Introduction
INTRODUCTION

Parkinson’s disease (PD) is the slow and progressive neurodegenerative movement disorder (Lees et al., 2009). Pathologic and anatomical hallmarks of PD are selective loss of dopamine (DA) synthesizing neurons in the substantia nigra pars compacta (SNpc) and Lewy body inclusions (Di Monte, 2003). The cardinal clinical symptoms for PD include resting tremor, rigidity, bradykinesia and postural instability (Chesselet et al., 2008; Jankovic, 2008), which are also accomplished by wide range of nonmotor symptoms affecting the patient’s quality of life drastically (Langston, 2006; Lees et al., 2009). Although a century old disease, the precise mechanism by which SNpc cell death occurs is still obscure. Considerable evidence suggests a multifactorial etiology contributed by age, genetic and environmental factors out of which only about 10%-20% of PD cases are attributed to familial PD and remaining are classified as sporadic/idiopathic PD i.e. PD with unknown cause (Dick et al., 2007; Weisskopf et al., 2010). Epidemiological and experimental studies have established heavy metals exposure as one of the major environmental risk factors for PD (Gorell et al., 1997; 1998; Singh et al., 2007; Kumar et al., 2010; 2012). Increased accumulation of Zinc (Zn) and iron found in SNpc of PD patients in autopsy studies implicated their role in PD pathogenesis (Dexter et al., 1989; 1991).

Zn is second most abundant transition metal inside brain. It is redox inert and has structural, catalytic, and regulatory roles in cellular biology (Daniel and Dieck, 2004; Karlin et al., 1997). Involvement of Zn has been established in several pathological conditions like excitotoxicity, including ischemia, epilepsy, and brain trauma (Sensi et al., 2009; 2011). Zn mobilization from intracellular pools is also a crucial contributor to neuronal injury (Hwang et al., 2008; Corona et al., 2011). The neurotoxic potential of exogenous Zn in neurons has been observed both in vivo (Cuajungco & Lees, 1996; 1997; Lees et al., 1990) and in vitro (Koh et al., 1996). Moreover, intranigral infusion and systemic exposure of Zn in rats was shown to induce apoptosis of dopaminergic neurons (Lin et al., 2001; 2003; Kumar et al., 2010; 2012).

Human exposure to excess Zn is common in real life situations as ZnSO₄ is globally preferred in the agricultural field by farmers to improve the quality and quantity of food grains production over other Zn formulations. In addition, people involved in paint manufacturing, electrometallurgy and mine and smelting industries are also
exposed to excessive level of Zn and might be prone to Zn-induced toxicity. ZnSO₄ is also used as anti-corrosive for lining water pipelines and may contaminate drinking water supply. Due to wide range usage in agriculture, industry and pharmaceuticals Zn is much sought metal which is looked for neurotoxic consequences.

Oxidative stress, mitochondrial dysfunction and compromised defense system are the key events in idiopathic and chemically-induced PD (Cristovao et al., 2009; Garrido et al., 2011). Although, previous studies have suggested the role of oxidative stress in Zn-induced dopaminergic neurodegeneration in rats (Lin et al., 2001, 2003; Kumar et al., 2010) its entire molecular mechanism is still elusive. Therefore, our first aim was to investigate the effect of Zn exposure on motor functions and its correlation with the oxidative stress indices in exposed animals.

The oxidative stress and nitrosative stress are main contributors in PD pathogenesis (Banerjee et al., 2009). Both reactive oxygen species (ROS) and reactive nitrogen species (RNS) produced via oxidative stress are involved in etiology of various neurodegenerative diseases, including PD. Among RNS, nitric oxide (NO)/nitric oxide synthase (NOS) plays very important role in normal physiology. The role of NOS (nNOS/iNOS) has been implicated in dopaminergic neurodegeneration in toxin based PD models. Mechanistic studies on MPTP and rotenone induced PD phenotypes have elucidates involvement of nNOS in dopaminergic neuronal death (Liberatore et al., 1999; Dehmer et al., 2000; Klivenyi et al., 2000; He et al., 2003), while iNOS is implicated in MB+PQ and LPS induced PD models (Gupta et al., 2010; Wang et al., 2002; Kim et al., 2000; Tikka et al., 2001). In vitro studies have reported heavy metals (Pb, Fe, Cu, etc) induced modulation of nNOS activity whereas role of nNOS is suggested in lead (Pb) mediated neurotoxicity in rats in vivo (Chetty et al., 2001). As far as, interaction of Zn with NOS is concerned, controversial results have been reported. The in-vitro studies with purified NOS enzyme has revealed that Zn binds to NOS at reductase domain and inhibits NOS catalysis (Persechini et al., 1995; Perry and Marletta, 1998; Perry et al., 2000). Conversely, another study reported Zn-induced activation of iNOS in cultured microglial cells (Kauppinen et al., 2008). Although earlier studies have implicated the role of oxidative stress in Zn-induced neurotoxicity, role of NO in Zn-induced dopaminergic neurodegeneration is not yet elucidated.
Proteins are the main effector molecules, which decide the fate of physiological and pathophysiological events. Analysis of differential protein expression at various stages of environmental exposure or disease progression may serve as a useful tool for understanding the mechanism of normal physiological processes and PD pathogenesis. Proteomics has emerged as a reliable technique for identifying the dynamic nature of the proteins expressed within a particular cell, tissue, or organism and provides many insights into PD (Sowell et al., 2009). In proteomics study, 2-D PAGE is a molecular tool, which play significant role to identify the differential proteins (Rabilloud, 2002). Proteomic studies have exposed many pathways that are linked with disease pathogenesis and it may lead to the development of potential therapeutic targets. Proteome analyses have led to identification of protein spots specific to a pathophysiological condition and facilitated in differentiating neurological disorders with other diseases (Finehout et al., 2007; Hu et al., 2007). The proteomic studies have been performed in sporadic and chemicals-induced PD and differentially expressed proteins were identified (Basso et al., 2004; Singh et al., 2011b; Patel et al., 2007; Sinha et al., 2009; Srivastava et al., 2010; Tribl et al., 2009). Study of proteome profile of various toxin based PD models and sporadic PD patients indicated damage to nigrostriatal tissues by excessive free radical generation, induction of oxidative stress, alterations in the status of toxicant responsive cytoplasmic and mitochondrial proteins leading to alteration of biochemical and molecular pathways, resulted in appearance of PD like symptoms (Basso et al., 2004; Singh et al., 2011b; Patel et al., 2007). Although proteomic analyses of the nigrostriatal tissues have been performed for various PD models no such study is available in case of Zn-induced Parkinsonism. Since Zn-induced Parkinsonism is environmentally relevant and mimics the real life situation, it would be worthwhile to study proteome profile of Zn-exposed animals to elucidate the molecular mechanism of Zn-induced PD and to establish its similarity with sporadic PD and toxin-induced PD phenotypes.

The present study was therefore, undertaken to investigate the mechanism of Zn-induced dopaminergic neurodegeneration in the rats using behavioral, biochemical and molecular approaches with the following proposed objectives:

➢ To investigate the effect of zinc on motor functions and neurobehavioral parameters in exposed animals.
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➢ To investigate zinc-induced alterations in the levels of dopamine and its metabolites, antioxidant enzymes and tyrosine hydroxylase immunoreactivity of the dopaminergic neurons in the nigrostriatal tissues.

