reducing the iron uptake and retention compared to 6-OHDA. In conclusion, pretreatment of EGCG (not co-treatment) protected against 6-OHDA induced neurotoxicity by regulating genes
and proteins involved in brain iron homeostasis. However, further studies are warranted to determine the mechanism of that regulation.

Key words: EGCG, Iron, 6-OHDA, Parkinson’s disease

Introduction

Iron plays a crucial role in many physiological functions (1) including DNA synthesis, oxygen transport, and mitochondrial respiration (2, 3). Being a cofactor of tyrosine hydroxylase, iron is also involved in brain functions, such as neurotransmission and myelination (4, 5). It is important to regulate intracellular iron uptake, transport and storage. Iron homeostasis can be interrupted by dysfunction of iron related proteins, leading to the increased iron accumulation. The accumulation of unbound free iron in the cell further induces oxidative damage on cellular organelles by producing free radicals via the Fenton reaction (6), resulting in chronic neurological disorder diseases, including PD (7, 8).

Iron homeostasis and metabolism are regulated by iron-related proteins. The divalent metal transporter 1 (DMT1) is one of the proteins responsible for intracellular iron uptake. DMT1 can be upregulated by neurotoxin, such as 6-hydroxydopamine (6-OHDA), resulting in intracellular iron accumulation, free radical formation and neurodegeneration (9-11). However, it is still unclear whether iron influx and subsequent free radical formation is a primary or secondary event in the neurodegenerative process. Hepcidin is a key iron regulatory protein that is responsible for normal iron homeostasis. Hepcidin is expressed in several organs, such as the liver, brain, spinal cord and is closely related to intracellular iron concentration. Its expression is also influenced by oxidative stress (12). Ferroportin 1
(Fpn1) is the iron exporter which exports intracellular iron. Hepcidin can bind to Fpn1, causing its internalization and degradation, promoting iron retention in the cell (13).

It has been confirmed that 6-OHDA can induce oxidative stress and cause severe neuron cell death. 6-OHDA can release iron from ferritin (14, 15), induce depletion of PKC kinases, stimulate inflammatory factors and cause cell membrane potential hyperpolarization, etc. However, the detailed mechanisms remain unclear. Since 6-OHDA is shown to alter DMT1 expression and oxidative stress (9), there is a possibility that 6-OHDA may exert its neurotoxicity by interrupting iron homeostasis and subsequent oxidative damage.

Green tea polyphenol has been shown to provide beneficial effects against cancer, inflammation, neurological disorders, including PD. (-)-Epigallocatechin-3-Gallate (EGCG) is the most abundant polyphenol in green tea, and due to its capability of scavenging free radicals and chelating iron, it has been shown to protect against neurotoxins in several cell culture models of PD (16, 17). EGCG is reported to have antioxidant activity to reduce oxidative stress. It also can affect protein kinase and cell apoptosis. Several in vitro studies have shown EGCG reduces 6-OHDA neurotoxicity, but the mechanism of the protection is not clear. We hypothesized that 6-OHDA and EGCG administration would affect the regulation of iron via iron-related proteins. We used immortalized rat mesencephalic dopaminergic neuronal cell line (N27 cells) to determine whether 6-OHDA disrupted iron metabolism by regulating iron transporters, such as DMT1, Fpn1 and hepcidin, etc. We also determined whether EGCG protected against the adverse effects of 6-OHDA by normalizing and maintaining iron homeostasis.
Materials and Methods

**Reagents.** EGCG, 6-OHDA, and thiazolyl blue tetrazolium bromide (MTT) were purchased from Sigma–Aldrich (St. Louis, MO). SYTOX® Green Nucleic Acid Stain was from Molecular Probes (Eugene, OR). Substrate for caspase-3, Acetyl-Asp-Glu-Val-Asp-AFC (Ac-DEVD-AFC), was obtained from MP Biomedicals (Solon, OH). The mouse TH⁺ antibody was obtained from Millipore (Temecula, CA); Alexa 680-conjugated anti-mouse secondary antibodies, RPMI-1640 medium, fetal bovine serum, l-glutamine, penicillin, streptomycin and neurobasal medium, B27 supplement, and Dulbecco’s modified Eagle’s medium (DMEM) were obtained from Invitrogen (Carlsbad, CA). Rabbit anti-rat-DMT1 polyclonal antibody, rabbit anti-rat-MTP1 polyclonal antibody and rabbit anti-rat-hepcidin polyclonal antibody were purchased from Alpha Diagnostic International (San Antonio, TX), β-actin mouse-anti-mouse-monoclonal antibody was from Sigma Chemicals (St. Louis, MO). Secondary antibody Goat-anti-rabbit-IgG-HRP and Goat-anti-mouse-IgG-HRP were obtained from Santa Cruz Biotechnology (Dallas, TX). Supersignal west femoto chemiluminescent substrate for western blotting was from Thermo Scientific (Rockford, IL). ⁵⁵Fe was purchased from Perkinelmer (Waltham, MA).

**Cell culture.** N27 cells were grown in RPMI-1640 medium containing 10% fetal bovine serum, 2 mmol/L l-glutamine, 50 U penicillin and 50 µg/mL streptomycin and maintained at 37°C in a humidified atmosphere containing 5% CO₂ as described in previous studies (18). We also used primary mesencephalic neuronal cultures to determine the neuroprotective effect of EGCG. Primary mesencephalic neuronal cultures were prepared from the ventral mesencephalon of gestational 14-day-old C57 black mice embryos as described previously (19). Mesencephalic tissues were
dissected and kept in ice-cold Ca\textsuperscript{2+}-free Hanks’ balanced salt solution. Cells then were
dissociated in Hans’ balanced salt solution containing trypsin, 0.25% EDTA for 30 min at 37°C. Cells were suspended in neurobasal medium with 2% neurobasal
supplement (B27) after 10% FBS inactivating enzyme activity in DMEM medium.
The cells were then plated at density of 0.5 \times 10^6 cells on 12-mm coverslips precoated
with 1 mg/ml poly-D-lysine. Cell cultures were maintained in 500 μM l-glutamine,
100 IU/ml penicillin, and 100 units streptomycin neurobasal medium, incubated in a
humidified CO\textsubscript{2} incubator (5% CO\textsubscript{2} and 37°C). Medium was replaced half every 2
days and assays were conducted using cultures at 7 days old. Primary mesencephalic
dopaminergic neuronal cells were exposed to 6-OHDA (25 μM) for 24 h in the
presence or absence of 2 h pretreatment of EGCG (1, 10 and 100 μM). Cells were
then fixed for immunocytochemical analysis.

**Sytox green assay.** Equal numbers of N27 cells grown in 24-well plates with 500 μl RPMI medium co-incubated with 1:1000 Sytox green dye. To determine the effects of 6-OHDA and EGCG and protective effect of dosage from EGCG, N27 cells were
pretreated with EGCG at concentrations of 1, 10, 50 and 100 μM for 2 h, and then
followed by 6-OHDA (100 μM) treatment for 6 h. The assessment of cell death was
conducted using Sytox green nucleic stain as described previously (19). After each
treatment, Sytox green signal was detected using a fluorescence microplate reader
(Bio-Tek microplate reader) at an excitation wavelength of 485 nm and an emission
wavelength of 538 nm. The intensity of fluorescence was directly proportional to the
number of dead cells. To quantify cell death, fluorescence intensity was monitored
after the experiments were conducted and fluorescence pictures were taken using a
Nikon inverted fluorescence microscope equipped with a SPOT digital camera
(Diagnostic Instruments, Sterling Heights, MI).
Cell viability. The N27 cells were pretreated and cotreated with EGCG (100 μM), followed by 6-OHDA treatment for 6 h, the cell viability was measured by MTT assay as described previously (20). Briefly, after treatments, cells were washed with PBS and then incubated with serum free RPMI medium containing 0.25 mg/mL MTT reagent for 3 h at 37°C. Isopropanol–HCl (300 μl) solution was added to dissolve intracellular purple formazan and absorbance was read at 570 nm with a reference wavelength of 630 nm using a microplate reader (Molecular Devices, Sunnyvale, CA).

Caspase-3 activity. Caspase-3 activity was measured to assess cell apoptosis as previously described (21). Briefly, N27 cells was pretreated with EGCG for 2 h, followed by 6-OHDA treatment for 6 h, then the cells were resuspended in lysis buffer (50 mM Tris-HCl, 1 mM EDTA, and 10 mM EGTA) containing 10 mM digitonin for 20 min at 37°C. Supernatants were treated with the fluorogenic substrates Ac-DEVD-AFC for 1 h at 37°C and fluorescence was measured at excitation at 400 nm and emission at 505 nm using a Gemini XS fluorescence plate reader (Molecular Devices Inc). The activity was measured as fluorescent units (FU)/mg protein.

Immunocytochemistry. After treatments, the primary cultures were processed for immunocytochemical analysis. Briefly, the cells were fixed with 4% paraformaldehyde and permeabilized, and nonspecific sites were blocked with 5% normal goat serum containing 0.4% BSA and 0.2% Triton-X 100 in PBS for 20 min. Cells were then incubated with antibodies directed against TH (1:500) overnight at 4°C followed by incubation with Cy3-conjugated (1:1000) secondary antibody for 1 h at room temperature. Secondary antibody treatments were followed by incubation with 10 μg/ml Hoechst 33342 for 3 min at room temperature to stain the nucleus.
Then the coverslips containing cells were washed with PBS, mounted on a slide, and viewed under a Nikon inverted fluorescence microscope (Model TE-2000U) and images were captured with a SPOT digital camera (Diagnostic Instruments, Sterling Heights, MI). TH\(^+\) cell count and neurite processes were measured as described (18). Briefly, the total number of TH\(^+\) cells averaged 14/coverslip in untreated mesencephalic cultures. For measurement of TH\(^+\) cell count, Metamorph Image analysis software was used. The images were first thresholded, and then neuronal count and volume were measured using the Integrated Morphometry Analysis (IMA) function. For measurement of neuronal processes, the lengths of the processes were marked by applying the region and length measurement function in the IMA.

