Neuroprotective effect of phytic acid in Parkinson's disease

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Neuroprotective effect of phytic acid in Parkinson’s disease

by

Qi Xu

A thesis submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Major: Toxicology

Program of Study Committee:
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Ames, Iowa
2007
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CHAPTER 1. GENERAL INTRODUCTION

Introduction

Parkinson’s disease (PD) is the second most common neurodegenerative disease, affecting more than 1% of the US population over 50 years of age and causing an estimated economic obligation of $25 billion annually (Scheife et al. 2000). The clinical features of PD include resting tremor, bradykinesia, rigidity, and postural instability. These symptoms result from selective degeneration of dopamine neurons arising in the substantia nigra and terminating in the striatum.

Recent research interest has been directed toward understanding the pathogenesis and the neuroprotective therapy of the disease. There have been many dopamine replacement strategies to neutralize the motor deficits resulting from PD. These strategies, however, are limited to symptomatic relief. Other limitations of these approaches include drug tolerance and drug-induced involuntary movements, and most importantly, such dopamine therapy does not attenuate the progression of the illness. Therefore, novel neuroprotective agents that effectively prevent the progression of the neurodegenerative process are needed.

Since excessive iron accumulation in substantia nigra was found in postmortem brains of PD patients, the role of iron in this disease has recently gained attention. Selective cell death in substantia nigra of the brain region is associated with oxidative stress, which may be exacerbated by the presence of excess iron, because of its pro-oxidative nature. Iron metabolism is disrupted in PD, such that iron accumulates in Lewy bodies and distribution of iron transport and storage proteins is altered. Increased iron levels without an increase in ferritin (an iron storage protein) levels has been suggested to provide “free iron” for free
radical generation (Berg et al. 2001; Bishop et al. 2002), thus causing oxidative damage. Studies showing the effectiveness of antioxidants and iron chelators in preventing and treating PD clearly support the involvement of oxidative stress and iron in the disease (Kaur et al. 2003; Zhang et al. 2005).

Phytic acid is present in high concentration in cereals and legumes and is generally considered an antinutrient by virtue of its ability to chelate divalent minerals and prevent their absorption (Reddy et al. 1996). Its unique chelating action with iron has been shown to inhibit hydroxide (•OH) formation and decrease lipid peroxidation in vivo (Rao et al. 1991). Phytic acid may also influence oxidative stress by altering cell signaling pathways or influencing the activity and expression of key enzymes in the antioxidant defense system (Shamsuddin et al. 1997).

Based on the positive effect of iron chelators in PD and the antioxidant property and iron chelating ability of phytic acid, the objective of this study is to determine the neuroprotective effect of phytic acid in the cell culture model of PD.

**Thesis organization**

This dissertation is written in an alternative thesis format. It contains a general introduction, two research papers, a general conclusion and an appendix of the research report. The list of references cited is included at the end of each chapter. Chapter 1, the general introduction, includes the research objectives and the background information related to the studies presented: (1) Parkinson’s disease (PD) and animal models of PD (2) Pathogenesis and risk factors of PD (3) The role of iron in PD (4) Treatment of PD (5) Alternate therapeutic strategies (6) Nutritional approaches to PD. Chapter 2 is the manuscript
“Neuroprotective effect of the natural iron chelator phytic acid in a cell culture model of Parkinson's disease” which will be submitted to The Journal of Nutrition. Chapter 3 is the second manuscript “Phytic acid protects against 6-hydroxydopamine and iron induced apoptosis in a cell culture model of Parkinson's disease” which will be submitted for publication in Neuroscience letters. Chapter 4 is the general conclusion. The appendix summarizes the experimental results on the effect of phytic acid in an animal model of PD.

The thesis contains the experimental results obtained by the author during her graduate study under the supervision of her major professors, Dr. Manju B. Reddy and Dr. Anumantha G. Kanthasamy.

**Literature review**

**Parkinson’s disease (PD) and animal models of PD**

Parkinson’s disease was first described in 1817 by a British physician, James Parkinson, as a neurological illness consisting of resting tremor and a peculiar form of progressive motor disability (Samii et al. 2004). Currently, this debilitating neurodegenerative disorder is the second most common neurodegenerative disorder in the US and is expected to impose an increasing economic burden on societies. In the US, PD affects more than 1% of the population over 50 years of age, and causes an estimated economic obligation of $25 billion annually (Scheiffe et al. 2000). The cardinal symptoms of PD include resting tremor, bradykinesia, and rigidity. The resting tremor is considered to be the most common symptom and is shown as the first sign in 70% of patients. Asymmetric at first, this tremor occurs in hands, arms, legs, jaw, and face. It usually occurs at rest but may also be present when the arms are raised. Bradykinesia refers to slowed movement including both nonvolitional and
volitional movements. It is the most disabling symptom of early PD and initially manifests with difficulties with fine motor tasks such as doing up buttons or handwriting and reduced arm swing while walking (Samii et al. 2004). The masked face of PD is an example of slowed nonvolitional movement. Rigidity produces a resistance to passive movement that is uniform throughout the range of motion of the limbs and joints. Various non-motor features in PD have also been recognized in the past ten years, including autonomic dysfunction, sensory symptoms, sleep disturbance, anxiety, depression, and dementia (Samii et al. 2004).

The basic anatomy of the basal ganglia will be reviewed, since it is so important for understanding PD pathology. Motor, cognitive, and affective activities are influenced by two major subcortical structures: the basal ganglia and cerebellum (Hoshi et al. 2005). The basal ganglia refers to the major anatomical telencephalic subcortical nuclei at the base of the forebrain and consists of striatum, globus pallidus, substantia nigra, and subthalamic nucleus. The basal ganglia and the cerebellum receive information from cerebral cortex projects and send their output right back to the cortex via the thalamus or directly to the motor systems in the midbrain and hindbrain. The output of the cerebellum is excitatory, whereas the basal ganglia output is inhibitory. The balance between these two systems allows for smooth and coordinated movement, and a disturbance in either system will cause movement disorders.

The pathological hallmark of PD is the degeneration of dopaminergic neurons whose cell bodies are located in the substantia nigra pars compacta (SNpc) and whose projecting axons and nerve terminals are found in the striatum (Przedborski 2005). The loss of nigral neurons in the substantia nigra results in severe dopamine depletion in the striatum, affecting the motor symptoms (Fig. 1.1). The disease is also considered as a tyrosine hydroxylase (TH) deficiency syndrome (Fig. 1.2), since TH catalyzes the formation of L-3, 4-
dihydroxyphenylalanine (levodopa), the rate-limiting step in the biosynthesis of dopamine in the striatum (Haavik & Toska 1998). In general, the appearance of disease symptoms is observed after loss of 80% of striatal dopamine and 50% of TH-immunoreactive dopaminergic neurons in the substantia nigra (Samii et al. 2004).

**Figure 1.1** Schematic diagram of nigrostriatal dopaminergic pathway (Betarbet et al. 2002).

**Figure 1.2** Main pathways of synthesis and metabolism of dopamine. The main enzymes involved are tyrosine hydroxylase (TH), aromatic amino acid decarboxylase (AADC), and catechol-O-methyltransferase (COMT). DA, dopamine; DOPAC, 3, 4-dihydroxyphenylacetic acid; HVA, homovanillic acid; levodopa, L-3, 4-dihydroxyphenylalanine.

The mechanism responsible for cell death in PD is still unknown. However, increasing
evidence suggests that neuronal death in the SNpc may be apoptotic (Lang & Lozano 1998). Apoptotic cell death is a morphologically and biochemically defined mode of cell death characterized by chromatin condensation and aggregation to the nuclear margin, cytoplasmic shrinkage, DNA fragmentation, and membrane blebbing. Blebbing of the membrane results in the cell budding off from itself into membrane-bound “apoptotic bodies”, which are phagocytized by neighboring cells without an inflammatory response (Andersen 2001). In vitro studies with isolated neurons and in vivo studies in animals treated with neurotoxin have provided strong evidence that substantia nigra cell death is predominantly from apoptosis (Kaul et al. 2003; Kostrzewa 2000). In addition, postmortem studies have also reported that dying neurons in the Parkinsonian brains displayed morphological characteristics of apoptosis including cell shrinkage, chromatin condensation, and DNA fragmentation (Andersen 2001; Anglade et al. 1997; Mochizuki et al. 1996).

Another important characteristic pathology of PD are lewy bodies, the intraneuronal inclusions of abnormal protein aggregation (Emborg 2004). The lewy bodies are small spherical inclusions that consist of neurofilaments and ubiquitin (Blum et al. 2001). Though the detailed mechanisms are still under investigation, the production of excessive misfolded proteins and the compromised ubiquitin-proteasome system are thought to play important roles in the formation of lewy bodies. The presence of lewy bodies is not restricted to PD; the inclusions are also observed in Alzheimer’s disease, suggesting the abnormal protein aggregation in neurons is ubiquitously involved in the pathogenesis of neurodegenerative diseases.
Neurotoxins induced PD

Since the cause and mechanism of PD remain unknown and investigating the etiology and pathogenesis of the disease in humans is difficult, neurotoxins are used to induce experimental models of PD. The most common neurotoxins used are 6-hydroxydopamine (6-OHDA), rotenone, lactacystin, and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Beal 2001; Zhang et al. 2005).

Neurotoxicity induced by MPTP, a toxic byproduct in the clandestine synthesis of a pethidine analogue, may be most useful for understanding PD in developing strategies. Numerous studies have investigated the mechanism of MPTP induced Parkinsonism. MPTP administration selectively damages dopaminergic neurons arising in the SNpc and causes the loss of dopaminergic nerve terminals in the striatum, which leads to impaired dopaminergic neurotransmission (Burns et al. 1983; Kalivendi et al. 2003). MPTP induced Parkinsonism in primates replicates all the clinical signs of PD, including tremor, rigidity, and bradykinesia. In addition, primates treated with MPTP show an excellent response to the dopamine precursor levodopa and to dopamine receptor agonists treatment. When administered to animals, MPTP crosses the blood brain barrier and is converted into its active metabolite, 1-methyl -4- phenylpyridinium (MPP⁺), by monoamine oxidase type B. MPP⁺ is taken up by the plasma-membrane dopamine transporter and accumulates in the dopaminergic neurons in the SNpc due to its similar structure to dopamine (Fig. 1.3). Intracellular MPP⁺ is taken up and accumulates in the mitochondria, where it inhibits the respiratory chain by inhibiting NADH dehydrogenase (Beal 2001). Since the transport of electrons down the electron transport chain (ETC) will release energy to create a proton and electrochemical gradient for ATP formation, disruption of the ETC may lead to the decrease in ATP which could affect
many processes in the neurons. Meanwhile, MPP⁺-induced inhibition of the complex I could increase the superoxide radical formation, as NADH could no longer transfer reduced equivalents to oxidized ubiquin-one, and the high energy electrons will react with oxygen to form superoxide (Fonck & Baudry 2003; Shults 2005). MPTP is thus considered a powerful compound to induce neurodegeneration in animals, and MPP⁺ is used in cellular models of PD to exert oxidative stress and induce apoptosis.

Figure 1.3 Schematic diagram of MPP⁺ intracellular pathway (Dauer & Przedborski 2003). 6-hydroxydopamine is also one of the most common neurotoxins used to induce experimental nigral degeneration in vitro as well as in vivo. 6-hydroxydopamine is a hydroxylated analogue of the natural dopamine neurotransmitter, exhibiting a high affinity for several catecholaminergic plasma membrane transporters including the dopamine transporters (DAT) and norepinephrine transporters. Consequently, 6-OHDA could enter both dopaminergic and noradrenergic neurons and damage both the peripheral and the central nervous systems (Bove et al. 2005). However, 6-OHDA could not cross the blood brain barrier (Schober 2004). In the experimental models of PD, 6-OHDA is injected directly into the striatum, the substantia nigra, or the ascending medial forebrain bundle to damage the nigrostriatal dopaminergic pathway. 6-hydroxydopamine selectively accumulates in
dopamine neurons, destroys nigral dopaminergic neurons, and depletes the dopamine neurotransmitter in the striatum. 6-hydroxydopamine is suggested to induce nigrostriatal dopaminergic lesions via the generation of hydrogen peroxide derived hydroxyl radicals since it could be oxidatively deaminated by monoamine oxidase to produce hydrogen peroxide (Blum et al. 2001). Furthermore, 6-OHDA has been shown to reduce striatal glutathione (GSH) and superoxide dismutase (SOD) enzyme activity and increase levels of lipid peroxidation products (Schober 2004).

Pathogenesis and risk factors of PD

Although the etiology of PD remains obscure, the disease may result from multi-factorial effects of age, genetic susceptibility, environmental exposure, infection, and oxidative stress.

Age

Age has been considered as a potential risk factor of PD because most patients develop PD after 50 years of age. The prevalence of PD increases with age, from 20/100,000 overall to 100/100,000 at age 70 (Dauer & Przedborski 2003; Tanner & Goldman 1996). Pathologically, aging is associated with a decline of pigmented neurons in the SNpc. It is also suggested that the aging nigrostriatal system may be more vulnerable to damage caused by exogenous and endogenous toxins since the neurotoxin MPTP causes more severe pathological and neurochemical injury in older mice (McCormack et al. 2004).