➢ To investigate the effect of zinc on proteome profile in the nigrostriatal region of brain of control and zinc exposed rats and identification of differentially expressed proteins.
Chapter 1

Review of Literature
1.1. Parkinson’s disease

Parkinson’s disease (PD) is a progressive neurodegenerative motor disorder. It is characterized by loss of dopamine, which is the key player in motor function regulation. It is recorded as the second most common neurodegenerative disorder, first being Alzheimer’s disease. The clinical symptoms of PD are motor dysfunctions such as bradykinesia, resting tremor, muscle rigidity and postural instability. Apart from motor impairment, a number of non-motor symptoms also appear that include autonomic, olfactory, cognitive and psychological disturbances (Menza et al., 2006; Litteljohn et al., 2011). The anatomical and pathophysiological marker of PD is Lewy bodies and Lewy neuritis produced due to aggregation of α-synuclein (Redeker et al., 2012; Fomo 1996). There are mainly two types of PD- sporadic PD whose cause is unknown and it is about 90% in prevalence and familial PD is explained on the basis of pedigree which is usually rare in prevalence. Although the exact mechanism of PD is still unclear, considerable scientific evidences suggest that it is the result of interplay between age, environmental and genetic factors. Epidemiological studies have implicated exposure to pesticides and heavy metals in the evolution of PD (Dick et al., 2007; Weisskopf et al., 2008). Recent studies in molecular genetics have identified several genes causing PD. These are α-synuclein, leucine-rich repeat kinase 2 (LRRK2), Parkin, DJ-1 and PTEN-induced kinase 1 (PINK1) and many of them implicated in mitochondrial function.

1.2. Factors contributing to PD pathogenesis

PD is reported since a century but the exact etiology of PD is still elusive. It is considered to be a multifactorial disease (Cicchetti et al., 2009; Orth and Tabrizi, 2003) mainly contributed by aging, genetic predisposition and environmental factors alone or in combination (Tanner et al., 2011; Di Monte et al., 2001, 2002, 2003; McCormack et al., 2002). PD is generally a late-onset neurodegenerative disorder that occurs most commonly in a “sporadic” (idiopathic) form, without a clearly defined genetic basis and only a vaguely delineated pathogenesis. Nevertheless, it is believed that sporadic PD is caused by prolonged environmental exposures that are
superimposed on an individual’s composite genetic susceptibility but this hypothesis has not been tested adequately.

1.2.1. Age

Age is an indisputable risk factor for the PD. PD is divided into three major groups, on the basis of time of onset-idiopathic PD (>40 years), young-onset PD (21–40 years) and juvenile PD (<20 years). Although all these PD show similar clinical symptoms, however the onset of disease and some pathological features are distinct. Age-related neurodegenerative diseases are an increasing burden to an aging population. PD almost effects 1.5 million elderly individuals in U.S., or approximately 2% of the population over the age of 50, and the prevalence increases to 5% by the age of 85 (Lee et al., 2009; Lang and Obeso et al., 2004).

1.2.2. Gender bias

Males are more susceptible to PD as compared to females. This is due to hormonal disparity i.e. reduced estrogen levels in male. The gender disparities in PD patients might be the outcome of the changes in the estrogen levels during the female lifecycle. The role of estrogen in neuroprotection has been studied in animal models particular to PD (Shulman, 2007).

1.2.3. Genetic predisposition

Genetic models of neurodegenerative diseases have been quite helpful in understanding the pathogenesis of a variety of neurodegenerative disorders (Dawson et al., 2010). The underlying principle for studying genetic mutations of a disease is the belief that the clinical similarities between the inherited and sporadic forms of the disease share a common mechanism that can lead to the identification of molecular and biochemical pathways involved in the disease pathogenesis (Bleza et al., 2012). Often the initial models only replicate a portion of the disease in humans, but as models are refined, key features of most diseases can be replicated (Dawson et al., 2010). Genetic mutations in PD are rare and represent only about 10% of all PD cases (Dauer et al., 2003). Genetic studies have enabled the identification of 18 gene loci, named PARK 1–18, that result in autosomally dominant or recessive inherited forms of PD (Martin et al., 2011).
α-Synuclein is an abundant presynaptic phosphoprotein. Genetic discovery of α-synuclein describes its prominent role in PD that led to the discovery that α-synuclein was the major structural moiety of Lewy bodies and neurites (Goedert, 2001). Three point mutations in α-synuclein (A53T, A30P, and E46K) cause familial PD (Gasser, 2009). Mutations in α-synuclein increase the propensity for misfolding. α-Synuclein exists in a variety of higher-ordered structures, including oligomers, protofibrils, fibrils, and filaments where protofibrils and fibrils seem to be the most toxic forms (Lee and Trojanowski, 2006). Stabilization of these higher-ordered structures may be central to the pathogenesis of PD. Toxic misfolded forms of α-synuclein appear to play a prominent role in cell death (Cookson, 2005; Gupta et al., 2008; Lee and Trojanowski, 2006).

LRRK2 is a large multidomain protein that contains GTPase and kinase domains, in the same open reading frame (Biskup and West, 2009). Mutations in LRRK2 are the most frequent genetic cause of autosomal dominant PD (Zimprich et al., 2004). It causes gain of function mutations. It also appears to play a prominent role in sporadic PD as well, since mutations in LRRK2 account for up to 4% of sporadic PD (Albrecht, 2005). The most common mutation so far reported is G2019S. In cellular models, overexpression of disease-causing mutations of LRRK2 are toxic, and the toxicity is kinase and GTP-binding dependent (Greggio et al., 2006; Smith et al., 2006; West et al., 2007). Thus, LRRK2 kinase inhibitors and modulators of GTP binding may be therapeutic strategy (Dawson et al., 2010).

Parkin, an E3 ubiquitin ligase, participates in the ubiquitin proteasome system (Shimura et al., 2000), cause a recessive, early-onset, slowly progressive Parkinsonism. Mutations in parkin were first identified as the genetic cause of autosomal-recessive juvenile Parkinsonism in Japanese families (Kitada et al., 1998). More than 100 mutations in parkin have been reported. As an E3 ubiquitin ligase, parkin catalyzes the transfer of ubiquitin to target proteins to either mark them for degradation by the ubiquitin-proteasome system or for signaling purposes.

PINK1 is a conserved serine/threonine mitochondrial kinase. Mutations in PINK1, the second most common autosomal recessive mutation following parkin contributes to almost 1% – 7% of early-onset PD (Gasser, 2009). It leads to loss of function (Valenteet et al., 2004). Most mutations occur in or near the kinase domain and
consequently disrupt the kinase activity of the protein. PINK1 is localized to the mitochondrial intermembrane space. Since its kinase domain faces towards the cytosol, PINK1 kinase substrates may need to reside in the cytosol. PINK1 may regulate mitochondrial calcium dynamics (Gandhi et al., 2009). PINK1 recruit parkin from the cytoplasm to the mitochondria to initiate the process of mitophagy (Vives-Bauza et al., 2010).

DJ-1 is a member of the family of molecular chaperones (Gasser, 2009; Moore et al., 2006). Mutations in DJ-1 play a small but important role in early-onset, rare form of Parkinsonism (Bonifati et al., 2003). DJ-1 is a redox active protein expressed predominantly in astrocytes. In cellular models, it regulates redox dependent kinase signaling pathways and acts as a regulator of antioxidant gene expression (Kahle et al., 2009). DJ-1 functions in vivo as an atypical peroxiredoxin-like peroxidase, where it protects against oxidative stress in mitochondria (Andres and Mateos, 2007). There is no consensus as to the precise mechanism through which DJ-1 orchestrates such protection. Despite these uncertainties, DJ-1 represents the third "mitochondrial" protein (after parkin and PINK1) whose loss of function results in a Parkinsonian syndrome.