**Quantitative real-time PCR analyses.** The mRNA expression levels of DMT1, Fpn1, hepcidin, transferring receptor (TfR) and H-ferritin were quantified using real-time PCR as described previously (22, 23). Briefly, N27 cells were pretreated with EGCG and followed by 6-OHDA for 24 h. Total RNA was isolated from the cells using absolutely RNA miniprep kit (Stratagene, Santa Clara, CA) and reverse transcribed by High Capacity cDNA Reverse Transcription Kits (Applied Biosystems, Foster City, CA). Quantitative real-time RT-PCR was performed using Brilliant SYBR Green QPCR Master Mix kit and the Mx3000P QPCR system (Stratagene, Santa Clara, CA). The 25 μl PCR reaction mixture included 1 μl of cDNA (produced by 100 ng of RNA), 12.5 μl of 2 × master mix, and 100 nM each primer (listed in Table 1). Cycling conditions contained an initial denaturation at 95°C for 10 min, followed by 40 cycles of amplification 95°C for 30 s for denaturation, 60°C for 30 s for annealing, and 72°C for 30 s for extension. Fluorescence was detected during the annealing/extension step of each cycle. Dissociation curves were used to verify the specificity of the PCR products. GAPDH gene was used as internal control. A
comparative threshold cycle method was used to analyze the data. All reactions were performed in triplicate.

**Western blot analyses.** DMT1, hepcidin and Fpn1 expression were evaluated by Western blot assay. N27 cells were pretreated with EGCG and followed by 6-OHDA for 24 h. Then the cells were washed with cold PBS and homogenized in RIPA buffer containing 1% Triton X-100, 0.1% SDS, 1 mM phenylmethanesulfonyl fluoride (PMSF) and protease inhibitors (pepstatin 1 mg/ml, aprotinin 1 mg/ml, leupeptin 1 mg/ml), and then sonicated on ice. After a centrifugation at 13,000 rpm for 15 min at 4°C, the supernatant was collected. Aliquots of supernatants were assayed for protein concentrations. Forty μg of protein extract was mixed with an equal volume of 5 × sample buffer (0.35 M Tris–HCl, 10% SDS, 30% glycerol, 0.012% bromophenol blue), and loaded onto a 12 % or 15 % SDS-polyacrylamide gel or Urine-gel, electrophoresed, and then transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA). The membrane was blocked with 5% nonfat dry milk in TBST and then incubated with rabbit anti-rat DMT1 with IRE polyclonal antibody (1:200), rabbit anti-rat Fpn1 polyclonal antibody (1:500) or rabbit anti-rat hepcidin polyclonal antibody (1:500), followed by horseradish peroxidase-conjugated goat anti-rat IgG antibodies (1:3000). To ensure even loading of the protein samples, β-actin (1:2000) was used as internal control, with the incubation of the corresponding goat-anti-mouse-IgG-HRP secondary antibody (1:3000). All western blot assays were performed triplicately in two separate experiments.

**Cellular iron uptake.** N27 cells were grown in 12-well plates. After 2 h pretreatment with EGCG, N27 cells were exposed to 6-OHDA, and ferric chloride (0.5 μCi/ml 55Fe) was also added to the media and incubated at 37°C for 24 h. Cells were then washed in cold 1 × PBS twice and lysed in 250 μl of lysis buffer (RIPA
buffer). After centrifuging at 13,000 rpm for 10 min at 4°C, 50 μl of supernatant was used to measure the $^{55}$Fe radioactivity in a scintillation counter mixed with the 10 ml cocktail scintillation fluid. The $^{55}$Fe uptake was calculated by dividing the number of counts per minute (CPM) and the values were normalized to cell protein concentration. The experiments were performed in duplicate.

**Statistics analyses.** Data were analyzed with Prism 4.0 software (Graph Software, San Diego, CA). All values are expressed as mean ± SEM and represented as percentage of the respective controls. ANOVA with Tukey’s multiple comparison test was used to detect the differences among treatments. Student t-test was used to compare the difference between 6-OHDA with EGCG pretreatment and 6-OHDA alone groups in iron related genes expression. The mean differences were considered significant at $p<0.05$.

**Results**

**Effects of 6-OHDA and EGCG on cell death**

The Sytox green assay showed the dose response of EGCG protected against 6-OHDA induced cell death (Fig. 1). 6-OHDA significantly increased cell death by 196% ($p<0.0001$) compared to control. Pretreatment of EGCG at a concentration of 50 and 100 μM for 2 h decreased the cell death by 31 % ($p<0.0001$) and 55% ($p<0.0001$) compared to 6-OHDA alone group. There was no significant decrease in EGCG pretreatment at the concentration of 1 and 10 μM.

**EGCG protected against 6-OHDA induced decreased cell viability**

As showed in Fig 2., cell viability was decreased to 59% ($p<0.001$) after 6 h treatment with 6-OHDA, but EGCG pretreatment for 2 h partially protected against this toxicity significantly by 21% ($p<0.01$). In contrast, only 10% ($p>0.05$) increase in
cell viability in EGCG co-treatment group, suggesting pretreatment of EGCG shows
the protective effect.

**EGCG protected against 6-OHDA induced cell apoptosis**

Caspase-3 activity was used to determine the cell apoptosis (Fig. 4). Compared to
control, 6-OHDA significantly increased caspase-3 activity by ~ 12-fold (p<0.0001),
while pretreatment of EGCG for 2 h decreased the caspase-3 activity by 49%
(p<0.0001) compared to 6-OHDA. There was no change in caspase-3 activity in
EGCG treatment alone group, suggesting EGCG pretreatment protected against cell
apoptosis.

**EGCG decreased TH^+ neuronal loss from 6-OHDA in primary mesencephalic cultures**

Treatment with 6-OHDA for 24 h induced ~ 80% (p<0.0001) loss of TH^+ cell count
(Fig. 5A), compared to control. Pretreatment of EGCG (1, 10, 100 μM) for 2 h
significantly increased TH^+ cell count by 155% (p<0.01), 205% (p<0.0001) and 290%
(p<0.0001), respectively (Fig. 5B). The average lengths of TH^+ neuronal processes in
EGCG pretreated cells were significantly longer than the processes of neurons with
only 6-OHDA treated. We also found that 6-OHDA significantly decreased the TH^+
neurite length by 93% (p<0.0001), while pretreated with EGCG (1, 10, 100 μM) for 2
h significantly increased the neurite length by ~ 10-fold (p<0.0001), 12-fold
(p<0.0001), and 12-fold (p<0.0001), respectively (Fig. 5C). In addition, 6-OHDA
significantly decreased Hoechst activity, while pretreatment of EGCG (1, 10, or 100
μM) increased Hoechst staining (Data not shown), suggesting the increased cell
availability. Overall, EGCG protected against 6-OHDA toxicity in primary cultures.

**EGCG altered the 6-OHDA regulation on the mRNA expression of iron-related genes**
As shown in the Table 2, 6-OHDA significantly increased the mRNA expressions of DMT1+IRE, hepcidin, TfR2, H-ferritin and decreased Fpn1 and TfR1. However, EGCG altered the effects by decreasing DMT1+IRE by 60% (p<0.001), hepcidin by 54%, (p<0.05), H-ferritin by 53%, (p<0.05), TfR2 by 27%, (p<0.05), while increasing Fpn1 by 70%, (p<0.01) and TfR1 by 96%, (p<0.01), compared to 6-OHDA, may indicate the reduced iron burden in the cell.

**EGCG altered the 6-OHDA regulation on the iron-related protein expressions**

The regulation on iron related proteins DMT1+IRE, hepcidin and Fpn1 was assessed by western blot. We found that 6-OHDA significantly increased DMT1+IRE expression by 201% (p<0.05), hepcidin by 177% (p<0.05) and decreased Fpn1 by 61% (p<0.0001) compared to the control. Pretreatment of EGCG altered these effects by decreasing DMT1+IRE by 66% (p<0.01), hepcidin by 43% (p<0.05) and increasing Fpn1 by 82% (p<0.01) compared to 6-OHDA alone.

**EGCG reduced iron burden induced by 6-OHDA**

As shown in Fig. 7, 6-OHDA significantly increased $^{55}$Fe uptake to 196% (p<0.01). Pretreatment of EGCG significantly decreased $^{55}$Fe in the cell by 27% (p<0.05) compared to 6-OHDA, supporting the results of and mRNA and protein expression.

**Discussion**

Neurotoxins, such as 6-OHDA, have been widely used to establish PD model both in vitro and in vivo studies (9, 24, 25). Due to the abilities to induce oxidative stress, mitochondrial dysfunction and iron dyshomeostasis, 6-OHDA has been reported to cause significant neuronal cell death, including SH-SY5Y, PC12. High dose of 6-OHDA treatment causes severe insult to cells and is effectively used for short-term and acute pathological research. It was shown that the treatment of 6-OHDA at 100
μM for 6 h caused a complete loss of cell viabilities (26). In our study, we also found that 6-OHDA at the same condition significantly increased dopaminergic N27 cell death approximately 3-fold, cell apoptosis ~ 12-fold and decreased cell viability by ~40%. Relative lower concentration of 6-OHDA induced a smaller, but significant decrease in cell viability and increase in apoptosis, which benefits the clarification of mechanism of toxin induced neurodegeneration (27). In our study, we used a lower dose of 6-OHDA (25 μM) to determine the regulation on iron-related mRNA and protein expression, as well as the intracellular iron uptake. Although higher amounts of 6-OHDA (200 μM) has been reported to induced rat primary nigral culture death (28), lower concentrations (<50 μM) are used by most primary neuron studies due to sensitivity of neuron (29, 30). In our study, we used 10 μM 6-OHDA to show the significant TH+ cell loss after 24 h. Overall, the dose of 6-OHDA used in the study depends on the cell types and the study aims.