Genetic susceptibility

Although most PD is sporadic, the genetic component is suggested to be an important risk factor. Epidemiological studies have identified a positive family history of Parkinson
disease as one of the most important risk factors for the disease (Allam et al. 2005). Results of one study among 20,000 male twins suggest that genetic factors appear to be an important factor in PD with onset before age 50 years (Tanner et al. 1999). Discovery of at least nine gene loci in familial PD further demonstrates the genetic contribution to this disorder (Samii et al. 2004). The identification of these responsible genes for familial PD will provide important clues for understanding the molecular pathogenesis of the disease (Kubo et al. 2006). 

**Environment**

The environmental factor hypothesis for PD emerged following the discovery of the Parkinsonian toxin MPTP. MPTP was recognized as a neurotoxin early in 1982, when several young drug addicts mysteriously developed a parkinsonian syndrome after the intravenous use of synthetic heroin analogue contaminated by its byproduct, MPTP (Przedborski et al. 2001). This episode supported the concept that exogenous chemicals can induce PD symptoms. Since then, several epidemiological studies have suggested that exposure to different environmental agents including pesticides, insecticides, metals, and microbial toxins may increase the risk of PD (Di Monte 2003; Kanthasamy et al. 2005). Dieldrin, one of the most persistent bioaccumulative and toxic chemicals, is associated with increased incidence of PD (Kanthasamy et al. 2005). Metals like manganese, copper, iron, lead, and aluminum have been linked to PD in various etiological studies (Gorell et al. 2004; Tanner & Goldman 1996). Conversely, tobacco, caffeine, and tea seem to protect against development of PD due to their antioxidant properties, based on case-control studies (Checkoway et al. 2002; de Lau & Breteler 2006; Gorell et al. 2004; Hellenbrand et al. 1997; Ross et al. 2000).
Inflammation

Inflammation has also been implicated in the pathogenesis of PD. Studies have shown that many cases of PD are accompanied by brain inflammation with a dramatic proliferation of reactive microglial cells (Arai et al. 2006; McGeer et al. 1988a; McGeer et al. 1988b). Pro-inflammatory cytokines such as interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) may induce microglia activation and cause microglia related injury to neurons. Cytokines could also bind to the receptors on the dopaminergic neurons and trigger intracellular death in signaling pathways (Arai et al. 2006). An association between the low risk of PD and the use of the non-steroidal anti-inflammatory drug ibuprofen has also been reported. Compared with nonusers, the PD risks were 0.73 for ibuprofen users of fewer than 2 tablets/week, 0.72 for 2 to 6.9 tablets/week, and 0.62 for 1 or more tablets/day, suggesting the role of inflammation in PD (Chen et al. 2005).

Oxidative stress

Oxidative stress induced cell damage, including apoptosis, may play a prominent role in neurological degeneration associated with PD. It is widely known that age is a major risk factor for PD and oxidative stress increases with age. Brain tissues are susceptible to oxidative stress since the brain 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 since the mitochondrial ETC is the main source of ATP in the mammalian cells and electrons could leak to form oxygen free radicals during energy transduction (Valko et al. 2007). It is also suggested that brain cells have low antioxidant capability (Crichton et al. 2002). For example, the antioxidant enzymes SOD and catalase
concentrations were 7- and 140-fold lower in the brain than in the liver (Crichton et al. 2002; Reddy & Clark 2004). Nigral dopaminergic neurons are particularly exposed to oxidative stress because dopamine is inactivated by the enzyme monoamine oxidase (MAO), a reaction yielding hydrogen peroxide (H₂O₂) and other reactive oxygen species (ROS), which could cause increased formation of oxidized GSH and impair the major antioxidant system in the body (Jenner 2003; Olanow & Tatton 1999; Yoritaka et al. 1996). Hydrogen peroxide could also react with iron in the substantia nigra to produce highly toxic hydroxyl radicals. In addition, dopamine could be decomposed by autooxidation and form quinines and semiquinones, which may lead to generation of ROS (Koutsilieri et al. 2002). Levodopa, the major therapeutic agent for the treatment of PD, increases oxidative stress and plays a role in disease progression due to its increased production of free radicals during oxidative metabolism of levodopa and its product dopamine (Jenner 2003; Mayo et al. 2005; Shulman 2000).

The occurrence of oxidative stress in PD is also supported by human postmortem studies. There is evidence that levels of basal oxidative stress in the substantia nigra in the brain are increased in PD. The deletion of reduced GSH and impairment of the antioxidant system were also found in substantia nigra in PD cases, which was associated with a significant increase in oxidized GSH (Sofic et al. 1992).

Oxidative stress causes damage to proteins, DNA, and lipids and induces apoptotic death in dopaminergic neurons (Fig. 1.4). Protein carbonyls were found to be twofold higher in the SNpc compared to other regions in normal postmortem brains, suggesting susceptibility to oxidative damage (Floor & Wetzel 1998). Furthermore, protein carbonyl concentration was higher in PD patients compared to age matched controls (Alam et al. 1997a; Floor & Wetzel
Similarly, increased levels of the lipid peroxidation products malondialdehydes and lipid hydroperoxides were found in the substantia nigra in the PD patients (Olanow & Tatton 1999; Yoritaka et al. 1996). In addition, indicators of DNA damage, such as 8-hydroxyguanine, were also increased in the substantia nigra as well as some other brain regions in the PD patients (Alam et al. 1997b; Jenner 2003). All these results suggest that oxidative damage is involved in PD.

![Figure 1.4](image)

**Figure 1.4** Pathway of ROS formation, the role of antioxidant and oxidative damage. CAT, catalase; GPX, glutathione peroxidase; SOD, superoxide dismutase; MAO, monoamine oxidase.

**The role of iron in PD**

Metals may contribute to the pathogenesis of PD by involvement in protein aggregation and oxidative stress (Gaeta & Hider 2005). Copper-mediated ROS production may play a role in increased oxidative stress burden in aging and neurodegeneration (Sayre et al. 1999). Copper and iron were also found to stimulate α-synuclein fibril formation, the major constituent of intracellular protein inclusions in PD (Uversky et al. 2001). The involvement of metals in the etiology of PD was also demonstrated from epidemiological study. Long-
term occupational exposure to certain metals such as copper, manganese, lead, mercury, zinc, aluminum, and iron may increase the risk of PD (Gorell et al. 1999; Gorell et al. 2004; Uversky et al. 2001). Exposure to high levels of manganese could cause neurotoxicity and induce a Parkinson’s-like syndrome known as manganism (Latchoumycandane et al. 2005; Olanow 2004).

Iron is an essential nutrient for all living organisms since it plays many important roles in the body, including electron transport, activation of molecular oxygen, oxygen transport, collagen synthesis, tissue growth, neurotransmitter synthesis, and many catabolic processes (Pollitt & Leibel 1976). Iron deficiency can cause changes in many metabolic processes, such as glucose metabolism, neurotransmitter synthesis, and protein synthesis (Beard 1990; Beard et al. 1990; Brooks et al. 1987).

Iron also plays a deleterious role due to its ability to catalyze the production of highly reactive hydroxyl radicals through the Haber-Weiss and Fenton reactions (Braughler et al. 1986), which could cause oxidative damage to membranes, nucleotides, and proteins (Fig. 1.7 A).

**Haber-Weiss reaction**  \[ \text{H}_2\text{O}_2 + \text{O}_2^- \rightarrow \text{O}_2 + \text{OH}^- + \text{OH}^* \]

**Fenton reaction**  \[ \text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \text{OH}^* + \text{OH}^- \]

Under physiological conditions in vivo, iron is in the form of iron-protein complex and may not participate in the above reactions. However, under iron overload or other pathological conditions, iron may be involved in the free radical generation and can be toxic to cell survival. For example, iron from transferrin may be released at pH values that can be seen in atherosclerotic lesions (slightly lower than physiologic pH) to induce lipid oxidation (Reddy & Clark 2004).
Iron plays an important role in brain function since it is involved in electron transport in production of ATP. It is an essential component of cytochromes a, b, and c, cytochrome oxidase, and the iron-sulfur complexes of the oxidative chain (Connor et al. 2001). Iron is also involved in ribonucleoside reductase, the rate limiting enzyme of the first metabolic reaction in DNA synthesis, and succinate dehydrogenase and aconitase of the TCA cycle (Domingo J. Pinero 2000).

Iron requirement is high in the brain since the brain consumes high levels of ATP to maintain membrane ionic gradients, synaptic transmission and axonal transport. Iron is also an essential cofactor for many proteins that play essential roles in the normal function of neuronal tissues (Connor et al. 1992; Zecca et al. 2004). Iron is a cofactor for the enzymes TH and tryptophan hydroxylase, which are involved in neurotransmitter synthesis and catabolism, respectively (Beard & Connor 2003).

There are two categories of iron in the brain: heme iron as a part of hemoglobin and enzymes like peroxidases, and nonheme iron associated with proteins (Haacke et al. 2005). For the nonheme iron, transferrin and ferritin are considered key proteins in iron homeostasis (Fig. 1.5). Transferrin is a single chain, 80-KDa protein that contains two iron-binding motifs and is responsible for delivery of iron from/to peripheral tissues (Domingo J. Pinero 2000). Transferrin (Tf) carries iron from the blood into brain tissue via transferrin receptors (Tfr) located in the brain’s microvasculature (Haacke et al. 2005). Tf-Tfr complex could be taken up by cells via endocytosis and iron is released and exported into the cytoplasm by divalent mental transporter 1 (DMT1) (Domingo J. Pinero 2000). In the cytoplasm, iron is available for necessary metabolic processes or is incorporated into the storage pool with ferritin for future need. In most cells, the main regulator of iron homeostasis is iron regulatory protein
(IRP)/iron regulatory element (IRE) system. Changes in iron status such as iron overload or iron depletion lead to compensating changes in this system (Fig. 1.6). When iron is in excess, IRPs are in their inactive form and do not bind the IREs on the mRNAs of ferritin and TfR. As a result, the ferritin is synthesized and the TfR mRNA is degraded by nucleases. However, in conditions of iron depletion, IRPs binds to IREs on the mRNAs, preventing ferritin but allowing the synthesis of TfR by protecting the mRNA from degradation (Zecca et al. 2004).

**Figure 1.5** Cellular iron uptake (Domingo J. Pinero 2000).

**Figure 1.6** Translational regulation of the transferrin receptor and ferritin production (Zecca et al. 2004).
The brain is highly susceptible to excess iron since it is rich in polyunsaturated fatty acids (Schafer et al. 2000). The substantia nigra has the highest concentration of iron in the mammalian brain. The substantia nigra has 20 ng/mg iron during the first year of life, which increases to 200 ng/mg by the fourth decade (Zecca et al. 2001). Excess iron could interact with neuromelanin, a dark-colored pigment in the dopaminergic neurons, to induce oxidative stress and induce neurodegeneration (Double et al. 2000).

Since excessive iron accumulation in substantia nigra was found in postmortem brains from Parkinson's patients, the role of iron in PD has recently gained attention (Berg et al. 2002; Berg et al. 2001). A disruption in the iron metabolism occurs in PD, such that there is an accumulation of iron in Lewy bodies and altered distribution of iron transport and storage proteins (Fig. 1.7B). Recent studies also suggest that mis-regulation of iron metabolism, iron-induced oxidative stress, and free radical formation are major pathogenic factors in PD (Jellinger 1999). Ferritin is reportedly decreased in the substantia nigra of the PD brain compared to elderly controls. H ferritin and L ferritin levels are reported to be 75% and 37% of normal in the substantia nigra, respectively (Domingo J. Pinero 2000). Increased iron levels and low levels of ferritin allows for “free iron” that may be involved in free radical generation (Berg et al. 2001; Bishop et al. 2002). The neurotoxins MPTP and 6-OHDA release ferritin bound iron (Fig. 1.7C) in both in vitro and in vivo conditions (Grunblatt et al. 2000). Although the mechanism is not clear, MPTP disrupts iron metabolism through iron regulatory proteins by increasing transferrin receptors, and thus causing increased iron uptake by the brain (Blum et al. 2001; LaVaute et al. 2001).
**Figure 1.7** Hypothesized mechanism for the role of iron in PD.

**Treatment of PD**

Current therapeutic strategies for PD focus primarily on minimizing the disease associated motor deficits using dopaminergic medications. The dopamine precursor levodopa was first demonstrated to be effective in reducing these motor deficits 30 years ago and remains the most potent antiparkinson drug (Hubble 1999; Samii et al. 2004). This treatment can significantly attenuate the parkinsonian symptoms and improve the quality of life of parkinsonian patients. However, while levodopa therapy improves PD symptoms, but does not inhibit the progressive degeneration of dopaminergic neurons in the substantia nigra. Moreover, the majority of patients receiving 5 years of levodopa monotherapy will develop fluctuations and/or involuntary movements called dyskinesias (Schelosky & Poewe 1993). Patients feel levodopa effectiveness declines with time, and they become slower and more tremulous after long term treatment with levodopa (Samii et al. 2004). Furthermore, there is an “on-off” phenomena in the patients, with sudden fluctuations between mobility and immobility, with the “off” periods marked with severe akinesia that can extend over several hours (Singh et al. 2007). As a result, a variety of dopamine receptor agonists such as cabergoline, pramipexole, and ropinirole are used as adjuvant therapies to levodopa to
enhance the effectiveness of levodopa and reduce motor fluctuations in parkinsonian patients (Rinne 1981). Dopamine receptor agonists mimic endogenous dopamine and directly stimulate the presynaptic and postsynaptic dopamine receptors (Radad et al. 2005). In the early stages of PD, dopamine agonists are as effective as levodopa but delay the occurrence of motor complications associated with long term administration of levodopa. However, dopamine agonists lose efficacy over time (Bonuccelli & Pavese 2006). Most important, all these treatments are limited to symptomatic relief and do not attenuate the progress of the underlying pathology and the natural course of the disease.