The ATP13A2 gene (locus PARK9) encodes the protein ATP13A2, a lysosomal type 5 P-type ATPase that is linked to autosomal recessive familial Parkinsonism. Loss of P-type ATPase, ATP13A2/PARK9 function induces general lysosomal deficiency and leads to Parkinson disease neurodegeneration (Dehay et al., 2010).

Genes linked to rare forms of PD or the processes that they regulate are potential therapeutic targets (Gasser, 2009; Lees et al., 2009). Attempts have been made to develop PD models in animals or cellular systems using genetic manipulations. All genetic causes of PD ultimately lead to loss of DA neurons in the nigrostriatal pathway, and there are likely to be some common molecular mechanisms. This is done by transgenic overexpression of mutant genes for autosomal dominant genes such as α-synuclein and leucine rich repeat kinase 2 (LRRK2) and knockout or knockdown models for autosomal-recessive genes such as Parkin, DJ-1, phosphatase and tensin homolog (PTEN)-induced novel kinase 1 (PINK1), which insight into the molecular mechanisms of this disorder are leading to new ideas about the pathogenesis of PD.
1.2.4. Lifestyle related factors

The modernization of the world has lead to a fast lifestyle including some dietary habits, and it exerts an influence over one’s risk of developing PD. There are various reports of an inverse, dose-dependent association between tobacco use and PD. There is evidence that nicotine alters various components of dopaminergic systems and may protect against dopaminergic cell death (Quik et al., 2008). Several studies reveal that intake of caffeine and tea is also associated with a dose-dependent decrease in the risk of developing PD (Singh et al., 2008; Ascherio et al., 2001).

1.2.5. Environmental factors

It is believed that we are surrounded by many toxic compounds, and it is dose that decides the extent of toxicity “dose does matter”. Environmental factors include, compounds in the air we breathe (solid, liquid and gas), the substances we ingest as in the form of solid or liquid and of course certain metabolic changes induced by activities we perform (Lai et al., 2002). List of environmental substances that may trigger PD phenotype include heavy metals, rural habitat, agrochemicals (mostly pesticides), microbial toxins; well water drinking and farming. Various studies have implicated a positive correlation of PD with exposure to heavy metals such as manganese (Mn), copper (Cu), Zn, iron (Fe), mercury (Hg), lead (Pb) and aluminum (Al) (Montgomery, 1995; Gorell et al., 1998). Epidemiological and experimental studies have established pesticides and heavy metals exposure as the major environmental risk factors for PD (Gorell et al., 1997; 1998; Singh et al., 2007; Kumar et al., 2010; 2012). PD is a dyshomeostasis of both redox-active and redox-inactive metal ions. In general, the loss of homeostasis of iron and copper in the brain is accompanied by severe neurological consequences. Redox inactive metal ions such as Zn may be pathogenic by virtue of their ability to displace redox-active metal ions from sites where redox activity of the latter is held in check (Sayre et al., 2005). Although Zn is redox-inactive metal ions, it may contribute to neurodegeneration due to role in protein aggregation or structural modification of protein and peptide. Transition metals, along with redox-inactive metal ions, may additionally contribute to neurodegeneration through their deleterious effects on protein and peptide structure, such as a pathological aggregation phenomenon.
1.3. Animal Models of Parkinson's disease

Animal models provide very useful predictive mechanism to approach the etiology of PD. The closer the similarity of a model to PD, the higher is the predictive value for clinical trials. An ideal PD model should present behavioral signs and pathology that resemble the human disease. These models can be valuable to define early and late processes associated with neuronal degeneration and evaluate neuroprotective strategies during mid or late stage degeneration (Meredith et al., 2008). An ultimate mouse model, relevant to address all PD-related questions, is yet to be developed. However, many of the existing models are useful in answering specific questions. Animal models are useful only to the extent to which they accurately simulate the behavioral, biochemical, histological and clinical feature of PD (Betarbet et al., 2002). Current standard criteria for an animal model should include following features, about 50% degeneration of dopaminergic neurons, the model should depict neurobehavioral deficits, and it should show the characteristic Lewy body neuropathology (Emborg, 2004). Human exposure to toxins mostly happens via ingestion, inhalation or direct skin contact. As a result, realistic animal models may not truly reflect human conditions. Each model has advantages and shortcomings, and none could be regarded as suitable to represent all aspects or to address all questions that pertain to PD (Bove et al., 2005).

1.3.1. Reserpine model

Reserpine, a rabbit model helped to elucidate the critical role of dopamine in the pathogenesis of PD. This is earliest developed PD models, which prevents the storage of dopamine in presynaptic terminals, resulting in dopamine depletion in the striatum (Carlsson et al., 1957; Steg, 1964). This finding led to the discovery of dopaminergic drugs such as L-DOPA. Limitation of this model is due to its non-specificity, because it does not exclusively deplete dopamine and norepinephrine but all monoamines in a transient fashion and also does not show the progressive dopamine depletion as the case of PD (Antony et al., 2011).

1.3.2. 6-Hydroxydopamine (6-OHDA) model

6-OHDA is the first neurotoxin model used for the study of mechanism of PD pathogenesis (Betarbet et al., 2002). It is structurally similar to dopamine and
norepinephrine (NE), and extensively used for both *in vitro* and *in vivo* investigations. With the help of dopamine transporters, 6-OHDA can be taken up into dopaminergic terminals. Inside the cell, it is metabolized, resulting in the production of reactive oxygen species, which causes neuronal death via mitochondrial dysfunction. By varying the position and extent of the lesion different stages of human PD could be modeled. 6-OHDA is generally administered unilaterally to the SN or striatum. The unilateral injections cause a typical asymmetric circling motor behavior and its magnitude depends on the degree of nigrostriatal lesion. This specific behavioral abnormality is most prominent after administration of drugs that stimulate dopaminergic receptors, such as apomorphine (rotation away from the lesion), or drugs that stimulate the release of dopamine, such as amphetamine (rotation toward the lesion), due to physiologic imbalance between the lesioned and the unlesioned striatum (Przedborski et al., 1995). The major drawback of this model system is that it cannot cross the blood-brain barrier and therefore needs to be directly delivered to the brain by stereotaxic injection.

### 1.3.3. MPTP model

In 1982, MPTP was accidentally discovered, when a group of young drug addicts in California developed subacute onset of severe Parkinsonism, caused by contamination of a synthetic opiate (heroin) with MPTP (Langston et al., 1983). The toxicity of MPTP is due to its metabolite 1-methyl-4-phenylpyridine (MPP⁺). Being an excellent substrate for the dopamine transporter, MPP⁺ is selectively transported into the dopaminergic neurons, where it is accumulated in mitochondria and inhibits respiration at the level of complex I (Greenamyre et al., 2001) thus representing a selective complex I toxin able to produce a parkinsonian syndrome (Langston, 1996). The inhibition of complex I resulted in reduced levels of ATP generation and increased free radical generation. MPTP is the best characterized toxin-based model of Parkinson’s disease because it replicates almost all of the PD symptoms. Most of the studies have shown that absence of Lewy body formation which is the anatomical hallmark of PD (Forno et al., 1996; Halliday et al., 2009) while few studies also demonstrated the production of Lewy body-like inclusions after MPTP administration (Kowall et al., 2000; Fornai et al., 2005).
1.3.4. Rotenone model

Rotenone, a plant derived pesticide, which is present naturally in the tropical areas (Blesa et al., 2012). The evident beauty of this model is that it replicates almost all of the hallmarks of sporadic PD including α-synuclein aggregation and LB formation (Sherer et al., 2003) however the drawback of rotenone is its non-specific nature, i.e. it causes deleterious effects on other neuronal populations also (Hoglinger et al., 2003). Cell culture studies suggest that it triggers intracellular dopamine release, which could in turn explain the toxic effect on dopaminergic neurons (Inden et al., 2011). Chronic exposure to low doses resulted in the uniform inhibition of complex I throughout the rat brain (Betarbet et al., 2000).