Although the mechanisms of 6-OHDA induced neurotoxicity remain unclear, several pathways have already been proposed, such as mitochondrial dysfunction, inflammation, etc. It has been reported that 6-OHDA is able to release iron from ferritin and alter DMT1 expression, suggesting that 6-OHDA might exert its toxicity by interrupting iron homeostasis (31, 32). We found the disruption of iron metabolism was closely related to the alteration of iron transporters and iron regulatory proteins. In our study, we showed 6-OHDA up-regulated DMT1+IRE, hepcidin and decreased Fpn1 expression, which might result in an excess iron condition. The alteration on other iron-related factors, such as ferritin, TfR1, TfR2 by 6-OHDA also indicated an intracellular iron excess status. Our ⁵⁵Fe uptake data further confirmed 6-OHDA caused high intracellular iron (~ 2-fold, p<0.01).
It was shown that the up-regulation of DMT1 by 6-OHDA occurred in an IRE/IRP-dependent manner (33, 34), indicating 6-OHDA may affect the activity of IRPs, altering the translation and stabilization of DMT1 mRNA. In our study, 6-OHDA did not alter the expression of DMT1 without IRE (data not shown), also suggesting the regulation on DMT1 via IRPs. The increase of DMT1 caused iron influx, leading to oxidative stress. However, further studies are required to clarify whether there is causal-effect relationship between DMT1 and oxidative stress.

Hepcidin is mainly involved in regulating iron homeostasis and can be regulated by iron influx and iron overloading (35, 36), oxidative stress and inflammation. In our study, we showed 6-OHDA significantly increased hepcidin expression in N27 cells. The regulation on hepcidin may depend on the excess intracellular iron, which was supported by the increased ferritin and $^{55}$Fe uptake. It was shown that TfR2 was positively associated with hepcidin expression and may be another hepcidin regulator (37, 38). Our data confirmed that increased TfR2 expression was accompanied by increased hepcidin level. Since hepcidin can bind to Fpn1 and induce its internalization and degradation, the increased hepcidin suppressed Fpn1 expression, resulting in iron retention. 6-OHDA can cause not only the decreased Fpn1 shown in our study, but also in primary mesencephalic neuron, MES23.5 cell (39, 40). The iron overloading can also decrease Fpn1 in PC12 (41). Other studies showed toxin can increase Fpn1 in primary cultured astrocytes, SH-SY5Y (40, 42). The difference of Fpn1 regulation may imply the significance of different cell type in neuronal iron homeostasis.

Since the alteration of iron level in the brain is linked to several neurological disorders, maintaining normal iron homeostasis seems to be an ideal strategy for neuroprotection (43). As iron chelator and antioxidant, EGCG has been reported to
protect against neurotoxicity in numerous studies (44-46). In this study, we found that EGCG protected N27 cell against 6-OHDA induced cell death. The EGCG pretreatment method was considered more effective than co-treatment. We also found that EGCG (10-100 μM) exerted protection both in N27 cell and primary cultures and the concentration at 100 μM showed the best protective effects, whereas higher dose (> 200 μM) was found to be toxic (supplement data). This finding matched the bell-shaped pattern of EGCG as a typical of antioxidants, showing neuroprotection at low concentrations, and being pro-oxidant at high concentration (16, 17). We also confirmed that EGCG (1 μM -100 μM) protected TH+ cells against 6-OHDA toxicity in primary cultures. Different dosages of EGCG were also reported in other primary cultures studies. It showed lower dose (<10 μM) of EGCG protected neurons against 6-OHDA (47, 48), while other studies reported higher (25 to 400 μM) EGCG protected SH-SY5Y cell apoptosis induced by 6-OHDA (24).

Several proposed mechanisms may attribute to the neuroprotection of EGCG. First, it may regulate the iron-related proteins, maintain normal iron homeostasis (49). In this study, we showed the pretreatment of EGCG counteracted the adverse effect on iron-related proteins by 6-OHDA, leading to less intracellular iron accumulation. The decreased iron accumulation by EGCG pretreatment was supported by our 55Fe uptake data, showing 27% (p<0.05) decrease in iron burden against 6-OHDA. Second, EGCG may chelate the iron and the complex form of iron cannot be used by the cell, reducing the iron induced ROS generation. We have shown the decreased 55Fe uptake and decreased ROS in another study (data not shown). Third, EGCG may also enhance the antioxidant system by up-regulating antioxidant enzyme (16, 50), and scavenging the free radicals directly. The decreased oxidative stress may also affect the iron homeostasis directly or indirectly. In this way, EGCG causes less iron and
maintains the iron homeostasis. In addition, since the inflammation is related to antioxidation and EGCG was shown to reduce inflammatory activity, it may indirectly affect antioxidation and iron related protein. Further studies are needed to investigate how EGCG regulates iron metabolism in neural cells.

In summary, 6-OHDA induced neurodegeneration may be via affecting iron metabolism. The increased DMT1 expression significantly caused iron influx into cell body, while decreased Fpn1 expression might have exacerbated iron accumulation. The increased hepcidin, ferritin and $^{55}\text{Fe}$ suggest the increased free iron concentration, resulting into oxidative stress. EGCG exerted the protective effects against 6-OHDA by decreasing DMT1, hepcidin, increasing Fpn1 and reducing cell iron uptake to maintain normal iron level. EGCG may protect against neurotoxicity by modulating iron homeostasis. Further studies are needed to elucidate the mechanisms of protection by EGCG in vivo.

**Literature Cited**


FIGURE 1 Effects of 6-OHDA and EGCG on cell death. The value are expressed as mean ± SEM, n=8. Labeled means without a common letter differ, * P<0.001. 6-OHDA, 100 μM.
FIGURE 2 Effects of 6-OHDA and EGCG on cell viability. Cell viability was evaluated by MTT assay and the values are expressed as mean ± SEM, n=6. Data are represented as % of control (no treatment). Labeled means without a common letter differ. 6-OHDA, 100 μM for 6 h; EGCG 100 μM. Pre-trt, EGCG pretreatment 2 h, Co-trt, EGCG cotreatment with 6-OHDA. *P<0.01. **P<0.001
FIGURE 3 Effects of 6-OHDA and EGCG on cell apoptosis. Cell apoptosis was measured as caspase-3 activity. The values are expressed as mean ± SEM, n=3. Labeled means without a common letter differ. 6-OHDA, 100 μM for 6 h; EGCG 100 μM. *P<0.001.
**FIGURE 4** EGCG prevented TH⁺ neuronal loss by 6-OHDA. Immunochemistry analysis was processed and measured with TH⁺ cell count (Green color) and neurite length. Hoechst nuclear staining (Blue color) was used to identify the live cells (A). Quantification data on TH⁺ number (B) and neurite length (C). The values are expressed as mean ± SEM, n=3. Labeled means without a common letter differ. 6-OHDA, 10 μM for 24 h; EGCG 1, 10, 100 μM pretreatment 2 h. *P<0.01, **P<0.001.
FIGURE 5 Effect of 6-OHDA and EGCG on iron-related proteins expression. Western blot analysis on (A) DMT1 (+IRE), (B) Hepcidin, (C) Fpn1 proteins expression which were normalized by β-actin in N27 cells. Quantitative data are shown in the bottom panels D, E, F, respectively. The values are expressed as mean ± SEM, n=3. Labeled means without a common letter differ. 6-OHDA, 25 μM for 24 h; EGCG 100 μM pretreatment 2 h. *P<0.01, **P<0.001.
FIGURE 6 Iron uptake were the amount of $^{55}$Fe taken by the cell, which was calculated by count per minute (CPM) normalized with protein concentration. The values are expressed as mean ± SEM, n=3. Labeled means without a common letter differ. 6-OHDA, 25 μM for 24 h; EGCG 100 μM. *P<0.05, **P<0.01.
**TABLE 1** Summary of the primers for iron-related gene expression

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<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
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<tr>
<td>Hep</td>
<td>GAA GGC AAG ATG GCA CTA AGC A</td>
<td>TCT CGT CTG TTG CCG GAG ATA G</td>
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<tr>
<td>Fp1</td>
<td>CCA CCT GTG CCT CCC AGA T</td>
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<td>CAG TGC TCT GTA CGT AAC CTG TAA GC</td>
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<td>H-fer</td>
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<td>TGC AGG AAG ATT CGT CCA CCT</td>
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<tr>
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<tr>
<td>Tfr2</td>
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<td>GAPDH</td>
<td>CCT GGA GAA ACC TGC CAA GTA T</td>
<td>AGC CCA GGA TGC CCT TTA GT</td>
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All the primers were synthesized from Integrated DNA Technologies.
TABLE 2 Effect of 6-OHDA and EGCG on the iron-related genes expression

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<th>EGCG+6-OHDA</th>
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<td>DMT1+IRE</td>
<td>2.3±0.2*</td>
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<td>Hepcidin</td>
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<td>0.8±0.2ns</td>
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<td>Fpn1</td>
<td>0.7±0.0*</td>
<td>1.2±0.2ns</td>
<td>&lt;0.05</td>
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<td>H-ferritin</td>
<td>2.4±0.4*</td>
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<td>Tfr1</td>
<td>0.7±0.0*</td>
<td>1.3±0.1ns</td>
<td>&lt;0.01</td>
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<td>Tfr2</td>
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Data were presented as mean ± SEM, n=3-6. Values represent the fold changes of the ratio of mRNAs/GAPDH in treatments compared with control. Symbol * indicated difference between 6-OHDA group and EGCG pretreatment group, and was determined using student t-test. * p<0.05; ns, no significance.
CHAPTER 4. THE PROTECTION OF EGCG AGAINST 6-OHDA-INDUCED OXIDATIVE STRESS AND INFLAMMATION

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Abstract

6-hydroxydopamine (6-OHDA) is classic neurotoxin being used for Parkinson’s disease research. 6-OHDA can increase intracellular reactive oxygen species (ROS) and cause cell damage, which can be attenuated by (-)-Epigallocatechin-3-gallate (EGCG) treatment. However, the mechanism by which EGCG protects against 6-OHDA toxicity remains unclear. In this study, we used CM-H₂DCFDA probe to determine the ROS level in N27 cells. A significant increase in ROS generation after 6-OHDA (25 μM) treatment was found, while EGCG (100 μM) attenuated the ROS generation. We evaluated the oxidative damage by determining thobartituric acid reactive substances (TBARS) and protein carbonyl content. 6-OHDA significantly increased TBARS by 82.7% (p<0.05) and protein carbonyl content by 47.8 (p<0.05), compared to the control. Pretreatment of EGCG decreased lipid peroxidation and protein carbonyls by 36.4% (p<0.001) and 27.7% (p<0.05), respectively, compared to 6-OHDA. We also used western blot to test the E2-related factor 2 (Nrf2), heme oxygenase-1(HO-1) and peroxisome-proliferator activator receptor γ (PPARγ) expression to evaluate the antioxidant and antiinflammation. 6-OHDA increased Nrf2 expression by 69.6% (p<0.001), HO-1 by 173.3% (p<0.001) and PPARγ by 122.7%
(p<0.001), compared to the control. Pretreatment of EGCG significantly normalized these alterations induced by 6-OHDA. In conclusion, our results suggested that the neurotoxicity of 6-OHDA in N27 cells was associated with ROS and inflammation pathways, whereas pretreatment of EGCG suppressed the ROS generation, resulting into the inactivation of Nrf2/HO-1 and PPARγ pathway.