**Alternate therapeutic strategies**

Alternate therapeutic strategies must be considered to identify novel neuroprotective agents that effectively prevent the progression of the neurodegenerative process. Based on the current knowledge regarding the etiology and pathogenesis of PD, research on the use of antioxidants, free radical scavengers, and inducers of cellular antioxidant systems as therapeutic adjuncts in the treatment of PD provides hope that more effective therapies will be developed that not only attenuate symptoms, but also slow the process of neurodegeneration. Indeed, a number of studies have examined whether antioxidants prevent or reduce the progression of PD. In a rat study, dietary supplementation with vitamin E and vitamin C were found to protect against 6-OHDA induced striatal damage (Prasad et al. 1999). Coenzyme Q10, a well-known electron acceptor for complex I and II and an important antioxidant with regard to its function in the reduced form (ubiquinol), could attenuate the MPTP induced loss of striatal dopaminergic neurons and has a protective effect on PD (Ebadi et al. 2001; Ernster & Dallner 1995). In addition, estrogen, which acts as an antiapoptotic
agent as well as an antioxidant, offers neuroprotection in an MPTP mouse model (Tripanichkul et al. 2006). Although there are many disputes about the efficacy of vitamin E in the prevention or treatment of PD, both moderate and high intake of dietary vitamin E may have a neuroprotective effect and attenuate the risk of PD (de Rijk et al. 1997; Etminan et al. 2005). Long term, high dose vitamin E dietary supplementation (e.g. 1600-2000 IU d-α-tocopheryl succinate) beginning in the third decade of life may serve as a successful therapeutic strategy for the prevention of PD (Fariss & Zhang 2003). In addition, combination of α-tocopherol and ascorbate in patients with early PD might delay the progression of the disease by an average of 2.5 years (Fahn 1992; Ricciarelli et al. 2007).

Iron chelation has also been suggested to be an effective therapy for prevention or treatment of PD due to the association between iron and neurotoxin mediated neurodegeneration. Iron chelation via either transgenic expression of the iron binding protein ferritin or oral administration of the metal chelator clioquinol (CQ) reduced susceptibility to MPTP for inducing PD in animals, suggesting that iron chelation may be an effective therapy for prevention and treatment of the disease (Kaur et al. 2003). Pretreatment with the iron chelator, desferrioxamine (DFO), significantly protected (approximately 60%) against 6-OHDA induced reduction in striatal dopamine content and resulted in a normalization of dopamine release in a rat study (Ben-Shachar et al. 1991; Zhang et al. 2005). In another study, DFO reduced abnormal protein aggregation and attenuated the lactacystin induced dopamine neuron loss (Zhang et al. 2005). Desferrioxamine also significantly inhibited iron accumulation in the brain and decreased the formation of reactive hydroxyl radicals and lipid peroxidation induced by excess iron and MPTP, thus resulting in a significant reversion of the reduction of striatal dopamine levels (Lan & Jiang 1997).
However, the currently used iron chelators may cause some side effects in patients. For example, DFO is the most potent iron chelator, but it must be administered at high dosage to overcome its low ability to cross the blood brain barrier and causes some toxic effects (Zhang et al. 2005). CQ has been demonstrated to chelate both ferrous and ferric iron and decrease total brain iron levels (Kaur & Andersen 2002). Oral administration of CQ may protect against loss of striatal dopamine induced by MPTP (Kaur et al. 2003). However, CQ administration may reduce the brain and serum vitamin B12 levels and causes B12 deficiency, which may cause pernicious anemia and damage the nervous system (Kaur & Andersen 2002; Yassin et al. 2000).

**Nutritional approaches to PD**

Studies of nutritional approaches to prevent the onset of PD are limited. Various food groups and specific nutrients have been investigated for their ability to reduce the risk of PD. Nutritional approaches have focused on antioxidants due to involvement of oxidative stress in the pathogenesis of PD. High dose vitamin E dietary supplementation, such as 1600-2000 IU d-α-tocopheryl succinate beginning in the third decade of life, may prevent PD (Fariss & Zhang 2003). A combination of vitamin E and vitamin C may be beneficial in patients with early PD (Prasad et al. 1999). Tea is an ancient beverage and green tea polyphenols are now thought to be therapeutic in PD since flavonoids possess iron chelating, antioxidant, and anti-inflammatory activities (Mandel et al. 2006; Weinreb et al. 2004). Green tea extract and its major polyphenol, (±)-epigallocatechin-3-gallate (EGCG), can protect against MPTP induced neurodegeneration in animal studies (Choi et al. 2002; Levites et al. 2001). Based on the iron chelating ability, the effect of phytic acid (IP6, myo-inositol hexakisphosphate) in PD was
under investigation in one study (Obata 2003).

Phytic acid was first described as an abundant form of phosphorus in plant seeds and other plant tissues and is present at 2% of the wet weight in whole grains, nuts, seeds, cereals, and legumes (Raboy 2003). Phytic acid was found to be a common constituent of eukaryotic cells and serves a number of functions such as signal transduction, cell proliferation and differentiation (Raboy 2003; Sasakawa et al. 1995; Shamsuddin et al. 1997).

Phytic acid is generally considered as an antinutrient by virtue of its ability to chelate divalent minerals and reduce their absorption (Reddy et al. 1996). It can form chelating conjugates with essential metal cations such as copper, zinc, iron, and manganese, and its affinity to bind these metals is as follows: Cu^{2+} > Zn^{2+} > Co^{2+} > Mn^{2+} > Fe^{2+} > Ca^{2+}. A function of IP6 as a natural antioxidant was described in 1984 (Graf et al. 1984). Its unique chelating action with iron inhibits •OH formation and decreases lipid peroxidation catalyzed by iron and ascorbate in human erythrocytes (Rao et al. 1991). This suppression of iron-catalyzed oxidative reactions was suggested to result from the ability of IP6 to form a unique chelate with Fe (III) occupying all of the coordinating sites (Graf et al. 1984). Phytic acid is one of the most effective agents for inhibiting oxidation in food materials. Large amounts of phytate in plant seeds could offer protection from oxidation caused by high unsaturated fatty acids in soybeans (Graf et al. 1987). The antioxidant ability of IP6 in vivo is not clear, but in a previous study it reduced by 60% lipid peroxidation and reduced liver iron stores by 48% in a genetically iron overloaded-mouse model. Consumption of IP6 containing soy protein for 6-weeks to postmenopausal women reduced body iron stores significantly compared with a low-IP6 soy protein (Hanson et al. 2006). However, IP6 may influence oxidative stress independent of iron-involved hydroxyl radical formation. It may alter cell signaling pathways
or may influence the activity and expression of key enzymes in the antioxidant defense system, which detoxifies the ROS (Shamsuddin et al. 1997).

In contrast to other iron chelators, IP6 is safe as a therapeutic and preventive agent and is non-toxic over long term administration (Vucenik et al. 1992). Although IP6 intake may suppress the absorption of metal ions such as zinc and calcium (Zhou & Erdman 1995), several studies indicate ingestion of large quantities of IP6 with a diet poor in oligoelements may result in poor bioavailability of minerals (Graf & Eaton 1993; Vucenik & Shamsuddin 2003). The lack of toxicity associated with long term administration in animal models makes IP6 appealing for prevention of iron associated pathogenesis (Vucenik et al. 1992).

Phytic acid is partially dephosphorylated to lower forms (IP1-5) in the gastrointestinal tract by phytates from three different sources including the diet, the intestinal wall, and the bacterial flora of the gut contents (Williams & Taylor 1985). The absorption of IP6 in humans is not well studied, but a very recent study has shown 28% absorption of IP6 (Agte et al. 2005). However, in rodents it is well absorbed and is distributed to various organs as early as 1-h after administration since rodents have phytase enzyme in the gastrointestinal (GI) tract to hydrolyze IP6 (Vucenik & Shamsuddin 2003). The highest concentration of IP6 was found in the brain of rats fed an IP deficient diet, suggesting IP6 may cross the blood brain barrier. Although IP6 is a highly charged molecule, IP6 could be transported inside the cells by pinocytosis, endocytosis, or by interfering with the PLC-coupled receptor and tyrosine kinase receptors (Shamsuddin et al. 1997).

Phytic acid has many beneficial effects related to chelation of various metal ions and reducing oxidative stress. Phytic acid attenuated myocardial reperfusion injury and reduced the risk of kidney stone formation (Curhan et al. 2004; Rao et al. 1991). And striking anti-
cancer properties of IP6 have been demonstrated in both in vivo and in vitro studies. Phytic acid had antitumor activity in colonic cancer, mammary carcinoma, murine transplanted and metastatic fibrosarcoma, and chronic myeloid leukaemias (Deliliers et al. 2002; Shamsuddin & Vucenik 1999; Vucenik et al. 1993; Vucenik & Shamsuddin 2003; Vucenik et al. 1992). Although the exact mechanisms of IP6 antitumor activity are not known, it may exert its anticancer function by decreasing oxidative stress of transition metals or through the intracellular phosphate pool and signal transduction pathways (Graf & Eaton 1993; Shamsuddin & Vucenik 1999). Though extensive work related to the anticancer effect of IP6 has been conducted, the utility of IP6 in PD treatment has not been determined. To our knowledge, suppression of MPP⁺ induced hydroxyl radicals generation by chelating iron in rat striatum with IP6 was reported recently in one study (Obata 2003). In that study, MPP⁺ (75 nmol/L) and iron (10 μmol/L) were infused into the rats’ striatum and 100 μmol/L of IP6 significantly suppressed MPP⁺ and iron induced hydroxyl radical formation trapped as 2,3-dihydroxybenzoic acid (DHBA).

In conclusion, current medical treatments of PD only provide symptomatic relief of motor and non-motor symptoms. No drug has been identified to slow or reverse the progression of the disease. Thus, development of alternate therapeutic strategies that can prevent the progression of the disease have become a primary goal of PD research. Nutrients with natural, antioxidant, brain-permeable, and non-toxic properties provide a promising approach for the treatment of PD. For example, tea flavonoids with potent iron chelating, radical scavenging, and anti-inflammatory activities are neuroprotective in both in vivo and in vitro PD models (Guo et al. 2005; Levites et al. 2001; Mandel et al. 2006). However, the studies investigating nutritional treatments for PD are limited. Phytic acid is a natural
antioxidant with iron chelating as well as antioxidant ability. Based on its antioxidant property and iron chelating ability, the objective of this study was to determine the neuroprotective effect of phytic acid in the cell culture model of PD.

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CHAPTER 2. NEUROPROTECTIVE EFFECT OF THE NATURAL IRON CHELATOR PHYTIC ACID IN A CELL CULTURE MODEL OF PARKINSON’S DISEASE

Manuscript to be submitted to The Journal of Nutrition

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Abstract

Disrupted iron metabolism and excess iron accumulation has been reported in the brains of Parkinson’s disease (PD) patients. Because excessive iron can induce oxidative stress subsequently causing degradation of nigral dopaminergic neurons in PD, we determined the protective effect of a naturally occurring iron chelator, phytic acid (IP6), on 1-methyl-4-phenylpyridinium (MPP+) induced cell death in immortalized rat mesencephalic/dopaminergic cells (N27 cells). Cell death was induced with MPP+ in normal and iron-excess conditions and cytotoxicity was measured by thiazolyl blue tetrazolium bromide (MTT assay) and trypan blue staining. Apoptotic cell death was also measured with caspase-3 activity, DNA fragmentation, and Hoechst nuclear staining. IP6 (30 µmol/L) increased cell viability by 19% (p<0.05) and decreased cell death by 22% (p<0.05), compared to MPP+ treatment. A 3-fold increase in caspase-3 activity (P<0.001) and a 2-fold increase in DNA fragmentation (P<0.05) with MPP+ treatment was decreased by 55% (P<0.01) and 52% (P<0.05), respectively with IP6. IP6 (30 and 100 µmol/L) increased cell survival by 18% (p<0.05) and 42% (p<0.001), respectively in iron-excess conditions. Based on caspase-3 activity, a 40% and 52% (P<0.001) protection with 30 µmol/L and 100 µmol/L
IP6, respectively was observed in iron excess. Similarly, a 45% reduction (P<0.001) in DNA fragmentation was found. In addition, Hoechst nuclear staining results confirmed the protective effect of IP6 against apoptosis. Collectively, our results demonstrate a significant neuroprotective effect of phytate in a cell culture model of PD.