1.3.5. Maneb and Paraquat model

Paraquat (PQ) is a widely used herbicide. It shows the structural resemblance to MPP⁺, hence it was reasoned that PQ should behave like MPP⁺ (Di Monte et al., 1986). PQ exerts its deleterious effects through oxidative stress mediated by redox cycling. In particular, the superoxide radical, hydrogen peroxide, and hydroxyl radicals lead to the damage of lipids, proteins, DNA and RNA (Przedborski et al., 2005). Maneb (manganese ethylenebisdithiocarbamate) has been shown to decrease locomotor activity and potentiate both the MPTP and the PQ effects (Takahashi et al., 1989; Kachroo et al., 2010). Combined PQ and manebo exposure results in development of PD like symptoms in rodents including mitochondrial complex I inhibition, significant loss of dopaminergic cell and accumulation of nigral α-synuclein inclusions (Thiruchelvam et al., 2000).

1.4. Heavy Metals in Neurotoxicity

There are 35 metals that concern us because of occupational or residential exposure. It is quintessential for nature to have a balanced concentration of heavy metals. Imbalance of heavy metals can cause toxicity that can result into lower energy levels, alterations in blood composition and damage to vital organs viz. lungs, kidneys, liver, etc. It also causes mental retardation and central nervous system malfunction.

Heavy metals can be divided into two groups depending on their requirement for normal physiological processes- essential and non-essential heavy metals. Non-essential heavy metals are those metals which are not required for maintenance and
regulation of normal physiological functions. Major non-essential heavy metals which are encountered commonly include cadmium, mercury, lead, chromium, arsenic, etc. Essential heavy metals are elements which are required in trace amounts for normal functioning of physiological processes. These mainly include copper, iron, manganese and Zn etc.

Heavy metal toxicity has been reported by various research groups and may contribute in manifestations of several disorders including neurological disorders like AD, PD, and Wilson disease. It has been found that Al, Fe, Zn, and Cu accelerate aggregation of β-amyloid protein which play role in AD. Its levels are significantly elevated in AD neuropil and finally these metals are significantly concentrated within the core and periphery of senile plaques (Sayre et al., 2005; Lovell et al., 1998). Mn\(^{2+}\) itself is inactive in Fenton chemistry, although it was once considered as a possible contributor to idiopathic PD. It is now clear that the PD-like syndrome induced by Mn poisoning may have little connection to nigrostriatal damage occurring in idiopathic PD. Mn-induced Parkinsonism is termed as Manganism. It can be differentiated from PD because accumulation of Mn and damage occur mainly in the basal ganglia (pallidum and striatum), rather than in the pars compacta of the SN (Olanow, 2004; Dobson et al., 2004; Sayre et al., 2005). The role of Cu in Wilson disease is also well documented. Various studies have implicated a positive correlation of PD with exposure to heavy metals such as Mn, Cu, Hg, Pb, Fe, Al and Zn (Wang and Fowler, 2009). Co-exposure of Pb, Cu and Fe is documented to trigger pronounced Parkinsonian features (Gorell et al., 1995; 1997). Levels of heavy metals such as Zn and Fe were found to be elevated in SNpc of PD patients in autopsy studies suggesting their involvement in PD pathogenesis (Dexter et al., 1989, 1991; 1992; Halliwell et al., 1984; Jenner et al., 1992).

1.5. Zinc and PD

Zn is one of the most common elements in the earth’s crust and it is found in air, soil, and water. It is also present in foods. Zn is mainly stored in the organ like muscle, bone, liver, skin, lung, brain, heart and pancreas (Riordan, 1976). Zn is a component of a large number of proteins involved in a variety of metabolic processes, ion channels, immune defense and signal transduction (Riordan, 1976; Daniel and Dieck, 2004) and also a cofactor for a wide range of enzymes, including those associated
with cell division, protein synthesis and carbohydrate metabolism (Cuajungco and Lees, 1997; Lewis et al., 2002). So for nutritionists Zn is an essential micronutrient; to biochemists, it is a component of enzymes and other proteins; whereas to environmentalists and marine biologists, free Zn in water is a toxic pollutant. To neuroscientists, Zn is not only a micro nutrient and a component of proteins but also an ionic signal. Role of Zn in brain can be compared with Janus, an ancient Roman God of doorways with two different faces (Konoha and Lee, 2006). Zn salts have been used in several varieties of preparation such as micronutrients in fertilizers, antiseptic, antifungal agent, astringent etc (Gossel et al., 1994). ZnSO₄ is globally preferred in the agricultural field by farmers to improve the quality and quantity of food grains production over other Zn formulations. In addition, people involved in paint manufacturing, electrometallurgy and mine and smelting industries are also exposed to excessive level of Zn and might be prone to Zn-induced toxicity. Zn is biphasic in nature. Zn possesses both antioxidant and pro-oxidant properties therefore; may be neuroprotective or neurotoxic depending upon the concentration. The effects of Zn in both deficiencies as well as in abundance have been well documented in humans and experimental animals.

Zinc excess

- Lethargy
- Local neuronal deficits
- Respiratory tract
  - Respiratory disorder after inhalation of zinc smoke
  - Metal fumes fever
- Gastrointestinal tract
  - Nausea/vomiting
  - Epigastric pain
  - Diarrhea
- Prostate
  - Elevated risk of prostate cancer

Zinc deficiency

- Decreased nerve conduction
- Neuro-psychiatric disorders
- Neuro-sensory disorders
- Mental lethargy
- Thymus
  - Thymic atrophy
- Skin
  - Skin lesions
  - Decreased wound healing
  - Acrodermatitis
- Reproductive system
  - Infertility
  - Retarded genital development
  - Hypogonadism

Systemic symptoms
- Copper deficiency and sequelae
- Altered lymphocyte function

Adopted from Int. J. Environ. Res. Public Health, Plum et al., 2010
1.5.1. Zinc pool the sources

It is useful to consider three distinct pools of cellular Zn in the CNS (Frederickson, 1989). (1) Immobile Zn - It is first largest fraction, 80% or more, exists in tight coordination with intracellular proteins and is generally considered immobile. (2) Vesicular Zn - It is second largest, about 10% of cellular totals, this pool of Zn is sequestered in the vesicles of certain neurons and is readily detected with metal sensitive histochemical stains. It is always co-localized with glutamate, can be released into the synapse with neuromodulatory effects. (3) Free Zn - The third pool of Zn is free in the cytoplasm as unbound, ionic form.

1.5.2. Zinc homeostasis

Intracellular free Zn concentration is tightly controlled by (a) extrusion with the help of Zn transporters (b) buffering with the help of metallothioneins (c) sequestration of Zn in mitochondrial systems (Bertoni-Freddari et al., 2008).

Zn transporters - The cellular homeostasis of Zn is mediated by two protein families-(a) Zn importer (Zip) family- about 14 proteins involve in Zn transportation into the cytosol. (b) Zn transporter (ZnT) family- comprising 10 proteins transporting Zn out of the cytosol (Lichten and Cousins, 2009). ZnTs, which are members of the cation diffusion facilitator (CDF) family, control the Zn extrusion from the cytosol. These transporters promote Zn movement from the cytosol to the extracellular space or induce sequestration into intracellular compartments. The activity of ZnT-1 and ZnT-3 is mainly important for brain Zn homeostasis.