Introduction

6-hydroxydopamine (6-OHDA) can induce neurological damage, which is similar to Parkinson’s disease (PD). One of the main features of the neurotoxicity of 6-OHDA is involving in reactive oxygen species (ROS) generation. An increase in intracellular ROS production would disrupt redox homeostasis, cause oxidative stress and result into the irreversible oxidative modification on DNA, lipids and proteins (1). The presence of excess intracellular Fe (II) and Fe (III) may promote the generation of hydroperoxyl radical (HO·) through the Fenton reaction. Our previous in vitro study showed 6-OHDA induced the intracellular iron burden in immortalized rat mesencephalic dopaminergic neuronal cell line (N27) cells. Therefore, the excess iron may be involved in ROS generation and PD progression.

The transcription factor nuclear factor E2-related factor 2 (Nrf2) and antioxidant response element (ARE) (Nrf2/ARE system) in cells can scavenge ROS and maintain the redox homeostasis. Nrf2 is the main factor to activate antioxidant enzymes, phase II detoxification enzymes (2, 3), such as heme oxygenase-1(HO-1). The activation of detoxification enzymes directly scavenge free radicals and decrease the toxicity (4). There are several ways to trigger Nrf2/ARE system to increase antioxidant enzyme activity. For example, oxidative stress and electrophiles are the major activators on Nrf2. 6-OHDA has been shown to induce Nrf2 expression due to the induced
oxidative stress. In addition, the activation of Nrf2 can also be positively managed by itself (5).

Inflammation has a major impact on toxin-induced pathogenesis for PD (6, 7). The increasing evidence showed the positive correlative relationship between dopaminergic neurodegeneration and neuroinflammation (8). It has been shown that microglia inflammation was activated in 6-OHDA treated rats, indicating the critical role of inflammation in PD progression (9). The intracellular anti-inflammatory system, such as the peroxisome-proliferator activator receptors (PPAR), is responsible for suppressing inflammation reaction and maintaining normal redox status. One of the isoforms, PPARγ has the anti-inflammatory abilities and its agonists can used to treat inflammation-related diseases, including PD (10). In addition, PPARγ can also interact with Nrf2/ARE antioxidant system (11-16). It has been reported that PPARγ is involved in regulating HO-1 expression (17, 18). The relationship between Nrf2 and PPARγ suggests that oxidative stress and inflammation are interacted. Nrf2 can regulate PPARγ expression under oxidative stress, thereby affecting anti-inflammatory activity (14, 19). Therefore, antioxidation and anti-inflammatory pathways might be simultaneously involved in neurodegeneration. However, detailed mechanisms of the interaction between Nrf2 and PPARγ are not clear at this time.

Since iron burden and ROS are attributed to neuronal damage, iron chelation and antioxidative strategies are useful to protect neurodegeneration. (-)-Epigallocatechin-3-gallate (EGCG) is an antioxidant used to counteract neurotoxin and oxidative damage due to its iron chelation and free radical scavenging capabilities. EGCG can induce the expression of several antioxidant enzymes, and eliminate ROS and electrophile generation in the progression of neurodegeneration. EGCG has also been shown to inhibit the inflammatory reactions against cancer and neurological
diseases (20). It has been shown that EGCG treatment can prevent against environmental toxin, such as 6-OHDA induced neurodegeneration both in vitro and in vivo. However, the mechanism by which EGCG exerts its neuroprotection remains unclear. In this study, we hypothesized that 6-OHDA increased ROS levels and inflammation reaction, and cause oxidative damage in N27 cells, and EGCG treatment may prevent the adverse effects by stabilizing the Nrf2, PPARγ system, and thus reducing the oxidative damage on lipids and proteins.

Materials and Methods

Reagents. EGCG, 6-OHDA, Hanks’ Balanced Salts (HBSS) used for cell culture were purchased from Sigma (St. Louis, MO). RPMI-1640 medium, fetal bovine serum, L-glutamine, penicillin, streptomycin and CM-H₂DCFDA oxidative indicator probe were obtained from Invitrogen (Carlsbad, CA). Trichloroacetic Acid (TCA) was purchased from Fisher Scientific (Pittsburgh, PA). 1, 1, 3, 3-tetramethoxypropane, 2-Thiobarbituric Acid (TBA) were purchased from Sigma (St. Louis, MO). Protein carbonyl was assessed by commercial kit from Cayman Chemical Company (Ann Arbor, MI). Protein concentration was determined by Pierce BCA protein assay kit (Rockford, IL). Rabbit anti-rat-Nrf2 polyclonal antibody, rabbit anti-rat-HO-1 polyclonal antibody and rabbit anti-rat-PPARγ antibody, secondary antibody goat-anti-rabbit-IgG-HRP and goat-anti-mouse-IgG-HRP were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). β–actin mouse-anti-mouse-monoclonal antibody was purchased from Sigma (St. Louis, MO).

Cell culture. N27 cells were grown in RPMI-1640 medium containing 10% fetal bovine serum, 2 mmol/L l-glutamine, 50 U penicillin and 50 μg/mL streptomycin and
maintained at 37°C in a humidified atmosphere containing 5% CO₂ as described previously (21).

**Treatments.** N27 cells were pretreated with EGCG (100 μM) for 2 h, followed by 6-OHDA (25 μM) treatment for 24 h. Untreated cells were used as controls. After treatments, cell lysate were collected used for the following assays.

**Measurement of intracellular ROS generation.** The formation of intracellular ROS was measured by using a fluorescent probe, 2, 7-dichlorofluorescein diacetate as described previously (22, 23). The N27 cells were plated as the density of 2×10⁴ cells/well on the 96-well plate. Medium was changed to HBSS before each treatment, and CM-H₂DCFDA was added to final concentration with 10 μM to each well. The fluorescence intensity of dichlorofluorescein (DCF) was continuously measured using fluorescence spectrophotometer with excitation wavelength of 485 nm and emission wavelength of 535 nm with 30 min interval for 8 h.

**Thobartituric acid reactive substances (TBARS) assay.** The TBARS assay was used to determine the lipid peroxidation and performed fluorometrically with a modified protocol (24). Briefly, cell lysates (200 μl) from different treatments were combined with ice cold 20% TCA (200 μl), and then incubated on ice for 5 min. Samples were centrifuged at 14,000 rpm at 4°C for 5 min. Then 200 μl supernatant was mixed with 200 μl 0.67% (w/v) TBA reagent, and incubated at 95°C for 60 min. Then 400 μl butanol/pyridine (15:1) was added to each sample and centrifuged at 5,000 rpm for 5 min. The fluorescence intensity of malondialdehyde (MDA) in the upper butanol/pyridine phase was quantified with an excitation wavelength of 520 nm and emission wavelength of 550 nm on a black 96-well plate. 1, 1, 3, 3-tetramethoxypropane was used as a standard to calculate the final sample concentration.
**Protein Carbonyl Content (PCC) assay.** The protein oxidation in the cell lysates samples were determined by measuring the PCC by using the DNPH (2,4-dinitrophenylhydrazine). According to the protocol provided by Cayman’s protein carbonyl assay kit (Ann Arbor, MI), the amount of protein-hydrozone product was quantified spectrophotometrically at wavelength of 363 nm on 96-well plate reader. Total protein content of cell lysates was determined by Pierce BCA protein assay kit (Rockford, IL). The PCC was calculated and normalized to the total protein content.

**Western blot analyses.** After treatments described above, the N27 cells were washed with ice-cold PBS, then was homogenized in RIPA buffer (containing 1% Triton X-100, 0.1% SDS, 1 mM phenylmethanesulfonyl fluoride (PMSF) and protease inhibitors, including pepstatin 1 mg/ml, aprotinin 1 mg/ml, leupeptin 1 mg/ml) and sonicated on ice 2 × 10 s. The supernatant was collected after maximum centrifugation at 13,000 rpm for 15 min at 4°C. Aliquots of supernatants were used to measure the protein concentrations. Forty μg of protein was mixed with 5 × sample buffer (0.35 M Tris–Cl, 10% SDS, 30% glycerol, 0.012% bromophenol blue), and loaded onto a 12% or 15% SDS-polyacrylamide gel, electrophoresed and then transferred to a nitrocellulose membrane (Bio-Rad). The membrane was blocked with 5% non-fat milk in TBST and then incubated with rabbit anti-rat Nrf2 polyclonal antibody (1:500), rabbit anti-rat HO-1 polyclonal antibody (1:500) or rabbit anti-rat PPARγ antibody (1:500), respectively. Horseradish peroxidase-conjugated goat anti-rat IgG antibody (1:3000, Santa Cruz, CA) was used as a secondary antibody. To ensure even loading of the protein samples, β-actin (1:1000) was used as internal control, with the incubation of the corresponding goat-anti-mouse-IgG-HRP secondary antibody (1:5000). All western blot assays were performed in triplicates.
Statistics analyses. Data were analyzed with Prism 4.0 software (Graph Software, San Diego, CA). All the measurements were normalized to the respective controls in each experiment. All values were expressed as mean ± SEM as percentage of the controls. One-way ANOVA with Tukey’s multiple comparison test was used to detect the differences among treatments and $P \leq 0.05$ was considered as significant.