**Key Words:** Parkinson’s disease, IP6, Iron, MPP⁺

**Introduction**

Parkinson’s disease is characterized by a selective degeneration of dopaminergic neurons in the substantia nigra, resulting in irreversible motor dysfunction. The cardinal symptoms of PD include resting tremor, bradykinesia, and rigidity. This debilitating neurodegenerative disorder affects more than 1% of the US population over 50 years of age, and causes an estimated economic obligation of $25 billion annually (1). Recent research has been focused on understanding the pathogenesis as well as neuroprotective therapy of the disease. There have been many dopamine replacement therapies developed to minimize the PD associated motor deficits. These therapies, however, are limited to symptomatic relief. Other limitations of these approaches include drug tolerance, drug-induced involuntary movements, and most importantly, dopamine therapy is ineffective in attenuating the progression of the illness (2). Therefore, alternate therapeutic agents that effectively prevent the progression of neurodegenerative processes are needed.

Total body iron concentration generally increases with age, as does the concentration in the brain. The substantia nigra has 20 ng Fe/mg tissue during the first year of life and increases to 200 ng/mg by the fourth decade of life (3). Since excessive iron accumulation in substantia nigra was found in postmortem brains from Parkinson's patients, the role of iron in
PD has recently gained attention (4, 5). Although the relevance of brain iron pathways to PD has been considered, a cause or effect relationship has not yet been determined (5). Brain tissue is rich in polyunsaturated fatty acids, and hence may be more susceptible to free radical mediated lipid peroxidation (6). Iron metabolism is disrupted in PD; iron accumulates in lewy bodies, and distribution of iron transport and storage proteins is altered (7, 8). A rise in iron levels without concomitant change in ferritin provides free iron which can be involved in radical generation (5, 9). It is well known that iron is involved in the Fenton to produce hydroxide (•OH), the most reactive oxygen species (ROS) that can damage tissues.

Oxidative stress induced cell damage, including apoptosis, may play a prominent role in neurodegeneration associated with PD (10-13). Research on the use of antioxidants, free radical scavengers, and inducers of cellular antioxidant systems as therapeutic adjuncts in the treatment of PD continues to provide hope that more effective therapies that not only treat symptoms, but also slow the process of neurodegeneration will be developed. Coenzyme Q10, a well known electron acceptor for complex I and II as well as an important antioxidant with regard to its function in the reduced form (ubiquinol), could attenuate the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) induced loss of striatal dopaminergic neurons and has a protective effect on PD (14-17). In addition, iron chelation via either transgenic expression of the iron binding protein ferritin or oral administration of the metal chelator clioquinol (CQ) reduced susceptibility to MPTP induced PD in animals, suggesting that iron chelation may also be an effective therapy for prevention and treatment of the disease (18). Based on this evidence, antioxidants or iron chelators are thought to have great potential as therapeutic agents for the early stages of PD.

Phytic acid (IP6, myo-inositol hexakisphosphate) is generally considered as an
antinutrient because of its ability to chelate divalent minerals and reduce their absorption (19). It is also considered as an antioxidant due to the same metal chelating property. Phytic acid was shown to inhibit •OH formation and decrease lipid peroxidation catalyzed by iron and ascorbate in human erythrocytes (20). This suppression of iron-catalyzed oxidative reactions was suggested to be due to IP6’s ability to form a unique chelate with Fe(III) occupying all of the coordinating sites (21). Recently, we also showed in a human study that feeding IP6 containing soy protein for 6-weeks to postmenopausal women reduced body iron stores significantly compared with a low-IP6 soy protein (22). Cell signaling pathways (23) or antioxidant enzymes that detoxify the ROS may also be effected by IP6. Protective effect of IP6 in cancer was studied extensively (23-25), however, studies are limited in PD treatment (26). Based on the positive effect of iron chelators and antioxidants in PD, as well as the antioxidant property and iron chelating ability of IP6, our objective was to determine the protective effect of IP6 against 1-methyl-4-phenylpyridinium (MPP+) induced neurodegeneration in a cell culture model.

**Materials and methods**

**Chemicals**

Phytic acid (sodium salt), MPP+, ferrous sulfate, nitrilotriacetic acid (NTA), and thiazolyl blue tetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO). Substrate for caspase-3, Acetyl-Asp-Glu-Val-Asp-AFC (Ac-DEVD-AFC), was obtained from MP Biomedicals (Solon, OH). Cell Death Detection (enzyme linked immunosorbert assay) Plus kit and Hoechst 33342 were purchased from Roche Diagnostics (Indianapolis, IN) and Molecular Probes (Eugene, OR), respectively. Trypan blue stain,
RPMI-1640 medium, fetal bovine serum, L-glutamine, penicillin and streptomycin were obtained from Invitrogen (Carlsbad, CA). All the solutions were prepared immediately before use.

**Cell culture**

The immortalized rat mesencephalic dopaminergic neuronal cell line (1RB3AN27, generally referred as N27) was obtained as a kind of gift from Dr. Kedar N. Prasad, University of Colorado Health Sciences Center (Denver, CO). Cells were grown in RPMI-1640 medium containing 10% fetal bovine serum, 2 mmol/ L L-glutamine, 50 units penicillin and 50 µg/mL streptomycin and maintained at 37°C in a humidified atmosphere containing 5% CO₂ as described in previous studies (13, 27). Cells grown for 24-h were used for the following experiments.

**Treatment**

Initially the cells were incubated with various concentrations of IP6 (30, 100, 300, 600, and 1000 µmol/L) or with iron (Fe: NTA 1:2 molar ratio; 20, 50, and 100 µmol/L) for 24-h to determine the cytotoxicity of IP6 and iron separately. For determining the protective effect of IP6 in normal iron conditions, cells were pre-treated with 30 µmol/L IP6 for 24-h, followed by a 300 µmol/L MPP⁺ treatment, a neurotoxin that produces ROS, prior to measuring apoptotic cell death. Iron-excess condition in the cells was induced by treating the cells with 50 µmol/L iron for 24-h prior to IP6 (30 or 100 µmol/L) and MPP⁺ treatment for another 24-h.

**Cell viability**

Cell viability was measured using MTT assay as described earlier (27). After each treatment, cells were washed once with PBS buffer and further incubated with serum free
RPNI medium containing 0.25 mg/mL MTT solution for 3-h at 37°C. Isopropanol-HCL (200 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). Cell viability was also determined using trypan blue stain. After each treatment, cells were harvested using trypsin-EDTA and washed once with PBS. Trypan blue stain (0.4%) was added to count the dead cells and the percentage of cell death was calculated based on the fraction of dead cells with respect to the total cell count.

**Caspase-3 activity**

Since caspase-3 plays a vital role in the regulation and execution of apoptotic cell death, we measured its activity as described previously (28). After each treatment, the cell pellet after centrifugation was lysed with Tris buffer (50 mol/L Tris-HCL, 1 mmol/L EDTA, and 10 mmol/L EGTA at pH 7.4) containing 10 µmol/L digitonin for 20-min at 37°C. Lysates were subjected to a quick centrifugation at 20,000 x g and cell-free supernatant was collected. After incubating with a specific fluorogenic caspase-3 substrate (Ac-DEVD-AFC, 50 µmol/L) for 1-h at 37°C, the caspase-3 activity was measured by microplate reader (Model: Gemini XS, Molecular Devices) with excitation at 380 nm and emission at 469 nm. The activity is expressed as fluorescent units (FU)/mg protein.

**DNA fragmentation**

Chromatin condensation and DNA fragmentation are hallmarks of apoptosis (27). DNA fragmentation assays were performed using Cell Death Detection ELISA Plus kit. After each treatment, the cell pellet obtained from centrifugation was incubated with 40 µl of lysis buffer for 20-min. The lysates were then centrifuged and 80 µl immunoreagent containing anti-histone-biotin and anti-DNA-POD were added to 20 µl supernatant in streptavidin-
coated 96 well microtiter plates. The plates were incubated at room temperature for 2-h and then washed twice with incubation buffer. After adding 100 µl of 2,2’-azino-di- [3-ethylbenzthiazoline sulfonate] (ABTS) solution to each well, the absorbance was measured at 490 nm and 405 nm using a microplate reader. The DNA fragmentation was measured by the difference in absorbance at 405 and 490 nm and expressed as absorbance units (AU)/mg protein.

**Hoechst nuclear staining**

A fluorescent DNA-binding dye, Hoechst 33342, was used to measure nuclear morphology and apoptosis. Cells were grown on poly l-lysine-coated cover slip for 24-h and then subjected to treatments followed by fixing with 4% paraformaldehyde. The nuclei were stained with Hoechst 33342 (10 µg/ml) dye for 5-min in the dark. Cells were examined under fluorescence microscope (Nikon, Tokyo, Japan) to perform image analysis. Nuclei of apoptotic cells exhibited strong staining in a heterogeneous patchy manner due to chromatin condensation, while nuclei of the nonapoptotic cells were stained in a diffused, homogenous manner when observed under microscope (13).

**Statistics**

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. The differences among the treatments were compared with ANOVA with Tukey's Multiple Comparison test and considered significant at $P \leq 0.05$.

**Results**

*Cytotoxic effect of iron and IP6 alone*
To determine the optimal dose of IP6 and iron for later experiments, the dose response cytotoxic effect of IP6 and iron was assessed separately using the MTT assay (Table 2.1). No significant cytotoxicity was found with 30, 100, 300, and 600 µmol/L IP6 treatments, with only 2-5% reductions in cell viability compared to control. However, 1 mmol/L of IP6 decreased cell viability significantly (P<0.05) by 13% compared to the control. With iron treatment alone at 20, 50, 100 µmol/L, a small but significant cytotoxic effect was observed with a 92% (P<0.01), 89% (P<0.001), and 91% (P<0.01) reduction, respectively, compared to the control. Based on these results, 30 and 100 µmol/L IP6 and 50 µmol/L iron were chosen for the experiments to determine the neuroprotective effect of IP6.

**Protection of IP6 against MPP⁺ induced cell death**

Protection of IP6 against MPP⁺ induced cell death was determined using the MTT assay and trypan blue staining. Cell viability was decreased to 53% (P<0.001) after treating with 300 µmol/L MPP⁺, but 30 µmol/L IP6 pretreatment partially protected against this toxicity by 19% (P<0.05) as measured with MTT assay (Fig. 2.1A). Similarly, cell death was 3.4-fold (P<0.001) higher with MPP⁺ and 2.6-fold (P<0.001) higher with MPP⁺ plus IP6 as compared to the control, showing a 22% protection (P<0.05) with IP6 (Fig. 2.1B).

**Protection of IP6 against MPP⁺ induced apoptosis**

The protective effect of IP6 against MPP⁺ induced apoptosis measured as caspase-3 activity (A) and DNA fragmentation (B) and Hoechst nuclear staining (C) is shown in Fig. 2.2. Caspase-3 activity was not affected when cells were exposed to 30 µmol/L IP6 for 48-h. However, MPP⁺ treatment increased caspase-3 activity by 3.3-fold (P<0.001), but it was reduced significantly by 55% (P<0.01) with IP6. Similarly, DNA fragmentation was 2-fold (P<0.05) higher with MPP⁺ treatment; however, IP6 counteracted the effect (P<0.05). The
Hoechst nuclear staining clearly shows apoptosis following MPP⁺ treatment, and supports the data on protective effect of IP6 against caspase-3 and DNA fragmentation.

**Protection of IP6 against MPP⁺ induced cell death in iron-excess condition**

Protection of IP6 against MPP⁺ induced cell death was further evaluated in the iron-excess condition (Fig. 2.3). Cell viability was decreased to 87% (P<0.001) and 53% (P<0.001) with 50 µmol/L iron and 300 µmol/L MPP⁺ treatments, respectively. Cell viability was further decreased to 37% (P<0.001) with the combined MPP⁺ and iron treatments. Cytotoxicity with iron and MPP⁺ co-treatment was significantly higher than with iron alone (57%, P<0.001) or MPP⁺ alone (30%, P<0.001). An 18% protection (P<0.05) with 30 µmol/L IP6 was observed for the iron +MPP⁺ treatment. However, a higher protection (42%) was found with 100 µmol/L IP6 (P<0.001).

**Protection of IP6 against MPP⁺ induced apoptosis in iron-excess condition**

Caspase-3 activity, DNA fragmentation and Hoechst nuclear staining in the iron-excess condition and the protective effect of 30 and 100 µmol/L IP6 was shown in Fig. 2.4. Caspase-3 activity (A) was not affected when cells were treated with 50 µmol/L iron or 100 µmol/L IP6 alone or iron plus IP6 for 48-h. A 6.2-fold (P<0.001) increase was found with MPP⁺ and further increased to 8.4-fold (P<0.001) by adding iron. A 40% (P<0.001) and a 52% (P<0.001) reduction of MPP⁺-induced caspase-3 activity was found with 30 and 100 µmol/L IP6 in the presence of excess iron. For DNA fragmentation (B) and Hoechst nuclear staining (C) experiments, only 100 µmol/L IP6 was tested. DNA fragmentation was increased to 2.1-fold (P<0.001) after MPP⁺ plus iron treatment, but IP6 treatment showed a complete protection. Again, Hoechst nuclear staining supports the protection of IP6 against MPP⁺-induced apoptosis even in the iron-excess condition.
Discussion

Parkinson’s disease is a progressive neurodegenerative disorder which is characterized by selective loss of dopaminergic neurons. Several animal studies on PD were performed by inducing neurotoxicity with 6-hydroxydopamine (6-OHDA), rotenone, lactacystin, and MPTP (29, 30). MPTP-induced neurotoxicity is the best available approach for testing neuroprotective and neurorestorative strategies for PD (29). Since MPTP is converted into its active metabolite, MPP⁺, by monoamine oxidase type B in the brain and accumulates in the dopaminergic neurons, we used MPP⁺ to induce oxidative stress and apoptosis in our cell culture study (13, 31).