Metallothioneins (MTs) - MTs are a group of low molecular weight metal binding proteins that play a major role in buffering cytosolic Zn. There are three isoforms in CNS and all show distinct patterns of expression. MT-1 and MT-2 are largely found in astrocytes and glia while MT-3 is abundant in neurons. MT-3 seems to be particularly relevant to neuronal Zn homeostasis in critical brain regions such as the hippocampus. Oxidative stress has been found to be a key regulator of Zn homeostasis by interfering with Zn binding to MTs. Cellular oxidants have been shown to promote Zn release from MTs, while reducing agents have been shown to facilitate Zn binding (Maret et al., 1999; Plum et al., 2010).
Mitochondrial Zn - Excess Zn is sequestered into the intracellular organelles like endoplasmic reticulum, Golgi and lysosome through ZnT. Zn uptake seems to be largely mediated by the Ca\(^{2+}\) uniporter (Malaiyandi et al., 2005). Mitochondria seem to have a high Zn uptake capacity therefore blockade of mitochondrial Zn sequestration, leads to a significant elevation of cytosolic Zn. Mitochondria are able to sequester cytosolic Zn and under resting conditions the sequestered Zn may be released into the cytoplasm in a Ca\(^{2+}\) dependent fashion (Sensi et al., 2003). Zn overload in mitochondria can also induce severe mitochondrial dysfunction and oxidative stress.

1.5.3. Source of neurotoxic Zn?

It has been documented that if Zn gets accumulated in brain it results into the death of neurons in various injury models, however, the source of this toxic Zn is not entirely...
clear. A number of metal transporters probably regulate Zn ion flux across the plasma membrane under physiological conditions (McMahon and Cousins et al., 1998), but Zn\(^{2+}\) accumulation during neuronal injury occurs chiefly through voltage-sensitive calcium channels, glutamate receptors, and sodium–calcium exchange (Sensi et al., 1997; Cheng and Reynolds, 1998). Initially, it was thought that excessive release of vesicular Zn is prime reason of in vivo Zn mediated injury, because this Zn translocated to postsynaptic neurons with toxic consequences (Koh et al., 1996). More recent evidence suggests that Zn apparently translocates into neurons from the extracellular space, it does not come from the histochemically reactive, synaptically released vesicular pool (Lee et al., 2000). Lethal Zn\(^{2+}\) may also be reached through the oxidation of intracellular Zn binding proteins (Aizenman et al., 2000), and several reports indicate that Zn is a critical mediator of oxidative damage in whole animal models (Cuajungco and Lees, 1998). Exogenous Zn exposure is also reported to result in increased level inside brain (Kumar et al., 2010; Opoka et al., 2008).

1.6. Mechanism of PD pathogenesis

1.6.1. Oxidative stress

The cause of PD is still elusive, but various reports suggested that oxidative stress is implicated as the one of the primary event in idiopathic and chemically-induced PD. Free radicals, including reactive oxygen species (ROS) and reactive nitrogen species (RNS) play a critical role in the PD. Both enzymatic and non enzymatic pathways involved in free radical generation. Enzymatic sources include NADPH oxidase, xanthene oxidase, myeloperoxidase and cytochrome P450 dependent oxygenases (Babior, 2002; Vignais, 2002). The non-enzymatic production of superoxide (O\(^{2-}\)) occurs when a single electron is directly transferred to oxygen by reduced coenzymes or prosthetic group. ROS generated by NADPH oxidase and GSH depletion, contributes to oxidative stress-mediated cell death resulting in PD phenotype in the experimental animals (Cristovao et al., 2009; Garrido et al., 2011; Kumar et al., 2011). Zn itself is a strong inducer of oxidative stress by promoting extra-mitochondrial production of ROS which involved increased activity of NADPH oxidase in cortical neurons (a multi-subunit enzyme widely expressed in central neurons) via protein kinase C (PKC) activation, which together with superoxide can produce injurious peroxynitrite (ONOO-) (Noh and Koh, 2000).
1.6.2. Impaired Energy Production and Mitochondrial dysfunction

The exact mechanism by which PD occurs is poorly understood, but various reports indicated that mitochondrial dysfunction as a key player in PD pathogenesis. It is due to the central role of mitochondria in energy production, oxidative stress, ubiquitin system impairment and excitotoxicity (Keane et al., 2011). Mitochondrial dysfunction with complex I deficiency and impaired electron transfer observed in sporadic form of PD (Keeney et al., 2006; Keane et al., 2011) while mutations in several mitochondrial proteins have been associated with familial forms of PD (Kitada et al., 1998; Valente et al., 2004; Bonifati et al., 2003; Keane et al., 2011). Oxidants like hydrogen peroxide and superoxide radicals which are byproducts of oxidative phosphorylation, making mitochondria the prime site of ROS generation within the cell (Drechsel and Patel, 2008). The various neurotoxins like MPTP, rotenone and PQ involved in PD pathogenesis by causing mitochondrial dysfunction in exposed animals (Davis et al., 1979; Greenmyre et al., 1999; Liou et al., 1997). Zn impaired at the each step {glycolysis, tricarboxylic acid (TCA) cycle and electron transport chain} of energy generating mechanism involved in cellular respiration. Zn-induced cortical neuronal death mediated by reduced cellular levels of nicotinamide adenine dinucleotide (NAD\(^+\)). This reduction in NAD\(^+\) leads to the reduced activity of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and the subsequent accumulation of dihydroxyacetone phosphate (DHAP) and fructose 1,6 bisphosphate. It resulted as reduction in ATP levels and subsequent neuronal death (Shelinen et al., 2010).

Several studies in isolated mitochondria indicate that the cation inhibits cellular respiration by interfering with the activity of the ETC. Zn inhibits complex III at cytochrome bc1 and complex 1 by inhibiting ketoglutarate dehydrogenase (KGDHG).

In neurons, Zn rises are able to promote potent generation of mitochondrial ROS and intriguingly, this Zn triggered ROS production persists longer than that induced by Ca\(^{2+}\) (Sensi et al., 2000). Mitochondria have an integral role in the apoptotic cell death pathway. Hence inhibition of energy production system may lead to decrease in ATP generation as result cells undergo death by apoptosis or other means.

1.6.3. Antioxidant defense system

Various reports suggested that the antioxidant mechanism system is compromised in disease conditions; including PD. Oxidative stress describes a condition in which
cellular antioxidant defenses are insufficient to keep the levels of ROS below the toxic threshold (Schulz et al., 2000). Cells have a complex network of defense mechanisms to neutralize excessive ROS accumulation. The cells possess both enzymatic and non enzymatic protective system. The non enzymatic protective system includes antioxidant compounds e.g., glutathione (GSH), arginine, creatine, Zn, vitamin E, vitamin C and vitamin A, whereas enzymatic protective system includes SOD, GST, catalase, glutathione reductase (GR), and glutathione peroxidase (GPx). Hence, with the help of these protective systems, cells are able to cope with the flux of ROS under physiological conditions.