Results

*EGCG attenuated ROS generation induced by 6-OHDA*

Intracellular ROS level was determined and shown in Figure 1. 6-OHDA increased intracellular ROS level by 19 % ($p<0.001$) after 6 h treatment, and by 33.1 % ($p<0.001$) after 8 h, compared to control group. Pretreatment of EGCG for 2 h following with 6-OHDA treatment reduced ROS by 24.9 % ($p<0.001$) at 6 h, and by 31 % ($p<0.001$) at 8 h, compared to 6-OHDA treatment group; and reduced ROS by 10.6% ($p<0.05$) at 6 h, and by 8.1% ($p>0.05$) at 8 h, compared to control. There was a decrease in ROS (14.3%, $p<0.01$) at 6 h, and (18.7%, $p<0.001$) at 8 h with EGCG alone compared with control group. Overall, EGCG pretreatment attenuated 6-OHDA induced ROS generation.

*EGCG protected against 6-OHDA-induced lipid peroxidation*

6-OHDA resulted in a marked increase (82.7%, $p<0.001$) in MDA concentration after 24 h treatment (Fig 2). Pretreatment of EGCG for 2 h decreased lipid peroxidation by 36.4% ($p<0.001$) compared to the 6-OHDA treatment. Although EGCG alone had lower (by 30.3%, $p<0.01$) MDA value than 6-OHDA + EGCG group, the value was not significant different compared to control.
*EGCG protected against 6-OHDA-induced PCC*

As shown in Fig. 3, 6-OHDA increased the PCC by 47.8% (p<0.05) after 24 h treatment compared to the control. Pretreatment of EGCG for 2 h decreased the PCC by 27.7% (p<0.05), compared to 6-OHDA group. No difference between control and EGCG alone suggested there was no effect of EGCG on PCC.

*EGCG regulated antioxidant (Nrf2 and HO-1) and anti-inflammatory (PPARγ) system induced by 6-OHDA*

The activities of Nrf2, HO-1 and PPARγ were assessed and shown in Fig 4. 6-OHDA increased Nrf2 by 69.6% (p<0.001), HO-1 by 173.3% (p<0.001) and PPARγ by 122.7% (p<0.001) after 24 h treatment, compared to control group. Pretreatment of EGCG for 2 h significantly decreased Nrf2 (by 36.7%, p<0.001), HO-1 (by 49.3%, p<0.001) and PPARγ (by 41.1%, p<0.01), compared to 6-OHDA group. Pretreatment of lower concentration of EGCG (10 μM) showed no decrease on HO-1 and PPARγ expression, but only significant decrease (by 18.9%, p<0.05) in Nrf2, compared to 6-OHDA group. Therefore, these results suggested high concentration of EGCG showed antioxidant and antiinflammation effects.

**Discussion**

6-OHDA is used as classical neurotoxin to induce neurodegeneration and to understand the treatment of PD. The neurotoxicity of 6-OHDA has been proposed by several mechanisms, including free radicals damage, mitochondrial dysfunction, and oxidative stress (25). Since iron is involved in PD due to its pro-oxidant nature, the interruption of iron homeostasis by 6-OHDA causes neuronal damage. 6-OHDA can alter the expression of iron-related protein, resulting into iron dyshomeostasis (26, 27).
6-OHDA can cause iron release from cytosolic ferritin and increase intracellular free iron concentration (28). The excess free iron could generate ROS via Fenton reaction, causing oxidative stress and cell death, especially in the substantia nigra (29, 30). In our previous study, we showed that 6-OHDA regulated iron-related protein expression, increasing cellular iron uptake and retention in N27 cells. Here, we confirmed that 6-OHDA significantly increased the intracellular ROS level and oxidative damage to lipids and proteins in N27 cells. Therefore, iron dyshomeostasis is critical to cause neuronal cell death and maintaining normal iron level is important to prevent PD progression.

In addition to oxidative stress, inflammation also plays a critical role in PD. It had been shown that neuroinflammation was significantly higher in PD brains, including numerous inflammation-related enzymes and cytokines within the SN, striatum, and cerebral spinal fluid. For example, tumor necrosis factors (TNF) and nitric oxide synthase (iNOS) were found significantly elevated in PD patients, compared to age-matched control (31). One of the proposed mechanisms of 6-OHDA toxicity is the induction of inflammation (32, 33). Inflammatory factors, such as TNFα could directly activate cell apoptotic pathway or indirectly suppress the mitochondrial function. Considerable evidence from in vitro and in vivo studies suggests that there is an association between inflammation and ROS in PD (34, 35), and increased ROS level could augment the inflammation process (36, 37), thereby exacerbating neuronal damage.

Since oxidative stress and inflammation links to the progression of neurodegeneration, antioxidation and antiinflammation would be useful as neuroprevention strategies against PD. Nrf2/ARE system is considered a self-defense antioxidant system to counteract the increased ROS and oxidative stress. Increased
ROS levels and oxidative stress can trigger Nrf2 expression and Nrf2-associated antioxidant enzymes expression (38, 39). In the present study, we showed that 6-OHDA increased not only ROS level, but also Nrf2 (by ~70%, p<0.001) and HO-1 expression (by ~173%, p<0.001) in N27 cells. However, the increased antioxidant enzymes may not be enough to counteract the 6-OHDA toxicity, since we found elevated oxidative damage to lipid (by ~83%, p<0.001) and protein (by ~48%, p<0.05). Meanwhile, PPARγ was associated with the anti-inflammatory system to inhibit inflammatory reaction, increase DA, alter mitochondrial bioenergetics, reduce oxidative stress and impede PD progression (10). In our study, we found the 6-OHDA significantly increased PPARγ expression, which may indicate the increased inflammatory activity along with the increased ROS level. Similar to Nrf2, the increased PPARγ could not completely counteract against 6-OHDA toxicity, which may indicate the inadequate anti-inflammatory effect. Although the Nrf2/ARE antioxidant system is closely related to PPARγ antiinflammation system (40), whether there is a causal relationship remains unknown. Therefore, further research is needed to explore the relationship between Nrf2 and PPARγ.

EGCG has been considered as an antioxidant to decrease oxidative stress and maintain the redox balance due to its high reduced potential and radical scavenging ability. As showed in Figure 1, EGCG significantly decreased ROS level even compared to the control group. EGCG is also considered as an iron chelator, therefore, it can also decrease the ROS by binding free iron without affecting the iron bioavailability. Several in vitro and in vivo studies have shown that EGCG treatment can prevent cancer, vascular diseases and neurological disorders (41, 42). We have previously shown that EGCG decreased iron uptake and retention in N27 cells and prevented against 6-OHDA neurotoxicity, which was supported by our results showed
that EGCG significantly reduced intracellular ROS, lipid peroxidation and protein carbonyls, which maybe attributed through iron accumulation. Several studies indicate EGCG can activate Nrf2 expression, which can induce antioxidant enzymes expression, improve antioxidant defense ability, such as HO-1(42-44). However, we were not able to show that EGCG increased either Nrf2 expression or PPARγ activity in our study. Both lower (10 μM) and higher (100 μM) concentration of EGCG showed no effects on Nrf2, HO-1 and PPARγ expression. One reason might be that EGCG directly decreased the ROS level by scavenging the free radicals or chelating excess iron. Therefore, the reduced ROS level and balanced redox potential would not activate Nrf2/ARE system and PPARγ activity. Another reason for the discrepancy of our results might be related to the difference of cell types. We used N27 cells which were much more sensitive to oxidative stress and complicated regulation pathways compared to non-neuronal cells.

In conclusion, our *in vitro* data suggested that the neurotoxicity of 6-OHDA was closely related to ROS, oxidative stress and inflammation. Pretreatment of EGCG protected against 6-OHDA toxicity by inhibiting the ROS, consequently inactivating Nrf2, HO-1 and PPARγ expression. To our knowledge, this is the first *in vitro* study of EGCG on Nrf2 regulation to prevent 6-OHDA induced neurotoxicity. Further studies *in vivo* are needed to clarify the antioxidant and anti-inflammatory mechanisms of EGCG protection in PD.

**Literature Cited**


Figures and Tables

**FIGURE 1** EGCG attenuated 6-OHDA-induced intracellular ROS generation in N27 cells. The fluorescence density showed in the time course of 6-OHDA increased ROS levels. The values are expressed as mean ± SEM, n=3. 6-OHDA (25 μM) was treated for 24 h, pretreatment of EGCG (100 μM) for 2 h. * p<0.001.
FIGURE 2 Effect of 6-OHDA and EGCG on lipid peroxidation. The values are expressed as mean ± SEM, n=2 from 3 individual measurements. Means with letters indicated comparison with control treatment. Symbols represented the comparison between treatments. 6-OHDA, 25 μM for 24 h, EGCG, 100 μM pretreatment for 2 h. * p<0.01, ** p<0.001.
**FIGURE 3** Effect of EGCG on PCC induced by 6-OHDA. Cells were pretreated with EGCG, followed by 6-OHDA treatment. The values are expressed as mean ± SEM from duplicate measurements for 3 individual experiments. Labeled means without a common letter differ. 6-OHDA, 25 μM for 24 h, EGCG, 100 μM pretreatment for 2 h. * p<0.05.
**FIGURE 4** Effects of 6-OHDA and EGCG on Nrf2, HO-1 and PPARγ protein expression. Cells were pretreated with EGCG, followed by 6-OHDA treatment. Western blot analysis (A) for Nrf2, HO-1 and PPARγ protein expression (normalized with β-actin in N27 cells), and quantification analysis (B). The values were expressed as mean ± SEM from 4-8 individual measurements. Means with letters indicated comparison with control treatment. Symbols represented the comparison between treatments. 6-OHDA, 25 μM for 24 h, EGCG, 10, 100 μM pretreatment for 2 h. *p<0.05, **p<0.01, ***p<0.001.
CHAPTER 5. BENEFICAL EFFECTS OF GREEN TEA CONSUMPTION IN PARKINSON’S DISEASE PATIENTS

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ABSTRACT: Antioxidants may offer protection on oxidative stress-associated Parkinson's disease (PD). The objective of this study was to assess the beneficial effect of green tea consumption (3 cups daily for 3 months) on quality of life, iron status, antioxidant status, and oxidative damage in PD patients. Fourteen subjects (51-79y) who were within the first five years of PD, on stable medication and not regular green tea consumers were recruited. PD rating scales were used to evaluate disease symptoms. Hemoglobin, serum iron, iron saturation and ferritin concentrations were used to assess the iron status. Antioxidant enzymes including catalase, superoxide dismutase (SOD), and glutathione peroxidase (GPx) were measured to determine the antioxidant status. Lipid peroxidation and protein carbonyls were used as oxidative damage markers. Wilcoxon matched pairs t-test and student t-test were used for statistical analysis between baseline and 3 month intervention data. Among the PD rating scales, we found a significant increase in Mini-Mental State Examination (MMSE) (p<0.05) and a decrease in United Parkinson's Disease Rating Scale (UPDRS) motor scores (p<0.05) after the intervention, suggesting an improvement in cognitive and motor status. No changes were found in iron status. Catalase and SOD activities were increased significantly
(28%, p<0.05 and 37%, p<0.01, respectively), indicating the improvement of antioxidant status. Both lipid peroxidation and protein carbonyls decreased by ~ 52% (p<0.01) after intervention. In conclusion, green tea consumption may slow down PD progression by improving the antioxidant status and reducing oxidative damages without affecting iron status.