Oxidative stress causing damage to proteins, lipids and DNA, plays an important role in PD. Protein carbonyls were found to be two-fold higher in the substantia nigra pars compacta compared to other regions in normal postmortem brains. Further, higher protein carbonyl concentrations were found in PD patients compared to age matched controls (32, 33). Increased levels of lipid hydroperoxides and DNA damage, such as 8-hydroxyguanine, were also found in the substantia nigra in PD patients (34, 35). Based on the relationship with oxidative stress and PD, studies have looked at the effect of antioxidants on reducing the progression of PD. Vitamin C and vitamin E were found to protect against intrastriatal 6-OHDA injection induced striatal damage in rats (36, 37). Although the efficacy of vitamin E in the prevention or treatment of PD is highly disputed, high dose dietary supplementation or parenteral administration of vitamin E may be a useful therapeutic strategy for the prevention or treatment of PD (38).

Iron is believed to be an important contributor in PD pathology, possibly by increasing oxidative stress. Free iron can be involved in hydroxyl radical formation and causes oxidative
damage to the cells through the Fenton reaction. The substantia nigra has the highest concentration of iron in the mammalian brain. Iron bound to proteins is not involved in free radical generation, but free iron can be cytotoxic. However, both MPTP and 6-OHDA were shown to release ferritin bound iron in both in vitro and in vivo conditions (39). MPTP disrupts iron metabolism by increasing transferrin receptor 2 which may cause increased iron uptake by the brain (40), or possibly altering iron regulatory protein regulation (41). Since evidence for an association between iron and neurotoxin mediated neurodegeneration is strong, iron chelation has been suggested to be an effective therapy for prevention or treatment of PD. The iron chelator, desferrioxamine (DFO), was reported to protect against 6-OHDA and lactacystin-induced degeneration in dopaminergic neurons (30, 42). In addition, another iron chelator, CQ, was also shown to protect against MPTP induced neurodegeneration (18).

However, the currently used iron chelators may cause some side effects in patients. For example, DFO is the most potent iron chelator, but it must be administered at a high dosage, to compensate for its low ability to cross the blood brain barrier, and consequently may cause some toxic effects (30). Hence, our study aimed to determine the protective effect of the natural iron chelator, IP6, which is a natural food component.

Phytic acid is a strong antioxidant that inhibits iron-driven hydroxyl radicals and decreases lipid peroxidation (43). Although IP6 is generally considered as an anti-nutrient due to its negative effect on mineral bioavailability (44), a number of studies have shown beneficial effects, such as anticancer effects and lowering blood lipids (45-49). Lack of toxicity following long term administration in animal models makes IP6 appealing for use against iron associated pathogenesis (49). The absorption of IP6 in humans is not well
studied, but a very recent study demonstrated a 28% absorption of IP6 (50). However, IP6 is well absorbed in rodents and was distributed to various organs as early as 1-h after administration (25). When rats were fed an IP sufficient diet, the highest concentration of IP6 was found in the brain suggesting the ability of IP6 to cross the blood brain barrier.

Many previous studies with IP6 mainly focused on anticancer effects, but data on its effect on neurodegenerative diseases are limited. To our knowledge, only one study reported the suppression of MPP\(^+\) induced hydroxyl radical generation in rat striatum with IP6 administration (26). In that study, MPP\(^+\) (total dose 75 nmol) with or without iron (10 µmol/L) was infused directly into the rats’ striatum to induce hydroxyl radicals, measured as 2,3-dihydroxybenzoic acid (DHBA). Similar to the rat study, we showed increased caspase-3 activity, DNA fragmentation, and decreased cell viability with 300 µmol/L MPP\(^+\) treatment in our study. On the contrary, 30 µmol/L IP6 pretreatment partially protected against MPP\(^+\) induced neurotoxicity in our study compared to no significant decrease of the levels of 2,3-DHBA products with 100 µmol/L (26). The difference may be explained by the use of different models to study neurotoxicity.

When the animal study was performed in the iron-excess condition (26), 2,3-DHBA products were significantly increased in iron plus MPP\(^+\) compared with the MPP\(^+\) alone treated animals but a significant suppression was found with IP6. Similarly, we found in our study that cytotoxicity resulting from iron and MPP\(^+\) co-treatment was significantly higher than with MPP\(^+\) alone. Although both 30 µmol/L and 100 µmol/L IP6 showed a significant protection, the latter dose was more effective. Our results suggest that a higher dose of IP6 is needed to protect N27 cells against MPP\(^+\) induced cell apoptosis in the iron-excess condition. The higher dose is still within the physiological range, since no significant IP6 cytotoxicity
below 600 µmol/L was found (Table 2.1).

Several studies with animal models have suggested iron chelators may be potential therapeutic agents to slow the progression of neurodegeneration. In a mice study, the iron chelator coenzyme Q10 caused a significant attenuation of the loss of striatal dopamine and dopaminergic axons caused by MPTP (51). The iron chelator, DFO, also significantly inhibited iron accumulation in the brain by inhibiting the generation of reactive hydroxyl radicals and lipid peroxidation induced by excess iron and MPTP, thus resulting in a significant reversion of the reduction of striatal dopamine levels (52). Since IP6 is also an iron chelator, it may be as effective as other chelators discussed above based on our cell culture study results. However, future in vivo studies are needed to confirm its protective effect.

In summary, in our cell culture study, IP6 protected dopaminergic neurons against MPP⁺ induced apoptosis, even in the iron-excess condition. Our findings suggest that IP6 may offer protective therapy, along with traditional drug therapy, to slow the progression and limit the extent of neuronal cell death in PD. Future animal and human studies would be useful to confirm our results.

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**TABLE 2.1** Dose response effect of IP6 and iron alone on cell viability

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<th>Cell Viability (%)</th>
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*Cell viability was measured using MTT assay as described in Materials and Methods section. The values (Mean ± SEM, n=4-6) represent percentage of the control (0 concentration of respective treatments). ANOVA with Tukey’s Multiple Comparison test was used to detect the differences among the treatments. *P<0.05; **P<0.01; ***P<0.001.*
**Figure 2.1** Protective effect of IP6 against MPP⁺ induced cell death. Cell death was measured with MTT (A; n=12-13) and trypan blue (B; n=5-6). The values (Mean ± SEM) represent percentage of the respective controls (no treatment). ANOVA with Tukey's Multiple Comparison test was used to detect the differences among the treatments. Means with letters indicate comparison with control treatment. Symbols represent the comparison between treatments. *P<0.05; †P<0.001.
Figure 2.2 Protective effect of IP6 against MPP+ induced apoptosis. Cell apoptosis was measured with caspase-3 activity (A; n=4-8) and DNA fragmentation (B; n=3). The values (Mean ± SEM) represent percentage of the respective controls (no treatment). ANOVA with Tukey's Multiple Comparison test was used to detect the differences among the treatments. Means with letters indicate comparison with control treatment. Symbols represent the comparison between treatments. *P<0.05; **P<0.01; bP<0.05; cP<0.001. Hoechst Nuclear Staining (C) was used to identify the live cells. The apoptotic cell nuclei were shown in the 20X and 60X magnification with MPP+ treatment.
Figure 2.3 Protective effect of IP6 at 30 µmol/L and 100 µmol/L concentrations against MPP⁺ induced cell death in the iron-excess condition. Cell viability (n=3-4) was measured with MTT assay. IP6 (30); 30 µmol/L IP6 and IP6 (100); 100 µmol/L IP6. The values (Mean ± SEM) represent percentage of the respective controls (no treatment). ANOVA with Tukey's Multiple Comparison test was used to detect the differences among the treatments. Means with letters indicate comparison with control treatment. Symbols represent the comparison between treatments. *P<0.05; **P<0.001; bP<0.001.
Figure 2.4 Protective effect of IP6 against MPP⁺ induced apoptosis in the iron-excess condition. Cell apoptosis was measured with caspase-3 activity (A; n=4-10) and DNA fragmentation (B; n=4). IP6 (30); 30 µmol/L IP6 and IP6 (100); 100 µmol/L IP6. The values (Mean ± SEM) represent percentage of the respective controls (no treatment). ANOVA with Tukey's Multiple Comparison test was used to detect the differences among the treatments. Means with letters indicate comparison with control treatment. Symbols represent the comparison between treatments. *P<0.05; **P<0.001; ***P<0.001. Hoechst Nuclear Staining (C) was used to identify the live cells. The apoptotic cell nuclei were shown in the 20X and 60X magnification with iron plus MPP⁺ treatment.
CHAPTER 3. PHYTIC ACID PROTECTS AGAINST 6-HYDROXYDOPAMINE AND IRON INDUCED APOPTOSIS IN A CELL CULTURE MODEL OF PARKINSON'S DISEASE

Manuscript to be submitted to Neuroscience letters
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Abstract

Iron is thought to play an important role in Parkinson’s disease (PD) since it could induce oxidative stress-dependent neurodegeneration. In this study, we determined whether the iron chelator, phytic acid, protects against 6-hydroxydopamine (6-OHDA) induced apoptosis in immortalized rat mesencephalic/dopaminergic cells (N27 cells). Cells were grown in normal and iron-excess conditions and neurotoxicity was induced with 6-OHDA by 6-h treatment. Caspase-3 activity and DNA fragmentation were measured to determine the neurodegeneration. Caspase-3 activity was increased about 6-fold after 6-OHDA treatment (compared to control; p<0.001) and 30 µmol/L IP6 pretreatment decreased it by 38% (p<0.05). Similarly, a 63% protection (p<0.001) against 6-OHDA induced apoptosis was found with IP6 pretreatment as measured by DNA fragmentation. Even in the iron-excess condition, caspase-3 activity was increased about 6-fold (p<0.001) with 6-OHDA. However, a 41% (p<0.01) decrease was observed with 30 µmol/L IP6 treatments, but no further significant decrease was observed with 100 µmol/L IP6. Similarly, DNA fragmentation was increased by 42% with 6-OHDA and it was reduced by 27% (P<0.05) with IP6 treatment. Our data suggest that IP6 protects against 6-OHDA-induced cell apoptosis in both normal
and iron-excess conditions, and IP6 may prevent the progression of PD. However, future animal studies are needed to study the mechanism of protection in vivo.

**Key Words:** Parkinson’s disease, IP6, Iron, 6-OHDA

**Introduction**

Parkinson’s disease (PD) is a progressive neurodegenerative disease affecting more than 1% of the US population over 50 years of age and causing an approximate economic loss of $25 billion annually [39]. The clinical symptoms of PD, resting tremor, bradykinesia, rigidity, and postural instability, result from selective degeneration of dopamine neurons arising in the substantia nigra and terminating in the striatum. The loss of nigral neurons in the substantia nigra results in severe dopamine depletion in the striatum, and clinical signs of PD appear when striatal dopamine is reduced by 80% [6].

Oxidative stress has been implicated in the neurological degeneration associated with PD since oxidative stress can cause damage to the proteins, DNA, and lipids and consequently induce apoptosis [9, 10, 19, 22, 31]. Oxidative stress results from increased production of reactive free radicals such as reactive oxygen species (ROS). Brain tissue is highly susceptible to oxidative stress because of its high polyunsaturated fatty acids content. In addition, based on low antioxidant concentration in brain comparable to other tissues, brain may have a high oxidative stress environment. For example, the antioxidant enzymes superoxide dismutase and catalase concentrations were reported to be 7-and 140-fold lower in the brain than in the liver [11, 37]. The high oxidative stress in PD is supported by human postmortem studies, which show higher levels of protein carbonyls, lipid hydroperoxides, and DNA damage, such as 8-hydroxyguanine, in PD brains compared to normal brains [2, 17,
Furthermore, the deletion of glutathione and impairment of the antioxidant system, found in postmortem brain tissues of PD, clearly suggest the involvement of oxidative stress in PD [43].

A major role of iron in the pathogenesis of the disease has gained attention recently because of its involvement in oxidative stress [4, 5]. Iron is an essential nutrient for all living organisms since iron plays an important role in a series of biological processes such as electron transport and oxygen transport [33]. On the other hand, if not appropriately shielded, excess iron can be involved in Haber-Weiss and Fenton reactions to produce hydroxyl radicals, which are highly damaging to tissues. In the brain, excess iron can interact with neuromelanin (NM), a dark-colored pigment in the dopaminergic neurons, to increase oxidative stress and cause neurodegeneration [15]. Under normal conditions, NM decreases free radical damage by directly inactivating free radical species or binding transition metals such as iron [14, 16]. However, increased iron content may saturate iron chelating sites on NM and result in increased oxidative damage [14]. Total iron levels in the substantia nigra of PD patients are higher than age matched healthy controls [7]. Alterations in iron metabolism, such as altered distribution of iron transport and storage proteins, have also been reported in PD [49]. Ferritin, the major iron storage protein, is decreased in the substantia nigra of the PD brain compared to elderly controls [13]. A rise in iron levels concomitant with low levels of ferritin may provide “free iron” for ROS production [5, 8]. Although it is still not clear whether iron accumulation is a cause or an effect, the use of antioxidant iron chelators as therapeutic adjuncts in the treatment of PD provides hope that the process of neurodegeneration in PD patients can be slowed.