Superoxide dismutase (SOD) is a ubiquitous family of enzymes. It catalyzes the dismutation of superoxide to hydrogen peroxide and oxygen.

\[
2O^{2-} + 2H^+ \rightarrow O_2 + H_2O_2
\]

There are three major families of superoxide dismutase, depending on the metal cofactor. (a) Cu-ZnSOD/SOD1- This binds both copper and Zn (b) Fe and MnSOD/SOD2 - which bind either iron or manganese and (c) Ni type/SOD3- which binds nickel. SOD1 is located in the cytoplasm, SOD2 in the mitochondria and SOD3 is extracellular. SOD protects oxygen-metabolizing cells against harmful effects of superoxide free-radicals. This $O^{2-}$ ion is formed by the univalent reduction of $O_2$ during various enzymatic reactions or by ionizing radiation. This $O^{2-}$ ion, which has been considered important in aging, lipid peroxidation and the peroxidative hemolysis of red blood cells (Halliwell et al., 1984). SOD1 is thought to be one of the major cellular defense enzymes that perform a vital role in protecting cells against the toxic effect of superoxide radicals. A recent report suggested that over expression of SOD1 and catalase could increase the average lifespan of the fly. Differential modulation of cytosolic and mitochondrial SOD is reported in PD patients. A study has shown increased SOD2 levels in the SN of the brain of sporadic PD patients while others have reported reduction of SOD1 in the SN of the brain of PD patients (Jenner et al., 1992; Saggu et al., 1989; Kunikowska et al., 2003). Extra cellular SOD (EC-SOD/SOD3) is the most recently characterized SOD. It plays an important role in maintaining vascular tone, lung function, and the metabolism of NO. Protective effect of SOD/catalase mimetic against chemically-induced experimental models of PD in animals by over-expressing Cu,Zn-SOD have established the protective role of SOD.
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against oxidative stress-mediated PD pathogenesis (Hung et al., 1998; Cadet et al., 1994; Peng et al., 2005).

Heme oxygenase-1 (HO-1) is an enzyme that catalyzes the degradation of heme resulted in production of biliverdin, iron, and carbon monoxide. HO-1 is an inducible isoform in response to stress such as oxidative stress. HO-1 is documented to play a pivotal role in antioxidative and anti-inflammatory defense against oxidative stress-induced diseases including PD. HO-1 is reported to protect against oxidative stress-induced damage via activation of Nrf-2 transcription factor, which results in promoting cell survival mechanisms (Minelli et al., 2009). HO-1 over-expression is reported to protect dopaminergic neurons against MPTP, rotenone and 6-OHDA-induced cell death (Schipper et al., 1998; Hung et al., 2008; Quesada et al., 2009).

Glutathione-s-transferase (GST) is a family of multifunctional enzymes that are involved in metabolic detoxication of a variety of electrophilic xenobiotics (Vos and Van Bladeren, 1990). GSTs, a family of phase II toxicant metabolizing enzymes is well documented in idiopathic PD and chemically-induced PD phenotypes (Patel et al., 2006; Smeyne et al., 2007; Shi et al., 2009). GSTs are known to facilitate the detoxification of reactive metabolites of toxicants formed during phase I reactions by conjugation with GSH. ZnCl₂ mediated decreased GST was also documented (Patricia et al., 2005; Kumar et al., 2012) however increased activity and expression of GSTA4-4 isoforms was observed in PD patients and PQ and Zn exposed rats (Kumar et al., 2010; Selley, 1998). GST- pi expression was found to be decreased in MPTP induced Parkinsonian model (Smeyne et al., 2007).

Glutathione (GSH) is the main non-enzymatic antioxidant. It forms the major line of defense against oxidative stress mediated damage, including PD (Garcia et al., 2000). Reduction in GSH content is reported in both sporadic and chemically-induced PD (Jenner et al., 1992, Kang et al., 2009). GSH depletion and protective role of GSH in Zn-induced neurodegeneration was also reported (Kumar et al., 2011). Zn has been reported to alter GR (Mize et al., 1962). This enzyme is responsible for cellular GSH redox cycling, which is crucial for the detoxification of endogenous peroxides (Dringen et al., 2002).
1.6.4. Autophagy

Autophagy refers to the global process by which intracellular components are degraded by lysosomes (Luzio et al., 2001). Proteasomal and autophagic proteolysis are the two major pathways involved in the degradation of cellular constituents in eukaryotic cells. The documented reports indicated that alterations in autophagic pathways may be preferentially involved in neurodegenerative diseases, including PD (Levine and Kroemer, 2008). Increased number of autophagosomes has also been observed in cultured cells intoxicated with Parkinsonian neurotoxins such as MPP⁺, rotenone and 6-OHDA (Chen et al., 2007; Zhu et al., 2007; Dagda et al., 2008) and in post-mortem PD brain samples (Anglade et al., 1997). Recently, evidence has indicated that lysosomal membrane permeabilization (LMP) is a key contributing mechanism in oxidative and Zn²⁺ induced hippocampal neuronal death (Hwang et al., 2008) however its role in dopaminergic cell is still unknown.

1.6.5. Toxicity through ion channels and receptors

Modulation of various ion channels like voltage activated calcium channels (VACC) and K⁺ channels has been implicated in mouse model of PD (Chan et al., 2007). Differential cellular expression of neuronal VACC isoforms has been implicated in the neurodegeneration in PD (Hurley and Dexter, 2012). Exogenously applied Zn profoundly affects the activity of glutamate, GABA, and glycine ionotropic receptors. Zn²⁺ is co-released with glutamate in many excitatory synapses and this synaptic Zn can eventually enter into neurons through channels associated with glutamatergic post-synaptic receptors such as NMDA, calcium-permeable AMPA/kainate receptors and Zn transporters (Sensi et al., 2009). Ca²⁺ influx into cells activates a number of enzymes, including phospholipase, endonuclease and protease such as calpain. These enzymes go on to damage cell structures such as components of the membrane and DNA. Excitotoxicity may be involved in various neurodegenerative diseases such as multiple sclerosis, AD and PD. ROS-induced Zn accumulation promotes activation of specific K⁺ channels leading to K depletion, a key event in neuronal apoptosis (McLaughlin et al., 2001). Zn binds to the γ-aminobutyric acid (GABA) receptors and noncompetitively inhibits GABA-mediated responses (Kumamoto & Murata, 1995). Zn can also potentiate glycine-mediated currents (Trombley et al., 1996) and regulate
sodium and chloride channels (Harrison et al., 1994) however its role in Zn-induced dopaminergic neurodegeneration is still unclear.

1.6.6. Neuronal death and apoptosis

Apoptosis is a natural process in which cell undergoes programmed cell death (PCD). It occurs during the development and for defense against infected or damaged cells e.g. rotenone and other neurotoxicants induced apoptosis (Armstrong et al., 2001; Isenberg and Klaunig 2000). However, excessive PCD can cause unwarranted cell death, which might lead to diseases such as immunodeficiency and neurodegenerative disorders. Initially, the demonstration of increased numbers of TUNEL-positive dopaminergic neurons in the brain of patients with PD has been used to support the occurrence of apoptosis in this disease (Mochizuki et al., 1996). Zn may be involved in the pathogenesis of PD by activating apoptotic pathway, although the mechanisms underlying Zn induced apoptosis of dopaminergic neurons remain unclear. Intranigral infusion of Zn in rats was shown to induce apoptosis of dopaminergic neurons (Lin et al., 2003). Reports indicated that accumulation of intracellular Zn, either as a consequence of exogenous administration or release from intracellular stores by reactive oxygen species or nitrosation, activates pro-apoptotic molecules like p38 and potassium channels, leading to cell death (Kim et al., 1999, Wiseman, 2006). Increased intracellular Zn levels may also induce cell death by inhibition of the energy metabolism (Sheline et al., 2000). In context of its apoptosis-inducing properties, Zn has been shown to increase the expression of bax, leading to a decrease in the bel-2/bax ratio (Feng, 2008). As a consequence, dissipation of the mitochondrial membrane potential leads to the release of cytochrome c from mitochondria into the cytosol (Dineley, 2003; Bitanihirwe and Cunningham, 2009). It has been also demonstrated that cytosolic poly (ADP-ribose) polymerase (PARP) activation leads to decreased NAD$^+$ levels, an event that triggers the release of AIF from mitochondria. Cytosolic AIF eventually promotes the collapse of mitochondrial membrane potential, prompting the release of cyt c, and initiating the apoptotic cascade (Jang et al., 2001).