**Key Words:** Parkinson’s disease; antioxidant enzymes; oxidative stress; green tea consumption

Parkinson’s disease (PD) is now considered the second most common neurodegenerative disorder after Alzheimer’s disease, affecting approximately 1 percent of the population over 50 y.o. PD patients show classical motor symptoms including bradykinesia, rigidity, and resting tremors due to the depletion of dopamine in brain. Several factors, such as ageing, genetics, environment, oxidative stress, and inflammation are involved in PD progression. Among these factors, oxidative stress is one of the major causes in initiating and promoting neurodegeneration. Since brain tissue has high levels of polyunsaturated fatty acids, it is prone to oxidative damage. Excess iron accumulation in the brain leads to free radical formation and reactive oxygen species (ROS) generation via the Fenton reaction, contributing to oxidation damage of lipids and protein, and promoting cell death. Significant elevation of iron was found in the substantia nigra (SN) of PD patients compared to the age-matched control,\(^1,\,2\) indicating the critical role of iron in PD progression.

Antioxidants counteracting the detrimental effects of ROS offer a promising approach to protect neuronal cells. The antioxidant defense system is responsible for removing free radicals, scavenging ROS or their precursors, maintaining redox homeostasis, and decreasing oxidative damage.\(^3\) The antioxidant defense system can
be classified as exogenous (natural or synthetic), such as ascorbic acid, lipoic acids, polyphenols, and carotenoids, or endogenous, such as catalase, SOD, glutathione reductase (GSH), and glutathione peroxidase (GPx). Evidence has shown the beneficial effects of antioxidants in cell culture models.\textsuperscript{4-7} \textit{In vivo}, higher levels of antioxidants are required to result in protective effects on the central nerve system (CNS), which is extremely sensitive to redox changes and oxidative dyshomeostasis due to the high level of oxidative metabolism. Insufficient antioxidants and antioxidant enzyme activities may not be able to offset the oxidative modifications and damages. Several studies indicate that increased oxidative damage and reduced antioxidant activities might be responsible for the onset and progression of PD.\textsuperscript{8-10} Therefore, the activation of antioxidant enzymes is considered to be a necessary strategy to counteract the detrimental effects of ROS and restore the cellular redox balance.\textsuperscript{11}

Many antioxidants such as vitamin E, vitamin C, carotenoids, and flavonoids can improve antioxidant status and decrease oxidative stress, therefore preventing neurological disorders.\textsuperscript{12-14} Iron chelator, (i.e., desferrioxamine (DFO)) has shown neuroprotective effects in \textit{in vitro} and \textit{in vivo} studies.\textsuperscript{15,16} However, the side effects of DFO limit its usefulness. Catechin polyphenols can act as antioxidants by scavenging free radicals and chelating excess metal ions.\textsuperscript{17,18} They may also indirectly reducing oxidative stress inhibiting redox-sensitive transcription factors, nuclear factor-κB, and pro-oxidant enzymes, such as inducible nitric oxide synthase, and inducing phase II antioxidant enzymes, such as glutathione S-transferases and SOD. (-)-Epigallocatechin-3-Gallate (EGCG) is the most abundant polyphenol and iron chelator present in green tea,\textsuperscript{19} which has been shown to be an antioxidant \textit{in vitro} by trapping peroxyl radicals and inhibiting lipid peroxidation, contributing to the
neuroprotective effects of green tea in several PD model cells.\textsuperscript{20} EGCG can better represent the antioxidant function of green tea \textit{in vivo}, due to its bioavailability and metabolism. Higher green tea consumption is related to the reduced risk of PD\textsuperscript{21-23} because it may offer a promising dietary source of neuroprotection. However, data showing whether or not green tea directly or indirectly improves the PD patients’ status is limited. The purpose of this study is to identify the beneficial effect of green tea consumption in PD patients. We hypothesized that green tea consumption may improve quality of life of PD patients by improving antioxidant status, thus, reducing oxidative damage to lipids and proteins.

**Patients and Methods**

**Study Design and Participants**

Patients (n=15) (9 males, 6 females) were recruited from Parkinson’s disease and Movement Disorder Center at University of Kansas Medical Center (KUMC). The inclusion criteria included patients within the first five years of PD, aged between 50 to 80 y, willing to drink green tea and were on stable medication regiment with no changes in medication throughout the study. The exclusion criteria included premenopausal women, Parkinsonism resulting from other causes including toxicity, drugs and head trauma, and patients with uncontrolled chronic diseases, smokers, and current green tea drinkers (≥ 3 cups/day). Subjects were provided with tea bags (Lipton\textsuperscript{®} 100\% Natural Green Tea) in batches at each visit and requested to drink 3 cups of green tea per day (1 green tea bag steeped in 1 cup of boiling water for 4 minutes).
Assessment of PD Status at Baseline and Post-intervention

At baseline and at the 3 month visit, the United Parkinson's Disease Rating Scale (UPDRS) was completed to assess the PD-related disability and impairment. 39-item Parkinson’s disease questionnaire (PDQ-39) was used to assess the daily quality of life. Various other measures were used to evaluate PD symptoms, such as depression (Beck Depression Inventory), anxiety (Beck Anxiety Inventory), sleepiness (Epworth Sleepiness Scale), mental status (Mini Mental State Examination) and fatigue (Fatigue Severity Scale). Hoehn and Yahr (H&Y) scale was used to assess the stage of PD and Schwab and England Activities of Daily Living Scale (S&E) was used to test severity of the disease. All the questionnaires were included in the appendix 2. Blood was collected at baseline and 3 month intervention. The antioxidant enzyme activities in erythrocytes, including catalase, SOD, and GPx were determined by using commercial assay kits (Cayman Chemical Company, Ann Arbor, MI, USA). Serum ferritin was measured with RIA kit (Ramco Laboratories, Houston, TX, USA). Hemoglobin, serum iron saturation, and serum iron were determined by the commercial clinical laboratory (LabCrop, Kansas city, MO, USA). Serum malondialdehyde (MDA) concentration was estimated by thiobarbituric acid reactive substances (TBARS) assay and used to determine lipid peroxidation. Protein carbonyls in plasma were measured by using the commercial kits (Cayman Chemical Laboratories, Houston, TX, USA).
Statistical Analysis

Wilcoxon matched pairs t-test was used to compare the changes in PD rating scales and student t-test was used to compare the changes in antioxidant enzymes, TBARS, protein carbonyls, and iron status between baseline and post-intervention. The mean differences between baseline and post-intervention were considered significant at p<0.05.

Results

Subject Description

A total of 15 subjects were included in the study. One subject was withdrawn during the study period. The median of age of the 14 subjects was 61 y.o. with a range of 51 to 79 y.o. Median BMI at baseline was 28.2 kg/m$^2$ (overweight) with a BMI range of 18 kg/m$^2$ to 41.8 kg/m$^2$. At 3 months intervention, median BMI was 28.6 kg/m$^2$ (overweight) with a range of 18.5 kg/m$^2$ to 45.6 kg/m$^2$. Six of the subjects were classified as obese (BMI $\geq$ 30 kg/m$^2$) and the remaining 8 participants had a BMI between 18.5 kg/m$^2$ to 28.7 kg/m$^2$ following green tea consumption, classifying them as normal to overweight (BMI 18.5 kg/m$^2$ to 29.9 kg/m$^2$).

Changes in PD Rating Scales

The data from PD rating scales for all subjects are listed in Table 1. Values are expressed as mean $\pm$ SD. Mean MMSE increased significantly (p<0.05) from 29.5$\pm$0.7 to 29.9$\pm$0.4, indicating improved mental status following green tea consumption. The UPDRS motor exam score decreased significantly (p<0.05) from 21.2$\pm$7.5 to 18.1$\pm$9.2, indicating an improvement in motor status. On the contrary, total activities of daily living (ADL) score from the UPDRS and the mobility score
from the PDQ-39 increased significantly (p<0.05) from $9.2\pm 5.7$ to $9.9\pm 5.7$, and from $13.9\pm 15$ to $23.2\pm 26.9$ respectively, showing a decline in ability to perform ADL as well as mobility status. There were no changes in total UPDRS and total PDQ-39 scores, suggesting the absence of change in PD symptoms and quality of life. There were also no changes in the H&Y scale score, S&E scale score, indicating no stage or severity changes in PD after green tea consumption.

**Changes in Iron Status**

Details of iron status are shown in Table 2. The values are expressed as mean ± SD. No significant changes were found in hemoglobin, serum iron, iron saturation, and serum ferritin at baseline or post-intervention. At baseline and post-intervention, average hemoglobin concentration was $14.3\pm 1.5$ g/dL and $14.2\pm 1.5$ g/dL, average serum iron concentration was $85.9\pm 28.5\mu g/dL$ and $87.5\pm 38.2 \mu g/dL$, average iron saturation was $25.1\pm 8.7\%$, $26.5\pm 12.6 \%$, and average ferritin concentration was $61\pm 50$ ng/mL and $59\pm 46$ ng/mL. Overall, iron status was not affected by green tea consumption.