Phytic acid (IP6, myo-inositol hexakisphosphate) is a food component that is considered
as an antinutrient due to its ability to chelate divalent minerals and prevent their absorption [38]. Phytic acid is also recognized as a physiological antioxidant due to its unique chelating ability with iron and complete inhibition of iron-catalyzed hydroxyl radicals formation [46]. Phytic acid may also influence oxidative stress by altering cell signaling pathways or influencing the activity and expression of antioxidant enzymes [42]. Studies have demonstrated that IP6 could prevent a variety of malignancies including breast, colon, liver, leukemia, prostate, sarcomas, and skin cancer [18, 41, 42, 45]. However, its role in neurodegenerative disease is not well known. To our knowledge, IP6 was reported to protect against neurotoxin1-methyl-4-phenyl-1,2,3, 6-tetrahydropyridine (MPTP) induced hydroxyl radical generation by chelating iron in rat striatum in only one study [30]. Although our previous study (unpublished) showed the protection of IP6 against 1-methyl-4-phenylpyridinium (MPP⁺) induced apoptosis in a cell culture model of PD, the current study was designed to determine IP6’s neuroprotective effect on 6-hydroxydopamine (6-OHDA) induced PD in a cell culture model, since MPP⁺ and 6-OHDA induce neurotoxicity by different mechanisms.

**Materials and methods**

**Chemicals**

The immortalized rat mesencephalic dopaminergic neuronal cell line (1RB32AN27, generally referred to as N27) was a kind gift from Dr. Kedar N. Prasad, University of Colorado Health Sciences Center (Denver, CO). Ferrous sulfate, 6-OHDA, IP6, and nitrilotriacetic acid (NTA) were purchased from Sigma-Aldrich (St. Louis, MO). Substrate for caspase-3, Acetyl-Asp-Glu-Val-Asp-AFC (Ac-DEVD-AFC), was obtained from MP
Biomedicals (Solon, OH). Cell Death Detection (enzyme linked immunosorbent assay) Plus kit and Hoechst 33342 were purchased from Roche Diagnostics (Indianapolis, IN) and Molecular Probes (Eugene, OR), respectively. RPMI-1640 medium, fetal bovine serum, L-glutamine, penicillin, and streptomycin were obtained from Invitrogen (Carlsbad, CA). All the solutions were prepared fresh prior to assay.

**Cell culture**

Cells were grown in RPMI-1640 medium containing 10% fetal bovine serum, 2 mmol/L L-glutamine, 50 units penicillin, and 50 µg/ml streptomycin and maintained at 37°C in a humidified atmosphere containing 5% CO₂ as described in previous studies [24, 26]. Cells grown for 24-h were used for the following experiments.

**Treatment**

Based on the cytotoxic effects of phytic acid and iron in the previous study (unpublished), 30 and 100 µmol/L IP6 and 50 µmol/L iron were selected in the experiments. For determining the protective effect of IP6 in normal iron conditions, cells were pre-treated with 30 µmol/L of IP6 for 24-h, followed by a 100 µmol/L 6-OHDA treatment for 6-h. Iron-excess condition in the cells was induced by treating the cells with 50 µmol/L iron for 24-h prior to IP6 (30 or 100 µmol/L) followed by 6-OHDA treatment for another 6-h. Treatments without iron, IP6 and 6-OHDA served as controls for all the experiments.

**Caspase-3 activity**

Caspase-3 was measured as described previously [48]. After each treatment, the cell pellet after centrifugation was lysed with Tris buffer (50 mol/L Tris-HCL, 1 mmol/L EDTA, and 10 mmol/L EGTA at pH=7.4) containing 10 µmol/L digitonin for 20-min at 37°C. Lysates were subjected to a quick centrifugation at 20,000 x g and cell-free supernatant was
collected. After incubating with a specific fluorogenic caspase-3 substrate (Ac-DEVD-AFC, 50 µmol/L) for 1-h at 37°C, the caspase-3 activity was measured by microplate reader (Model: Gemini XS, Molecular Devices) with excitation at 380 nm and emission at 469 nm. The caspase-3 activity was expressed as fluorescent units (FU)/mg protein.

**DNA fragmentation**

DNA fragmentation assays were performed using Cell Death Detection ELISA Plus Kit as described previously [26]. After each treatment, cell pellet was incubated with 40 µl of lysis buffer for 20-min at room temperature. The lysates were then centrifuged and 80 µl immunoreagent containing anti-histone-biotin and anti-DNA-POD were added to 20 µl supernatant in streptavidin-coated 96 well microtiter plates. The plates were incubated at room temperature for 2-h and then washed twice with incubation buffer. After adding 100 ul of 2,2’-azino-di- [3-ethylbenzthiazoline sulfonate] (ABTS) solution to each well, the absorbance was measured at 490 nm and 405 nm using a microplate reader. DNA fragmentation was measured by the difference in absorbance at 405 and 490 nm and expressed as absorbance units (AU)/mg protein.

**Hoechst nuclear staining**

A fluorescent DNA-binding dye, Hoechst 33342, was used to measure apoptosis. Cells were grown on poly l-lysine-coated cover slips for 24-h and then subjected to treatments followed by fixing with 4% paraformaldehyde. The nuclei were stained with Hoechst 33342 (10 µg/ml) dye for 5-min in the dark. Cells were examined under fluorescence microscope (Nikon, Tokyo, Japan) for image analysis. Nuclei of apoptotic cells were identified as heterogeneous patchy inclusions due to chromatin condensation, while nuclei of the nonapoptotic cells were identified as diffused and homogenous manner after staining [24].
Statistics

Data were analyzed with Prism 4.0 software (Graph Software, San Diego, CA). Caspase-3 activity and DNA fragmentation were expressed as percentage of the respective controls. The differences among the treatments were compared with ANOVA with Tukey's Multiple Comparison test and considered significant at P≤0.05.

Results

Protection of IP6 against 6-OHDA induced apoptosis

The protective effect of IP6 against 6-OHDA induced apoptosis was measured with caspase-3 activity and DNA fragmentation, as shown in Fig. 3.1. Caspase-3 activity (A) was about 6-fold (P<0.001) higher with 6-OHDA treatment, but significantly (P<0.05) reduced by 38% with IP6. Similarly, DNA fragmentation (B) was about 3-fold (P<0.001) higher with 6-OHDA treatment compared to control, and IP6 pretreatment reduced it by 63% (P<0.001). The Hoechst nuclear staining (Fig. 3.2) clearly shows cell apoptosis with 6-OHDA and supports the caspase-3 and DNA fragmentation data on the protective effect of IP6.

Protection of IP6 against 6-OHDA induced apoptosis in iron-excess condition

Caspase-3 activity, DNA fragmentation, and Hoechst nuclear staining in excess-iron conditions and the protective effect of IP6 at both concentrations are shown in Fig. 3.3. Caspase-3 activity (A) was increased about 6-fold (P<0.001) with 6-OHDA and iron. A 41% (P<0.01) protection was found with 30 μmol/L IP6, and no additional protection was shown at the high concentration (32%, P<0.05). DNA fragmentation (B) was increased by 42% (P<0.05) after 6-OHDA and IP6 counteracted the effect (P<0.05). Again, Hoechst nuclear staining (Fig. 3.4) supports the protection of IP6 against 6-OHDA induced apoptosis in iron-
excess conditions.

Discussion

PD is a slow, progressive neurodegenerative disorder characterized by the degeneration of dopaminergic neurons in the substantia nigra. Oxidative stress has gained attention in the pathogenesis of nigral cell death in PD due to the high levels of free radicals generated during oxidative metabolism of dopamine [32]. The accumulation of iron, dysfunction of mitochondria, and decreased levels of the antioxidant nutrient glutathione found in PD clearly suggest the involvement of oxidative stress in PD [4, 5, 34, 35]. Generally, iron in the body is bound to proteins such as ferritin or transferrin. However, H ferritin and L ferritin are decreased in substantia nigra of PD patients [13]. Increased iron levels along with low levels of ferritin, result in free iron available for ROS generation. Iron may promote the oxidation of dopamine to release hydrogen peroxide and give rise to highly toxic hydroxyl radicals through the Fenton reaction [25]. Iron may also increase intracellular alpha-synuclein, the abnormal protein aggregation associated with the pathophysiology of PD [21, 44]. Alpha-synuclein is a major component of lewy bodies, the pathological hallmark of PD, and accumulation of alpha-synuclein may be central to the development of PD [32]. Based on the involvement of oxidative stress and iron in the pathogenesis of PD, identifying the compounds with antioxidant as well as iron chelating properties may represent a novel approach for slowing the progression of PD.

The cause of PD is not clear, and it is difficult to study the mechanism of the disease in humans. Therefore, neurotoxins induced experimental models are commonly used to investigate the pathogenesis and therapeutic strategies of PD. MPTP and 6-OHD are classic
neurotoxins used to study PD. MPTP, converted to its active metabolite MPP⁺ in the
dopaminergic neurons, damages dopaminergic neurons by inhibiting mitochondrial function
and generating free radicals [40]. 6-hydroxydopamine induces nigral degeneration in vitro as
well as in vivo via the generation of hydrogen peroxide and hydroxyl radicals in the presence
of iron [9]. Phytic acid is a natural antioxidant that can bind iron and reduces free iron
available for free radical generation [36]. Based on the interaction of 6-OHDA and iron, as
well as antioxidant and chelating ability of IP6, we designed our study to determine the effect
of IP6 on 6-OHDA and iron induced neurotoxicity.

Studies have demonstrated that 6-OHDA may induce apoptosis via caspase-3 dependent
activation [12, 20]. Exposure of 100 µmol/L 6-OHDA for 24-h increased caspase-3 activity
by 2.5-fold and DNA fragmentation by 93% in one cell culture study [23]. Similarly, we
showed a 6-fold increase of caspase-3 activity and 3-fold increase of DNA fragmentation
with the same dose but shorter exposure (6-h) of 6-OHDA. Toxicity induced by 6-OHDA
was similar in the iron-excess condition, and caspase-3 activity was increased by about 6-fold
and DNA fragmentation was increased by 42%. The protective effect of 6-OHDA induced
apoptosis in the iron-excess condition was also tested with a higher concentration of IP6 and
no extra protection was found for caspase-3 activity. We did not use a higher concentration
of IP6 in the DNA fragmentation experiment because 30 µmol/L IP6 completely
counteracted the increase of caspase-3 activity induced by 6-OHDA. In addition, the dose of
30 µmol/L is within the physiological range since we have shown that there was no
cytotoxicity induced by IP6 at under 600 µmol/L in our previous study (unpublished). In our
study, we also showed 6-OHDA induced apoptosis characterized by phase-bright nuclear
fragmentation by Hoechst nuclear stain. However, cells co-treated with IP6 and 6-OHDA
showed a round intact nucleus in a diffused, homogenous manner.

In addition to inducing apoptosis associated with hydrogen peroxide derived hydroxyl radicals in the presence of iron, 6-OHDA may also release iron from ferritin and promote ferritin-dependent lipid peroxidation [28, 29]. Therefore, iron chelators may be particularly effective in attenuating 6-OHDA induced neurotoxicity. Indeed, the iron chelators desferrioxamine and catechin, the major polyphenol in green tea, were used to study the protective effect against 6-OHDA induced toxicity in both in vivo and in vitro studies [3, 29]. Intracerebroventricular injection of 250 mg 6-OHDA caused an 88% reduction in striatal dopamine and a 2.5-fold increase in dopamine release. However, prior injection of the iron chelator desferrioxamine resulted in a 60% protection against 6-OHDA induced striatal dopamine content reduction and a normalization of dopamine release [3]. In another cell culture study, catechin at the concentration of 3.4 and 34 μmol/L significantly protected against 6-OHDA (200 μmol/L) induced oxidative damage and cell death [29]. Our results suggest that IP6 might offer similar protection against 6-OHDA induced neurotoxicity as desferrioxamine and catechin. However, desferrioxamine may cause some side effects in patients since it must be administered at a high dosage to overcome its low ability to cross the blood brain barrier [49]. Phytic acid is a natural iron chelator and is non-toxic over the long term administration [47]. Furthermore, the dose used in our study is in within the normal physiological range.

In conclusion, IP6 prevented against 6-OHDA induced apoptosis in our cell culture model in both normal and iron-excess conditions. However, the metabolism of IP6 in humans is controversial. Phytic acid is degraded by gut phytase, an enzyme that catalyzes the stepwise hydrolysis of phytate in the animals [27]. However, IP6 may not be hydrolyzed in
humans since humans lack phytase in the gastrointestinal tract, and a recent study showed a 28% absorption of IP6 in humans [1]. Since the dose of IP6 required to reach the target sites in the brain and enter the dopaminergic neurons remains to be established, future study is warranted to determine the effect of IP6 in an animal model of PD.