1.6.7. Misfolding and aggregation of proteins

The anatomical hallmark of PD is Lewy body and Lewy neuritis in SN region and it appears due to abnormal aggregation and accumulation of α-synuclein proteins
(Giasson et al., 2000; Spillantini et al., 1997; Dinis-Oliveira et al., 2006). In normal condition, it occurs in monomeric unstructured form while in case of PD patient its form aggregates due to duplication or triplication of α-synuclein (Singleton et al., 2003; 2005). Overexpression of α-synuclein causes cellular toxicity by interfering with vesicular transport between the endoplasmic reticulum and golgi complex. Zn is also documented to confer conformational changes of α-synuclein and therefore increased propensity for its aggregation (Kim et al., 2000).

1.6.8. Microglial activation and Inflammation

Microglia, which serve as the brain’s own specialized immune cells. In activated condition, it acquires an “amoeboid” morphology. Microglia has been implicated in the production of ROS and pro-inflammatory factors that are responsible for selective degeneration of SN neurons caused by MPTP and other neurotoxicants in animal models and in vitro experimental paradigms (Kauppinen et al., 2008; Purisai et al., 2007). They are primary cytokine producers, and the synthesis of these cytokines was augmented by head injury, stroke and neurotoxins (Clausen et al., 2009). The activated microglial cells, which are important mediators of neuroinflammation are abundant in the SN and striatum of patients with idiopathic PD (McGeer and McGeer, 2004; Ouchi et al., 2005). Epidemiological studies found a lower risk for PD associated with regular use of non-steroidal anti-inflammatory drugs. The production of ROS by microglial NADPH oxidase is an innate response to infection and other stimuli.

1.7. Role of Nitric oxide (NO) in neurodegenerative disorders

1.7.1. Nitric oxide (NO) and Nitric oxide synthase (NOS)

NO is free radical gas molecule. It is a small, lipophilic, diffusible and highly reactive molecule. It was assigned the molecule of the year in 1992 by Science and was the subject of the Nobel Prize in 1998 (Ignarro, Nitric oxide: biology and pathobiology, Academic Press, SanDiego, 2000). It is involved in numerous physiological and pathophysiological processes. NO has been implicated in the relaxation of vascular smooth muscle, the inhibition of platelet aggregation, neurotransmission and immune regulation. NO can acts as both pro-apoptotic as well as anti-apoptotic. This complexity is a due to consequence of the rate of NO production and the interaction
with biological molecules such as metal ion, thiol, protein tyrosine, and reactive oxygen species. Nitric oxide is synthesized from L-arginine by nitric oxide synthase (NOS). There are three types of NOS. It includes endothelial NOS (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS). NOS are a homodimeric protein i.e. made up of two domains (one oxygenase and one reductase) per polypeptide chain. The oxygenase domain stoichiometrically binds protoporphyrin IX type heme and (6R)-5,6,7,8-tetrahydro- L-biopterin (H₄B). Together with the heme and H₄B cofactors, the oxygenase domain forms the active site of the enzyme (Marietta, 1994). It is also known that each reductase domain binds one equivalent of FMN and one equivalent of FAD (Hevel and Marietta, 1992; Stuehr et al., 1991). The reductase domain shuttles NADPH derived electrons to the active site heme to support catalysis in the oxygenase domain. The oxygenase and reductase domains of NOS are linked by a polypeptide that recognizes Ca²⁺ bound calmodulin (CaM) (Perry, 2000).

1.7.2. Nitric oxide (NO) modulators

NO acts like messenger which modulate neuronal function. The use of NO donors’ and NOS inhibitors acts as a pharmacological tools to study the role of NO in various physiological function (Prast and Philippu, 2001). The protective role of NOS inhibitors (7-NI and L-NAME) has been established in various pesticide model of PD (Singh et al., 2005; Jeener, 1998). To know the effects of NO on the cell survival, or without the involvement of NOS, NO-releasing compounds (NO donors) are valuable tools (Noack and Murphy, 1991). They preserve NO in their molecular structure and exhibit biological activity after decomposition. These chemicals exhibit considerable variation in their structure, stability, and biological activity. Examples of NO donors are 3- morpholinosydnonimine (SIN-1), sodium nitroprusside (SNP), S-nitrosothiols-e.g. S-nitrosoglutathione (GSNO), S-nitroso-N-acetylpenicillamine-amine (SNAP), and S-nitrocysteine (CysNO), as well as compounds that contain the NO-functional group, such as the diethylamine-nitric oxide compound.

Both SNP and SNAP have been widely used in research studies as NO donor to understand the role of NO in physiological as well as pathophysiological conditions. It performs various roles like vasodilator, smooth muscle relaxant, lymphocyte activator and activates soluble guanylyl cyclase.
Sodium nitroprusside (SNP)  S-nitroso-N-acetyl-D,L-penicillamine (SNAP)

1.7.3. Role of Nitric oxide (NO)

NO has also been implicated in the etiology and sequel of numerous neurodegenerative diseases, including PD (Espey et al., 2002). NO can prevent or induce apoptosis depending on its concentration, cell type and the oxidative environment. Both neuroprotective as well as neurotoxicant role of NO has been documented. Both nNOS and iNOS have been implicated in dopaminergic neuronal death induced by MPTP (Hantraye et al., 1996; Liberatore et al., 1999; Dehmer et al., 2000; Przedborski, 1996). However, a recent report indicated that MPTP does not affect iNOS activity in the striatum (Rubio-Osornio et al., 2009). Rotenone, a mitochondrial inhibitor, selectively induced nNOS expression in the striatum, and increased NOS activity in both the striatum and SN (He et al., 2003). iNOS mediated neurotoxicity in LPS and MB+PQ model has also been documented (Wang et al., 2002; Kim et al., 2000; Tikka et al., 2001; Gupta et al., 2010). Overproduction of NO, acts as a pro-apoptotic modulator, activating caspase cascade through the release of mitochondrial cytochrome c into cytosol, up-regulation of the p53 expression, and alterations in the expression of apoptosis associated proteins, including the bcl-2 family (He et al., 2003; Chung et al., 2010; Singh et al., 2010c).

Various studies had shown that Pb exposure resulted in decrease the expression and activity levels of nNOS in both the hippocampus and the cerebellum of developing rat brain (Chetty et al., 2001). Chronic lead (Pb) exposure results in cognitive deficits in children. It has also been reported in purified enzyme system that NOS bind to transition metals stoichiometrically and the rate of catalysis is enhanced by non-heme iron while other divalent transition metals, including Cu and Zn inhibit NOS catalysis (Persechini et al., 1995; Perry, 2000). Activation of inducible nitric oxide synthase
iNOS resulted in increased nitric oxide (NO) production was also reported in ZnCl₂ treated microglial cell culture (Kauppincn et al., 2008) however, in vivo reports are still unknown. NO as a neuroprotectant with reference to PD is also accumulating in the literature. NO scavenge hydroxyl radical (-OH) as well as its potent reactivity with peroxyl radicals, heme protein, provides this radical with the potency to act as a good neuroprotector (Mohanakumar et al., 1998; Rauhala et al., 1996; 1998; Hogg et al., 1993). It protects against the neurotoxin-induced dopaminergic neurotoxicity (Mohanakumar et al., 2002; Tsai & Lee, 1998; Tsai et al., 1998; Genc et al., 2001). It also shields cells from oxidative stress (Wink et al., 1993; Suschek et al., 2001; Wenk et al., 2004). NO also inhibits apoptosis and inflammation by S-nitrosylation of the cysteine present at active site of caspases. NO increases the gene transcription of protective proteins, these include heat shock protein, HO-1, and NOS (Ignarro et al., 1987; Nathan, 1992).