**Changes in Antioxidant Enzymes**

Compared to baseline, the mean activity of SOD in erythrocytes increased by 37% (p=0.0025) (Fig. 1A) post-intervention. Additionally, mean catalase activity increased by 28% (p=0.0483) (Fig. 1B). Although there was a 13% increase in the mean activity of GPx after green tea consumption, the change was not statistically significant (p>0.05) (Fig. 1C). Overall, antioxidant enzymes activities were improved following green tea consumption.
Changes in Oxidative Damage

The mean value of MDA concentration decreased significantly (p<0.01) from 2.5±0.9 μM to 1.2±0.8 μM after green tea consumption (Fig. 2A), indicating a decrease in lipid peroxidation. Protein carbonyl concentration also decreased significantly (p<0.01) from 0.82±0.21 nmol/mg to 0.39±0.27 nmol/mg (Fig. 2B), suggesting a decrease in protein damage.

Discussion

PD rating scales have been developed to measure the impact of PD on patients. These scales not only identify and monitor PD symptoms, but also assess severity and disease progression. Additionally, PD rating scales are useful for determining a patient’s eligibility for participation in research trials. UPDRS is the most commonly used scale to assess motor disability, motor impairment, mental dysfunction, and motor or non-motor complications. H&Y and S&E scales are commonly used to determine the PD stage and severity.9 PDQ-39 and other PD symptom measures, such as the MMSE, are also widely used PD rating scales. Our data indicated no change in total UPDRS score, total PDQ-39 score, and PD symptoms measures, (i.e., BDI, BAI), suggesting no disease progression following green tea consumption. We found that there was approximately a 20% (p<0.05) decrease in motor examination scores and a significant (p<0.05) increase in MMSE, representing an improvement in motor abilities. There was no change in H&Y scale scores, as well as in S&E scale scores, suggesting no significant change in the stage or severity of PD. However, changes in total daily living score of UPDRS (~8%, p<0.05) and mobility score (~ 67%, p<0.05) of PDQ-39 may indicate a worsening of PD status. Our results conflict with various qualitative questionnaires used to assess PD status. In a recent study, motor
assessment was quantitatively evaluated by full-body motion capture data with a deep brain stimulator. This method enables us to devise more effective ways to track the progression of neurodegenerative movement disorders other than the currently used PD rating scales. Therefore, revising and designing new appropriate measurement scales can improve the evaluation on PD.

Iron plays a critical role in CNS functioning and is closely related to the progression of neurodegenerative diseases. Excess iron can induce the ROS generation and cause oxidative damage once it overrides the biological detoxification ability. Significantly higher amount of iron in the brain were reported in PD patients compared to age-matched controls. On the other hand, low circulating iron levels have also been reported in PD, suggesting iron deficiency or problems with iron storage in tissues such as liver and brain, thus, contributing to PD progression. In a study conducted by Qureshi (2006), it was shown that there was no change in serum iron levels, but a significantly higher level of iron in cerebrospinal fluid in PD patients, compared to a healthy control. In our study, iron status values at the baseline were in the normal range and none of the values indicated iron deficiency anemia or iron excess. Although there is a concern that polyphenols can inhibit iron absorption and alter overall iron status, no changes in iron status after 3 months of green tea consumption was observed in our study. Our iron status results are similar to the iron status results reported in the previous study. Therefore, the iron relocation within specific regions in brain might become an important issue to trigger the progression of PD rather than affecting overall body iron status.

Oxidative stress can cause dopaminergic cell death and neurodegeneration. Increased oxidative stress and decreased antioxidant enzymes have been shown in several human studies with PD patients. Since PD is related to oxidative stress
and iron accumulation in brain, maintaining normal iron homeostasis and enhancing antioxidant capabilities seem to be the ideal strategies to prevent PD. Green tea polyphenols are shown to prevent not only neurological diseases, but also cancer and inflammatory processes.\textsuperscript{32-34} EGCG is the main catechin component, contributing to the beneficial effects of green tea due to its iron chelation, antioxidant, and antiinflammation capabilities.\textsuperscript{35} EGCG has shown to protect neurotoxin-induced dopaminergic cell death in both \textit{in vitro} and \textit{in vivo} models.\textsuperscript{36,37} EGCG is also found to enhance the activities of antioxidant enzymes, catalase, and SOD in the striatum of mice in a neurotoxin induced PD model.\textsuperscript{38} Our results support the previous studies, since green tea polyphenols demonstrated a protective effect in PD patients. Although iron status remained unaltered, there was a significant increase in catalase and SOD activity and a decrease in oxidative damage in lipids and proteins, suggesting that green tea polyphenols could potentially be used as therapeutic supplements. We believe that the beneficial effects are due to green tea consumption since patients did not change their medication use during the study and there was no indication of changes in dietary consumption during the 3 month trial period.

Although epidemiological studies have shown a negative correlation between green tea consumption and the risk of neurological disorders including PD,\textsuperscript{23,39} limited human data directly shows its effects against PD progression. A cross-sectional study indicated that inverse relationship between green tea consumption and cognitive impairment, but not cognitive decline, may be due to a small number of participants in the study.\textsuperscript{39} Chan (2009) showed that a green tea polyphenol (0.4 g, 0.8 g, and 1.2 g daily) intervention for early PD patients over a span of 6 months can improve UPDRS scores.\textsuperscript{40} In our study, participants consumed 3 cups of green tea daily for 3 months, with a total polyphenol concentration of approximately 550 mg per 3 tea bags. This
dosage resulted in an improvement in antioxidant status and reduced oxidative damage on PD patients after the 3-month intervention.

One limitation of our study was the lack of an age-matched control group without PD and/or a control group including PD patients who didn’t consume green tea. During the 3-month green tea intervention, we did not find significant changes in the total UPDRS score, PDQ-39 score, and most of the PD measure scales, suggesting the need for a long-term study and a large group of PD patients. While we used a realistic, dietary approach to green tea consumption, using a purified EGCG supplement may prove to be more accurate for determining the beneficial effects on Parkinson’s disease patients in a future clinical study. Although our study had a few limitations, it also had several strengths. Our 3-month green tea intervention significantly improved antioxidant status and reduced oxidative damage in early PD patients without affecting their iron status. Based on this pilot study, a future study including a large number of subjects, the use of an EGCG supplement, and age-matched control group is needed to clarify the effectiveness of EGCG in preventing the progression of PD.

References


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Figures and Tables

**TABLE 1.** PD rating scales in subjects at baseline and after 3 mo green tea consumption

<table>
<thead>
<tr>
<th></th>
<th>Mean (SD)</th>
<th>Median (Range)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PDQ-39</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mobility *</td>
<td>13.9 (15.0)</td>
<td>10 (0-45)</td>
</tr>
<tr>
<td>Activity of Daily Living</td>
<td>17.6 (15.1)</td>
<td>16.7 (0-33.3)</td>
</tr>
<tr>
<td>Emotional well-being</td>
<td>14.9 (11.2)</td>
<td>10.4 (0-33.3)</td>
</tr>
<tr>
<td>Stigma</td>
<td>20.5 (18.4)</td>
<td>18.8 (0-50)</td>
</tr>
<tr>
<td>Social support</td>
<td>10.1 (15.4)</td>
<td>0 (41.7)</td>
</tr>
<tr>
<td>Cognition</td>
<td>15.2 (12.7)</td>
<td>12.5 (0-18.8)</td>
</tr>
<tr>
<td>Communication</td>
<td>12.5 (15.2)</td>
<td>8.3 (0-41.7)</td>
</tr>
<tr>
<td>Bodily discomfort</td>
<td>32.1 (19.0)</td>
<td>25 (8.3-75)</td>
</tr>
<tr>
<td><strong>PDQ Total Score</strong></td>
<td>16.4(11.5)</td>
<td>15.7 (2.6-42.9)</td>
</tr>
</tbody>
</table>

**UPDRS**

<table>
<thead>
<tr>
<th></th>
<th>Mean (SD)</th>
<th>Median (Range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mentation, Mood, and Behavior</td>
<td>0.7 (1.2)</td>
<td>0 (0-4)</td>
</tr>
<tr>
<td>Activities of Daily Living *</td>
<td>9.2(5.7)</td>
<td>9.5 (0-22)</td>
</tr>
<tr>
<td>Motor Examination *</td>
<td>21.2(7.5)</td>
<td>20 (8-38)</td>
</tr>
<tr>
<td>Total Score</td>
<td>31.1(12.6)</td>
<td>31.5 (8-58)</td>
</tr>
</tbody>
</table>

**Beck Depression Inventory** | 7.1(5.8) | 5.5 (0-21) |

**Beck anxiety inventory** | 10.3(6.7) | 9.5 (1-20) |

**Epworth Sleepiness Scale** | 7.8(4.4) | 6.5 (3-17) |

**Mini Mental State Examination** | 29.5(0.7) | 30 (28-30) |

**Fatigue Severity Scale** | 28.4(13.0) | 27 (13-55) |

Wilcoxon matched pairs t-test is used to compare the changes on PD rating scales between baseline and post-intervention. Values are mean (SD) and median (range), n=14, * p<0.05. B, baseline; P, post-intervention.
TABLE 2. Subject description and iron status at baseline and after 3 mo green tea consumption

<table>
<thead>
<tr>
<th>Subject #</th>
<th>Height (M)</th>
<th>Weight (Kg)</th>
<th>BMI (Kg/M$^2$)</th>
<th>Hemoglobin (g/dL)</th>
<th>Serum Iron (µg/dL)</th>
<th>Iron Saturation (%)</th>
<th>Serum Ferritin (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B</td>
<td>P</td>
<td>B</td>
<td>P</td>
<td>B</td>
<td>P</td>
<td>B</td>
</tr>
<tr>
<td>Mean</td>
<td>1.7</td>
<td>87</td>
<td>87.5</td>
<td>28.9</td>
<td>29.1</td>
<td>14.3</td>
<td>14.2</td>
</tr>
<tr>
<td>± (SD)</td>
<td>0.1</td>
<td>(20.5)</td>
<td>(22.5)</td>
<td>(6.0)</td>
<td>(6.7)</td>
<td>(1.5)</td>
<td>(1.5)</td>
</tr>
</tbody>
</table>