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Figure 3.1 Protection of IP6 against 6-OHDA induced apoptosis. Cell apoptosis was measured as caspase-3 activity (FU/mg protein, A; n=10) and DNA fragmentation (AU/mg protein, B; n=3). ANOVA with Tukey's Multiple Comparison test was used to detect the differences among the treatments. Means with letters were compared with the control treatment. Symbols represent the comparison between treatments. \(^{a}P<0.01; \(^{b}P<0.001; \(^{c}P<0.05; \(^{**}P<0.001.\)
Figure 3.2 Protection of IP6 against 6-OHDA induced apoptosis measured as apoptotic cell nuclei stained by Hoechst nuclear staining. Stained nuclei represent live cells.
**Figure 3.3** Protection of IP6 against 6-OHDA induced apoptosis in the iron-excess condition. Cell apoptosis was measured as caspase-3 activity (FU/mg protein, A; n=4-5) and DNA fragmentation (AU/mg protein, B; n=3-4). ANOVA with Tukey's Multiple Comparison test was used to detect the differences among the treatments. Means with letters were compared with the control treatment. Symbols represent the comparison between treatments. \(^{d}P<0.05; ^{c}P<0.01; ^{b}P<0.001; ^{*}P<0.05; ^{**}P<0.01.\)
**Figure 3.4** Protection of 30 µmol/L IP6 against 6-OHDA induced apoptosis in the iron-excess condition measured as apoptotic cell nuclei stained by Hoechst nuclear staining. Stained nuclei represent live cells.
CHAPTER 4. GENERAL CONCLUSION

General discussion

Parkinson’s disease (PD) is the progressive neurodegenerative disorder and characterized by the selective degeneration of dopaminergic neurons in the substantia nigra. The role of iron has gained attention in PD since disrupted iron metabolism and excess iron accumulation were found in the brains of PD patients (Berg et al. 2002; Lee et al. 2006). Excess non-protein bound iron can be involved in hydroxyl radical formation through Haber-Weiss and Fenton reaction, and cause oxidative damage to membranes, nucleotides, and proteins. In addition, iron may be involved in the pathogenesis of PD by promoting the oxidation of dopamine to produce hydroxyl radicals and increase oxidative stress in the dopaminergic neurons (Kaur & Andersen 2002).

1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and 6-hydroxydopamine (6-OHDA) are classic neurotoxins used to induce PD in experimental models. The active metabolite of MPTP, 1-methyl-4-phenylpyridinium (MPP+) and 6-OHDA are suggested to induce neurodegeneration by inhibiting mitochondrial function and generating free radicals. In addition, MPTP and 6-OHDA have been shown to release ferritin bound iron both in vitro and in vivo conditions (Grunblatt et al. 2000), which could further enhance oxidative stress, as well as increase the intracellular α-synuclein aggregation and trigger the formation of lewy bodies in PD (Fig. 4.1).
Figure 4.1 Schematic overview of protection of IP6 against MPP$^+$ and 6-OHDA induced cell apoptosis.

Iron chelators have been suggested to be effective therapies for prevention or treatment of PD based on the involvement of iron and oxidative stress in the pathogenesis of PD. The iron chelators desferrioxamine and clioquinol have been demonstrated to prevent MPTP induced neurodegeneration in the animal models of PD (Kaur et al. 2003; Lan & Jiang 1997). However, the currently used iron chelators may cause some side effects in patients. Thus, the nutrients with natural, non-toxic, antioxidant and iron chelating property may serve as novel therapeutic agents in the treatment of PD. Use of phytic acid (IP6), a natural food component, to prevent the progression of the disease is appealing because of its antioxidant and iron chelating property. In our study, we found IP6 protected against MPP$^+$ and 6-OHDA induced cytotoxicity and apoptosis both in normal and iron-excess conditions in the cell culture models of PD. Since MPTP and 6-OHDA were found to increase free iron levels both in vivo and in vitro, the proposed mechanisms of protection may be 1) prevent MPP$^+$ and 6-OHDA
induced neurotoxicity by chelating free iron and 2) protect against MPP\(^+\) and 6-OHDA induced cytotoxicity and apoptosis by altering cell signal transduction or key enzymes in the antioxidant system (Fig. 4.1).

In conclusion, our studies demonstrate a significant neuroprotective effect of IP6 against both MPP\(^+\) or 6-OHDA induced cytotoxicity and apoptosis both in normal and iron-excess conditions. Although future animal and human studies are needed to confirm the results, our findings suggest that IP6 may offer a promising safer therapeutic approach, maybe along with traditional drug therapy, to slow down the progression of PD.

References


APPENDIX. PHYTIC ACID DOES NOT HAVE A NEUROPROTECTIVE EFFECT IN AN ANIMAL MODEL OF PARKINSON'S DISEASE

Abstract

Disrupted iron metabolism leading to excessive iron levels can promote oxidative stress and subsequently induce neurodegeneration in Parkinson’s disease (PD). Phytic acid is a unique natural iron chelator which inhibits iron-catalyzed hydroxyl radicals formation. In the present study, we determined the effect of phytic acid on 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP) induced PD in normal or iron overloaded mice. MPTP significantly decreased striatal dopamine and its metabolites 3, 4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) levels in both normal and iron-excess conditions. Although pretreatment with phytic acid significantly inhibited striatal iron accumulation or whole brain iron content, it did not protect against MPTP induced neurodegeneration as expected. Our results suggest that phytic acid may not cross the blood brain barrier or it is hydrolyzed to lower inositol phosphate, which is a less effective antioxidant, before entering the dopaminergic neurons to prevent against MPTP induced neurodegeneration.

Introduction

Parkinson’s disease (PD) is the second most common neurodegenerative disorder after Alzheimer’s disease and is characterized by a selective degeneration of dopaminergic neurons in the substantia nigra, resulting in irreversible motor dysfunction such as resting tremor, bradykinesia, and rigidity (1,2). Prevention of PD is a challenge since current therapeutic strategies are limited to symptomatic relief. Alternate therapeutic and
neuroprotective agents that effectively prevent the progression of the neurodegenerative process are badly needed.

Oxidative stress may play a prominent role in neurological degeneration associated with PD since it can damage to proteins, DNA, and lipids and induce apoptotic death in dopaminergic neurons (3-6). Reduced levels of glutathione, decreased activity of antioxidant enzymes including peroxidase, catalase, and glutathione peroxidase, and increased lipid peroxidation and protein carbonyls clearly suggest the involvement of oxidative stress in PD (7,8). Free radical formation is linked to the excessive iron accumulation in the substantia nigra since a significant increase in iron was found in the substantia nigra of postmortem brains in PD patients (9,10). Excess iron can react with hydrogen peroxide through the Haber-Weiss and Fenton reactions to produce hydroxyl radicals, the most reactive oxygen species (ROS) that can damage tissues (11). Iron metabolism is disrupted in PD, such that iron in Lewy bodies accumulates and distribution of iron transport and storage proteins is altered. Increased iron levels without a concomitant increase in the iron storage protein ferritin allow for free iron that can be involved in oxidative stress (9,12,13).

The involvements of oxidative stress and iron in PD have encouraged the use of antioxidants as well as iron chelators as new therapeutic strategies in the prevention and treatment of PD. The most potent iron chelator, desferrioxamine (DFO), has been found to reverse the reduction of striatal dopamine induced by excess iron and 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP) (14). Iron chelation via either transgenic expression of the iron binding protein ferritin or oral administration of the metal chelator clioquinol (CQ) protected against MPTP induced neurodegeneration (15).

Phytic acid (IP6, myo-inositol hexakisphosphate) is present in high concentration in
cereals and legumes and has antioxidant capability by inhibiting iron-driven hydroxyl radicals and decreasing lipid peroxidation (16). This suppression of iron-catalyzed oxidative reactions was suggested to have resulted from the ability of IP6 to form a unique chelate with Fe(III) occupying all of the coordinating sites (17). A number of studies have shown the beneficial effect of IP6 on cancer and lowering blood lipids (18-22), but the effect of IP6 on neurodegenerative diseases is not yet well defined. To our knowledge, one study reported that IP6 could suppress 1-methyl-4-phenylpyridinium (MPP+) induced hydroxyl radical generation through direct administration to the rat’s striatum (23). Our previous study (unpublished) showed the neuroprotective effect of IP6 against MPP+ and 6-hydroxydopamine (6-OHDA) induced PD in a cell culture model. However, the objective of this study was to determine the effect of IP6 on neurotoxin MPTP induced neurodegeneration in a mouse model.

**Materials and methods**

**Chemicals**

Phytic acid, DFO, MPTP, sodium ethylenediaminetetraacetate (Na₂EDTA), bovine serum albumin (BSA), iron dextran, 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-4’,4”-disulfonic acid sodium salt (Ferrozine), and sodium trichloroacetate (TCA sodium salt) were purchased from Sigma-Aldrich (St. Louis, MO). Perchloric acid, sodium metabisulfite (Na₂S₂O₅), and paraformaldehyde were purchased from Fisher Scientific (Pittsburgh, PA). The mouse anti tyrosine hydroxylase was purchased from Chemicon (Temecula, CA). The cy3-conjugated goat anti-mouse antibody was obtained from Jackson ImmunoResearch (West Grove, PA). Standard diet 2018 plus 2.5% carbonyl iron was purchased from Harlan
Teklad (Indianapolis, IN). All the solutions were prepared immediately before use.

**Animal treatment**

Male C57 black mice (~25 g) were purchased from Harlan Teklad (Indianapolis, IN). The mice were housed individually in a temperature/humidity controlled room with a 12-h light/dark cycle. Food and water were provided ad libitum. The mice were sacrificed by decapitation after each treatment. All the procedures were approved by the Institutional Animal Care and Use Committee at Iowa State University.

**Experiment 1. Oral administration of IP6**

The mice were divided into four groups: control (n=2), IP6 (n=3), MPTP (n=5), and MPTP+IP6 (n=7). The mice in the IP6 and MPTP+IP6 group were given IP6 (800 mg/Kg, twice a day) by oral gavage for 35 days. Starting from day 31, the mice in the last two groups were given MPTP by intraperitoneal injection (i.p, 30 mg/Kg) for 5 days and the remaining mice were given equal volume of saline.

**Experiment 2. IP administration of IP6**

The mice were divided into four groups: control (n=8), MPTP (n=9), MPTP+IP6 (n=9) and MPTP+DFO (n=9). The mice in the last two groups were given IP6 (i.p, 40 mg/Kg) or DFO (i.p, 125 mg/Kg), respectively, for 10 days. Starting from day 8, all mice except in the control group were given MPTP (i.p, 20 mg/Kg) for 3 days.

**Experiment 3. Protection of IP6 with iron dextran induced iron overloading**

Mice were divided into three groups: control (n=2), MPTP (n=2) and MPTP+IP6 (n=2). The iron-excess condition was induced in all mice by one dose of iron dextran (i.p, 25 mg/Kg). After one week, the mice in the MPTP+IP6 group were given IP6 (i.p, 40 mg/Kg) for 10 days. Starting from day 8, the mice in the MPTP and MPTP+IP6 groups were given...
MPTP (i.p, 25 mg/Kg) for 3 days to induce neurodegeneration.

**Experiment 4. Protection of IP6 with dietary iron overloading**

Mice were divided into six groups: control (n=9), MPTP (n=9), iron (n=8), iron+MPTP (n=8), iron+MPTP+IP6 (n=9), and iron+MPTP+DFO (n=7). All mice except control and MPTP groups were fed with 2.5% carbonyl iron diet for 30 days. Starting from day 21, other mice in the last two groups were treated with IP6 (i.p, 40 mg/Kg) or DFO by intramuscular injection (i.m, 250 mg/Kg) for another 10 days while continuing with a high iron diet. On the 30th day, the mice in the MPTP, iron+MPTP, iron+MPTP+IP6, and iron+MPTP+DFO groups were given one dose of MPTP (i.p, 30 mg/Kg). Remaining animals were injected with an equal volume of saline.

**Experiment 5. Protection of IP6 with higher dose MPTP in dietary iron overloading condition**

We repeated the experiment 4 to see the effect of longer administration of MPTP by giving the mice three doses of MPTP. All the mice were fed an iron rich diet for one week. The animals were divided into three groups: control (n=8), MPTP (n=7), and MPTP+IP6 (n=8). From the second week, the high iron diet was changed to normal diet and the MPTP+IP6 group was given IP6 (i.p, 60 mg/Kg) for another 7 days. From day 12, the mice in the last two groups were injected with MPTP (i.p, 25 mg/Kg) for three days. Remaining animals were injected with an equal volume of saline. Neurochemical analysis, tyrosine hydroxylase immunohistochemistry analysis, whole brain nonheme iron, and striatal iron measurements were performed in this experiment and the procedures are described below.

**Neurochemical analysis**

Neurochemical analysis was performed in all the experiments. The striatum was dissected on an ice-cold glass platform immediately after the brain was removed. The tissues
were weighed and stored at −80°C until analyzed. On the day they were analyzed, the striatal tissues were sonicated in PBS buffer containing 0.2 mol/l perchloric acid, 0.5 mg/ml Na₂EDTA, and 1 µg/ml Na₂S₂O₅ and centrifuged at 13200 x g for 25-min. The supernatants were analyzed for dopamine (DA) and its metabolites DOPAC and HVA by reverse-phase high performance liquid chromatography (HPLC) coupled with electrochemical detection (EC) as described earlier (24). The HPLC system included a pressure module, a solvent delivery system (Rainin Instrument Co. Inc., Woburn, MA), and an automatic AS-48 sampler (Bio-Rad Laboratories, Hercules, CA). Reversed-phase column (A C-18, Rainin Instrument Co. Inc.) was used to separate neurotransmitters isocratically with the mobile phase (PH 3.1, 0.15 mol/L monochloroacetic acid, 0.13 mmol/ L sodium octyl sulfonate, 0.67 mmol/L sodium EDTA, 0.12 mol/l sodium hydroxide, and 1.5% acetonitrile) at a flow rate of 1ml/min. The DA, DOPAC and HVA levels were normalized by the wet tissue weight.