Adopted from Neurobiology of Disease, Cuajungco and Lee, 1997
1.8. Tests and diagnosis for Parkinson's disease

PD diagnosis is based on medical history, signs and symptoms, neurological and physical examination of patients. The presence of Lewy bodies in the midbrain on autopsy is usually considered proof that the patient is suffering from PD. In computed tomography (CT) and magnetic resonance imaging (MRI) brain scans with PD usually appear normal. However, specific technique of MRI, diffusion MRI, has been reported to be useful in identification of PD (Brooks, 2010). More refined techniques show some promise, particular, the identification of changes in iron deposition in the SN using magnetic resonance imaging (MRI), and the use of single photon-emission computed tomography (SPECT) scanning to visualize dopamine transporters in the nigrostriatal system might be useful for diagnosing Parkinson's disease at an early stage.

It is very important to have a more reliable alternative to diagnose PD. Hence the search is on for a biomarker for early Parkinson's disease diagnosis. Such a biomarker would have several benefits: patients could be warned that they are likely to develop Parkinson's disease. Biomarkers must be sensitive enough to detect most cases of Parkinson's disease at an early stage. So there might be several ways to detect the disease. Some proteins have been shown to be differentially displayed in biological fluid of PD patients. Study of a large number of proteins, provide crucial information regarding PD. Therefore the proteomics became an essential tool to predict the molecular mechanism of the disease. Hence study of various proteins in blood, with the help of two-dimensional polyacrylamide gel electrophoresis (2-D PAGE), a molecular technique, provide very important insight to diagnose PD.

1.9. Proteomics and nigrostriatal dopaminergic neurodegeneration

Proteomic studies have exposed many pathways that are linked with disease pathogenesis and it may lead to the development of potential therapeutic targets. The most traditional and widely used proteomic method is 2-D PAGE (Rabilloud, 2002). The protein map obtained from a 2-D PAGE can also be used to identify post-translational modifications such as glycosylation, phosphorylation and carbonylation. The identification of carbonylated proteins is one aspect of the field of redox proteomics (Butterfield, 2004; Sowell et al., 2009; Tribl et al., 2006). Several genes are transcribed but not translated; therefore the information obtained from
transcription level may or may not properly reflect actual status of the pathological conditions (Ideker et al., 2001). Also, a RNA molecule may translate into various proteins through alternating splicing. Hence, proteins are the final effector molecules which decide the pathological fate of a disease (Aebersold and Goodlett, 2001; Tribl et al., 2006). It is thus equally important to study gene expression at the protein level. Study of a large number of proteins, provide crucial information regarding PD. Therefore the proteomics became an essential tool to predict the molecular mechanism of the disease. Liquid chromatography coupled with 2-D PAGE, mass spectrometry and database analysis offers a comprehensive overview of cellular proteins (LoPachin et al., 2003; Xun et al., 2007). The proteomics approach revealed the involvement of various proteins in neurodegenerative disorders including PD. A study of 2-D PAGE, in SN and striatum was separately reported, and 25 differentially expressed proteins including stathmin and SNAP-25 were obtained in MALDI-TOF and LC/MS study (Singh et al., 2011b). The altered expressions of 9 proteins, including peroxiredoxin II, mitochondrial complex III, ATP synthase D chain, complexin I, profilin, L-type calcium channel δ-subunit and fatty-acid binding proteins are reported in the SNpc of PD patients as compared with controls (Basso et al., 2004). Besides DJ-1 monomeric forms another four different isoforms of SDS-resistant DJ-1 was migrated on the two-dimensional SDS-PAGE gels, and confirmed that DJ-1 becomes oxidized in PD and AD (Choi et al., 2006). Another study showed differential expression of 16 proteins including ferritin-H, GST-M3 and glial fibrillary acidic protein (GFAP) in SNpc between PD and healthy control groups (Werner et al., 2008). Thirty proteins, including Cu, Zn superoxide dismutase, α- synuclein, ubiquitin-conjugating enzyme E2N, stathmin-1, calcineurin-B, cystatin-B, ATP synthase D chain, mitochondrial NADH dehydrogenase (ubiquinone) Fe-S protein 8, glial maturation factor-β and brain lipid binding protein-A are also found to be differentially expressed in the rat brain (Li et al., 2008). Furthermore, α-enolase, GMF-β and complexin-I are identified in the SNpc of MB+PQ induced mouse PD phenotypes (Patel et al., 2007). Proteomics studies conducted on animal models have shown significant correlation with human studies. Although proteomic analyses of the nigrostriatal tissues have been performed for various PD models, however, it remains untouched in case of Zn. So, it will be worthwhile to investigate the effect of Zn on the nigrostriatal proteome profile to decipher the involvement of various proteins in
the molecular mechanism of Zn-induced Parkinsonism. It can be summarized as below.

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1.10. Treatment of Parkinson’s disease

Levodopa (L-DOPA) has been the mainstay/gold standard of Parkinson’s disease therapy. Dopaminergic agonists such as pramipexol, carbergoline, lisuride, ropinirol etc., which mimic dopamine at its receptors, provide invaluable therapeutic support. Dopamine agonist L-DOPA is given in combination with carbidopa, as it inhibit the dopamine decarboxylase. Anticholinergics block mucaric cholinergic receptors and are helpful in managing tremor in early stage Parkinsonism (Lang and Obeso, 2004). Catechol-O-methyl transferase (COMT) inhibitors such as entacapone and tolcapone block peripheral conversion of levodopa to 3-O- methyl DOPA and increase both the plasma half life of levodopa and its availability in CNS. Selegiline and rasagiline

Adopted from Ageing Res Rev, Sowell et al., 2009
selectively inhibit monoamine oxidase B (MAO-B) and tends to stabilise levels of dopamine. In mammals, melatonin is secreted into the blood by the pineal gland in the brain. Melatonin and its metabolites are potent free radical scavengers and indirect antioxidants (Reiter et al., 2009; Hardeland et al., 2009). Other nonenzymatic antioxidants tested for their efficacy against dopaminergic neurodegeneration include Vitamin C, Vitamin D, and Vitamin E. Thus, for the past two decades, researchers have been hunting for drugs that slow, stop or, better still, reverse the disease pathology.

1.11. Future perspective

The ultimate goal of current research into Parkinson’s disease treatments is to develop novel therapeutic interventions that either slow or halt the degenerative process. For this to occur, it is vital to have an animal model that truly recapitulates the behavioral phenotype, neuropathology and pathophysiology of Parkinson’s disease. Today, we understand that the prodrome of PD can be initiated by genetic and environmental factors. However, the total numbers of involved genes are not yet known. One challenge is, therefore, to better understand the complex relationships between genetic factors and PD onset and manifestation. As such, creating an improved animal model is an ongoing frustration, and a major therapeutic, clinical and research need. A breakthrough has remained elusive, but there is increasing information about the mechanisms underlying neuronal death and regional vulnerability.