The height, weight, BMI and iron status values are expressed as mean ± (SD), n=14. No significant changes were found in hemoglobin, serum iron, iron saturation and serum ferritin at baseline and post-intervention. B, baseline; P, post-intervention.
FIG. 1. Antioxidant enzymes activities in erythrocytes at baseline and after 3 mo green tea consumption. (A) SOD, (B) catalase, (C) GPx. n=14, * p<0.05, ** p<0.01.
FIG. 2. Lipid peroxidation (A, measured as MDA concentration in serum) and protein damage (B, measured as protein carbonyl concentration in plasma) at baseline and after 3 mo green consumption. n=14, *p<0.01.
Appendix. PD Patients Demographic Data

<table>
<thead>
<tr>
<th>Subject #</th>
<th>Age (Y)</th>
<th>Gender</th>
<th>PD Meds</th>
<th>Con Meds</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>53</td>
<td>F</td>
<td>Sinemet 25/100 qid, Sinemet CR 50/200 qhs, Requip 3 mg tid</td>
<td>Flexeril, Lorazepam, Cymbalta, Protonix, Benadryl, Melatonin</td>
</tr>
<tr>
<td>2</td>
<td>68</td>
<td>M</td>
<td>Stalevo 150 qid, Sinemet 25/100 qid, Mirapex 0.25 mg qhs</td>
<td>Zocor, MVI, ASA, Tramadol, Caltrate, Cal-Mag-Zn liquid, Vit D3, Magnesium</td>
</tr>
<tr>
<td>3</td>
<td>55</td>
<td>F</td>
<td>Azilect 1 mg qd, Mirapex 0.25 mg qd - bid, Stalevo 50 qid</td>
<td>MVI, Fish Oil, Flax seed, Calcium, Niacin, ASA, Glycolax powder, Glucosamine, CoQ10,</td>
</tr>
<tr>
<td>4</td>
<td>66</td>
<td>M</td>
<td>Sinemet 25/100 5.5 tabs/day</td>
<td>Aricept, Wellbutrin, Alprazolam, Flomax, Advil, Tylenol</td>
</tr>
<tr>
<td>5</td>
<td>57</td>
<td>M</td>
<td>Azilect 1 mg qd, Requip 3 mg tid, Sinemet 25/100 tid</td>
<td>ASA</td>
</tr>
<tr>
<td>6</td>
<td>51</td>
<td>M</td>
<td>None</td>
<td>Zocor, MVI, Vit D, ASA,CoQ10, Curcumin, Gingko Biloba, Fish Oil</td>
</tr>
<tr>
<td>7</td>
<td>61</td>
<td>M</td>
<td>Sinemet 25/100 2 tabs qid, Requip 3mg tid</td>
<td>Evoxac, Synthroid, Simvastatin, Clonazepam, Zantac, MVI, Vit E, Fish Oil, Saw Palmetto</td>
</tr>
<tr>
<td>8</td>
<td>67</td>
<td>F</td>
<td>Sinemet 25/100 tid</td>
<td>Lipitor, Toprol SL, HCTZ, Enalapril, Celebrex, Omeprazole, Gemfibrozil, Sertraline, Omega 3, Calcium, ASA, Neurontin</td>
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</table>
Appendix. PD Patients Demographic Data (Continued)

<table>
<thead>
<tr>
<th>Subject #</th>
<th>Age (Y)</th>
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<th>PD Meds</th>
<th>Con Meds</th>
</tr>
</thead>
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<tr>
<td>9</td>
<td>66</td>
<td>F</td>
<td>Azilect 1mg qd, Requip XL 8mg qd</td>
<td>Simvastatin, Atenolol, Calcium +D, Fosamax</td>
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<tr>
<td>10</td>
<td>54</td>
<td>F</td>
<td>C/L 25/100 bid, Azilect 1 mg qd</td>
<td>Altace, Prempro, Synthroid, Zoloft, Vytorin, Oxprozen, Fish Oil, Women's MVI</td>
</tr>
<tr>
<td>11</td>
<td>79</td>
<td>M</td>
<td>Sinemet CR 50/200mg bid, Azilect 1 mg qd, Mirapex .5mg bid</td>
<td>Oxycodone, Hydrocodone, HCTZ, Optiva, Lidoderm Patch, Detrol LA, Tylenol, Motrin</td>
</tr>
<tr>
<td>12</td>
<td>56</td>
<td>M</td>
<td>Sinemet 25/100mg qid, Mirapex 0.5mg tid</td>
<td>Buspar, Remeron, Benicar, Azasite</td>
</tr>
<tr>
<td>13</td>
<td>59</td>
<td>M</td>
<td>Sinemet 25/100mg tid, Requip 2mg tid, Azilect 1mg qd</td>
<td>Amlodipine, Pravastatin, Losartran HCTZ</td>
</tr>
<tr>
<td>14</td>
<td>66</td>
<td>F</td>
<td>Azilect 1mg qd</td>
<td>Claritin 24HR, Fosamax, Aleve, Simvastatin, ASA, Fish Oil, B12, Cranberry, Cal/mg/Zinc with D, Glucosamine CH, Multi Vit &amp; Mineral, Biotin, D3</td>
</tr>
<tr>
<td>15</td>
<td>66</td>
<td>M</td>
<td>Selegiline 5 mg bid</td>
<td>Actos, Multi-Vit, Fish Oil, Tylenol</td>
</tr>
<tr>
<td>Median (range)</td>
<td>61 (51~79)</td>
<td></td>
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</table>
CHAPTER 6. GENERAL CONCLUSION

Parkinson’s disease (PD) is a progressive neurodegenerative disease causing severe dopamine depletion in the striatum. Numerous evidences indicated that iron played an important role in PD. Both in vitro and in vivo studies showed that excess iron was closely associated with neuronal cell damage. Interestingly, several neurotoxins, such as 6-OHDA can disrupt iron homeostasis by altering iron-related gene and protein expression, consequently induce iron accumulation, ROS generation, redox dyshomeostasis and oxidative stress. We also confirmed the irreversible oxidative damage to lipids and proteins. Therefore, maintaining iron homeostasis is a promising strategy to prevent PD.

Results from epidemiological, cell culture, animal and clinical studies supported green tea polyphenols as well as the main constituent EGCG as neuroprevention methods to counteract neurodegeneration. In vitro study, pretreatment of EGCG showed more effective protection on neuronal cell against neurotoxin-induced cell damage than co-treatment, suggesting the preventive method is an effective protection on PD. It is also important to maintain the effective dosage of EGCG to show the neuroprotection. In this study, we showed EGCG at a concentration range of 10 μM -100 μM could protect neuron against 6-OHDA-induced cell apoptosis and cell death, whereas higher dosage (200 μM) was toxic to N27 cells and primary mesencephalic neuron. EGCG protected against 6-OHDA toxicity by decreasing DMT1, hepcidin, increasing Fpn1 expression and reducing cell iron uptake to maintain normal iron level. The pretreatment of EGCG suppressed the ROS generation and oxidative stress, attenuated lipid peroxidation and protein carbonyl, and decreased cell apoptosis and death.
Green tea consumption may offer a prospective dietary source of neuroprotection on PD patients. However, limited data showed green tea directly or indirectly improves the PD patients’ status. In our study, we have measured the PD patients’ blood EGCG concentration around 1.3 μM after consuming one bag of green tea with the bioavailability less than 1%. The participants daily consumed 3 cups of green tea for consecutive 3 months and this amount of green tea consumption showed an improved antioxidant status, reduced oxidative damage to lipid and protein, and a slow-down progression of PD without affecting the iron status. The poor bioavailability of EGCG is the concern for the effective concentration. Human study showed oral intake 20mg/kg body weight EGCG can cause plasma maximum concentration (Cmax) at 78 ng/ml (1). Animal studies showed that rat has only 1.6% bioavailability of EGCG, mice has relative higher, 26.5% (2, 3). The concentration is far below the micromolar concentration usually required in vitro activity. Therefore, modification of EGCG structure ((-)EGCG octaacetate) is an effective way to improve the bioavailability, which has already been shown to inhibit breast tumor growth and prostate cancer in animal studies (4, 5).

Future studies are still needed to clarify the mechanism on neuroprotection. In the first study, we showed EGCG reversed the adverse effects from 6-OHDA on iron related gene and protein expression. However, whether the oxidative stress affected the iron related factors, leading to the iron influx, or the influx of iron caused oxidative stress remains unclear. Also, whether the protection effect of EGCG directly depended on reducing oxidative stress, regulating the iron related factors, resulting in the decreased iron intake is still unclear. In the second study, we confirmed that Nrf2/ARE pathway, including HO-1 expression and PPARγ anti-inflammatory pathway were involved in the 6-OHDA induced neurotoxicity. We also showed that
Nrf2 positively related to PPARγ in neurodegeneration. However, whether there is a cause-effect relation remains unclear. In the third study, although we showed the beneficial effects of green tea consumption on PD patients, the improvement of health care, modern quality of life and other medication may also contribute to the changes and improve the PD status. Therefore, age-matched control is required in future human study.

In conclusion, we showed the EGCG protection against 6-OHDA by normalizing its adverse effect on iron homeostasis in vitro. We also figure out the EGCG decreased the oxidative stress and inflammatory reaction which derived by 6-OHDA. These results are preliminary data for future in vivo study. The human study showed the beneficial effects of green tea consumption on PD patients, and this pilot human study added supportive data for future study, long-term and higher dosage, with modification on EGCG supplement will help us to clarify the therapeutic way to prevent PD.

References


2. Lambert JD, Lee MJ, Lu H, Meng X, Hong JJ, Seril DN, Sturgill MG, Yang CS. Epigallocatechin-3-gallate is absorbed but extensively glucuronidated following oral administration to mice. J Nutr. 2003;133:4172-7.


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