**Tyrosine hydroxylase (TH) immunohistochemistry analysis**

The immunostaining of the TH marker of dopaminergic neurons was performed as described in a previous publication (24). The brains were fixed in 4% paraformaldehyde solution for 24-h and then placed in a 30% sucrose solution overnight. Coronal sections (25 µm) were cut through the entire substantia nigra using a cryostat and were floated in 0.1 mmol/l PBS. Sections were permeabilized in PBS containing 0.2% Triton X-100, followed by blocking with PBS containing 0.2% Triton X-100 and 1% BSA for 1-h. The sections were incubated with a primary antibody, mouse anti tyrosine hydroxylase (1:500), at 4°C overnight, followed by incubation with a second antibody, cy3-conjugated goat anti-mouse (1:500), for 90-min and mounted. For quantitative evaluation, two to three sections were selected and TH-positive cells in the pars compacta of the substantia nigra were counted to reflect the total
TH positive neurons in the midbrain. Quantification was performed with sections at the caudorostal level of the third cranial nerve as described previously (25). The slides were visualized under fluorescent microscope (Nikon, Tokyo, Japan). The images were thresholded and the neuronal counts were measured using the integrated morphometry analysis software (Molecular Devices, Downingtown, PA).

**Brain iron measurement**

Whole brain nonheme iron content (n=4) was determined by the modified TCA protein precipitation method using ferrozine as a chromogen (26,27). Brain tissues were homogenized and the homogenates were incubated with 20% TCA/6N HCL (hydrochloric acid) at 65°C for 20-h. After centrifugation for 15-min, the supernatants were used to measure nonheme iron content with a microplate reader at an absorbance of 563 nm.

Total striatal iron content (n=4-6) was determined by inductively coupled plasma mass spectroscopy (ICP/MS) by the Iowa State University Soil and Plant Analysis Laboratory. The samples were wet ashed with nitric acid in a microwave prior to analysis.

**Statistics**

Data were analyzed with Prism 4.0 software (Graph Software, San Diego, CA). All measurements were normalized with the respective controls in each experiment. The differences among the treatments were compared with ANOVA with Tukey's Multiple Comparison test and considered significant at P<0.05.

**Results**

**Neurochemical analysis**

The effect of orally administered IP6 against MPTP induced neurodegeneration in
experiment 1 was determined by measuring striatal DA (Fig. 1A) and its metabolites DOPAC (Fig. 1B) and HVA (Fig. 1C). MPTP significantly decreased DA (P<0.001) and its metabolites DOPAC (P<0.05) and HVA, but pretreatment with IP6 had no protection on MPTP induced decrease of neurotransmitters.

Protection with IP6 or DFO administered by IP injection against MPTP induced decrease of neurotransmitters in experiment 2 was shown in Fig. 2. MPTP treatment caused a significant reduction in striatal DA (P<0.001) and HVA levels (P<0.01) but not DOPAC levels. Again, pretreatment with IP6 or DFO did not show a significant protection against MPTP induced decrease of neurotransmitter levels.

The protective effect of IP6 against MPTP induced neurodegeneration in the iron-excess condition induced by iron dextran (experiment 3) was shown in Fig. 3. Pretreatment with IP6 significantly reversed the decrease of striatal HVA (P<0.05) but had no effect on the DA and DOPAC decrease induced by MPTP in the iron-excess condition.

Since one dose of MPTP did not induce neurotoxicity (data was not shown), we repeated the experiment by giving three doses of MPTP and feeding the iron rich diet for a shorter time in experiment 5 (Fig. 4). MPTP treatment significantly reduced striatal DA (P<0.001) and its metabolites DOPAC (P<0.001) and HVA (P<0.05), but no protection was shown with IP6.

**Tyrosine hydroxylase positive neuron analysis**

The effect of IP6 against MPTP induced neurodegeneration in experiment 5 was determined by evaluating TH positive neurons (Fig. 5). Similar to the neurotransmitter analysis, MPTP treatment significantly (P<0.001) decreased TH immunostained neurons in the iron-excess condition and IP6 pretreatment did not show significant protection.
Brain iron analysis

Whole brain nonheme iron concentration (Fig. 6) did not significantly increase in MPTP treatment in the iron-excess condition (experiment 5). However, a 17% reduction was observed with IP6 pretreatment compared to MPTP treated mice.

Total striatal iron content (Fig. 7), was significantly (P<0.05) increased by 31% in iron+MPTP treated mice compared with iron alone treated mice in experiment 5. However, pretreatment with IP6 resulted in a 36% reduction.

Discussion

PD is a progressive neurodegenerative disorder characterized by selective loss of dopaminergic neurons. To date, traditional therapy has focused only on symptomatic relief and there is no cure for PD. Although several neurotoxins such as 6-OHDA, rotenone, lactacystin, and MPTP are used to induce PD in animal models (13,28), MPTP is commonly used for testing neuroprotective strategies of PD since it induces Parkinsonism that is identical to that seen in PD (28-30). MPTP is converted to its active metabolite MPP⁺, in dopaminergic neurons and impairs mitochondrial respiration and depletes neurotransmitters (6,15,31).

Based on the involvement of oxidative stress and iron in the pathogenesis of PD, use of an antioxidant as well as an iron chelator may be a reasonable approach for slowing the progression of PD. Desferrioxamine, a powerful iron chelator, was shown to significantly inhibit reactive hydroxyl radicals and lipid peroxidation induced by excess iron and MPTP, resulting in a significant reversion of the reduction of striatal dopamine levels (14). Green tea and its major polyphenol (±)-epigallocatechin-3-gallate (EGCG) protected against MPTP
induced neurodegeneration in animal studies, possibly due to their potent iron chelating and antioxidant properties (32,33). Phytic acid is also considered as an antioxidant and has iron chelating properties and can completely prevent iron-catalyzed hydroxyl radicals formation (34). A previously published study showed the protective effect of IP6 against MPP+ and iron induced hydroxyl radicals (23). A limitation of this study is that IP6 was directly infused to the rat’s striatum and other routes of administration were not tested. In our previous study, we found IP6 prevented neurotoxins MPP+ and 6-OHDA induced apoptosis in a cell culture model. In the current study, we tested the effect of oral or IP administered IP6 on MPTP induced neurodegeneration in normal and excess-iron conditions in a mouse model of PD.

In our first experimental trial, oral gavage of IP6 had no effect on MPTP induced neurodegeneration, possibly due to the degradation of IP6 by phytase in the mouse gut. Since endogenous phytase activity is greater in rodents than humans, IP6 was intraperitoneally injected to avoid the gastrointestinal phytase degradation in the second experiment. The iron chelator DFO was also used as a positive control in the study. However, neither IP6 nor DFO protected against MPTP induced neurodegeneration. Our results contradict the previous studies showing that IP6 suppressed MPP+ and iron induced hydroxyl radicals and DFO protected against MPTP induced neurodegeneration in iron overloaded mice (14, 23). Both previous studies were performed in an iron overloaded condition and our study was performed in a normal iron condition. Because we speculated that differential iron status accounted for the different results, we performed the subsequent studies in an iron overloaded condition.

When the mice were given one dose of iron dextran followed by IP6 treatment, no protection was found with IP6. Although iron dextran causes iron overload in various tissues
(35-37), data on brain iron content are limited. The lack of increased iron in the brain after iron dextran administration suggests that it may not cross the blood brain barrier (38). Thus, we also tried inducing iron overload by dietary iron in the following experiments. This experiment was based on the previous study demonstrating that treatment with DFO significantly protected against iron+MPTP induced neurodegeneration. In the published paper, one dose of MPTP significantly reduced DA and DOPAC levels. However, we found the same one dose of MPTP did not decrease dopamine or its metabolites in our study, and we do not know why. We repeated the experiment by increasing the doses of MPTP and feeding the mice for a shorter time with the iron rich diet. IP6 decreased the striatal iron or whole brain iron significantly in iron+MPTP treated mice, suggesting IP6 makes iron less available to cross the blood brain barrier. Surprisingly, instead of protection, IP6 further decreased striatal DA and HVA levels and did not show protection against loss of TH positive neurons induced by MPTP.

Since the ability of IP6 to enter the brain is not clear, the lack of protection found in our study may be due to its low ability to cross the blood brain barrier. It is also possible that IP6 is hydrolyzed to the lower inositol phosphates before entering the dopaminergic neurons, resulting in decreased iron chelating ability (39). A previous study found protection of IP6 against MPP7 and iron induced hydroxyl radicals, but IP 6 was directly infused into the rat’s striatum through a microdialysis probe to avoid hydrolysis of IP6 and allow entry across the blood brain barrier (23). Therefore, IP6 may be neuroprotective if it is administered directly into the brain but not following other routes of administration.

In summary, IP6 did not show protection against MPTP induced neurodegeneration in either normal or iron-excess conditions, although IP6 could significantly decrease the iron
content both in the whole brain and striatum. Based on the neuroprotective effect of IP6 in
the cell culture model and the previous study with direct administration into the brain, IP6
may not cross the blood brain barrier and may hydrolyze to lower inositol phosphates before
entering the target site to prevent MPTP induced neurodegeneration.

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Figure 1 Effect of oral administration of IP6 on MPTP induced decrease of DA (A) and its metabolites DOPAC (B) and HVA (C). The values (Mean ± SEM) represent percentage of the respective controls (no treatment). ANOVA with Tukey’s Multiple Comparison test was used to detect the differences among the treatments. The bars sharing the same superscript are not significantly (P<0.05) different. n=2-7.
Figure 2 Effect of IP administered IP6 on MPTP induced decrease of DA (A) and its metabolites DOPAC (B) and HVA (C). The values (Mean ± SEM) represent percentage of the respective controls (no treatment). ANOVA with Tukey's Multiple Comparison test was used to detect the differences among the treatments. The bars sharing the same superscript are not significantly (P<0.05) different. n=8-9.
Figure 3 Effect of IP6 on MPTP induced decrease of DA (A), DOPAC (B), and HVA (C) with iron dextran induced iron overloading. The values (Mean ± SEM) represent percentage of the respective controls (iron alone treatment). ANOVA with Tukey's Multiple Comparison test was used to detect the differences among the treatments. The bars sharing the same superscript are not significantly (P<0.05) different. n=2.
Figure 4 Effect of IP6 with a higher dose of MPTP induced decrease of DA (A), DOPAC (B), and HVA (C) with dietary iron overloading. *The values (Mean ± SEM) represent percentage of the respective controls (iron alone treatment). ANOVA with Tukey's Multiple Comparison test was used to detect the differences among the treatments. The bars sharing the same superscript are not significantly (P<0.05) different. n=7-8.
Figure 5 Effect of IP6 on MPTP induced loss of dopaminergic neurons. “#The values (Mean ± SEM) represent percentage of the respective controls (iron alone treatment). ANOVA with Tukey's Multiple Comparison test was used to detect the differences among the treatments. The bars sharing the same superscript are not significantly (P<0.05) different. n=4.
Figure 6 Whole brain nonheme iron concentration. The values (Mean ± SEM) represent percentage of the respective controls (iron alone treatment). ANOVA with Tukey’s Multiple Comparison test was used to detect the differences among the treatments. The bars sharing the same superscript are not significantly (P<0.05) different. n=4.
Figure 7 The striatal iron content. The values (Mean ± SEM) represent percentage of the respective controls (iron alone treatment). ANOVA with Tukey's Multiple Comparison test was used to detect the differences among the treatments. The bars sharing the same superscript are not significantly (P<0.05) different. n=4-6.
ACKNOWLEDGEMENTS

I would like to take this opportunity to express my sincere thanks to my major professors, Dr. Manju B. Reddy and Dr. Anumantha G. Kanthasamy for their education, support and patience throughout my Master’s degree study and the writing of this thesis. They taught me a lot on how to conduct research, write papers and present academic posters. The research projects I completed were attributed to their insights and innovative ideas. I would like also thank Dr. Chad H. Stahl for being my committee member, for his input, support and valuable suggestions to improve my research work. Thank you to Dr. Vellareddy Anantharam and Dr. Arthi Kanthasamy, for their thoughtful and professional opinions for my research. Also thank you to Danhui Zhang and Amy Proulx, for many patient hours of teaching me laboratory techniques.

I also want to thank my colleagues, Danhui Zhang, Faneng Sun, Huajun Jin, Chunjuan Song, Hariharan Saminathan, Richard Gordon, Jenny Lin, Qinglin Li, Ying Zhou, Arunkumar Asaithambi, Hilary Afeseh Ngwa, Dustin Martin, Amy Proulx, Rebecca Lukac to make my study here a memorable one.

I also want to thank my husband, Zhenhui Shen for his support and encouragement. Last, but not least, I would like to thank my parents Songbin Xu and Yezhen Jing for their great love and confidence in me.

I feel that I was provided with an exceptional opportunity to learn a lot of new things during my study, I would like to express my gratitude to all those who helped me along the way.