Chapter 5

Effect of zinc on the expression of toxicant responsive proteins in nigrostriatal tissue of rats
5.1. INTRODUCTION

Parkinson's disease (PD) is a progressive, neurodegenerative disorder characterized by degeneration of dopaminergic (DA) neurons in the substantia nigra pars compacta (SNpc) region of mid brain (Lee et al., 2009; Dickson et al., 2009). Although the disease was scientifically described in 1817, the precise mechanism of dopaminergic neurodegeneration is poorly understood. Clinical and experimental evidences have shown mitochondrial dysfunction as the primary culprit leading to defective metabolism, oxidative stress, ubiquitin-proteasomal system impairment and excitotoxicity (Keeney et al., 2006; Schapira et al., 1990; Kitada et al., 1998; Keane et al., 2011). Meta-analyses have established a strong association between metals exposure and increased of PD (Dick et al., 2007; Weisskopf et al., 2010; Gorell et al., 1997; Singh et al., 2007). Post-mortem studies have demonstrated an increased accumulation of Zn in the SNpc of PD patients implicating its role in PD pathogenesis (Jenner et al., 1992; 1989). While role of oxidative stress in Zn-induced neuronal cell death in vivo and in vitro systems has been reported (Kumar et al., 2010; Pong et al., 2002; Choi et al., 1998; Kim et al., 1999; Oteiza et al., 2004), the complete mechanism of Zn-induced dopaminergic neurodegeneration is not yet completely established. 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. Among differential protein expression profiles techniques, two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) used for studying proteome profile at a given time has emerged as a valuable tool, which plays significant role to explore proteome profile.

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). Proteomic approaches identify the differentially expressed proteins in sporadic and metal-induced PD and explain the roles of identified protein involved in the pathophysiological conditions of PD (Basso et al.,
Proteome expression profiling followed by detection of protein spots specific to a pathophysiological condition is a potential tool, which selectively and effectively differentiates various neurological diseases (Finehout et al., 2007; Hu et al., 2007). 2-D PAGE in combination with mass spectrometry (MS) and western blotting offer a comprehensive overview of cellular proteins involved in various neurodegenerative disorders, including PD (LoPachin et al., 2003). Several proteomics studies have been performed in sporadic and chemicals-induced PD and a number of differentially expressed proteins have been 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). A study based on 2-D PAGE, in the nigrostriatal system of cypermethrin exposed rats is reported recently. In this study, it is demonstrated that differential expressions of 25 proteins of varies biological pathways regulate the onset and progression of PD (Singh et al., 2011b). Additionally differential expressions of peroxiredoxin-2 and profilin-2 are also reported in the substantia nigra of PD patients (Basso et al., 2004; Werner et al., 2008). Although proteomic analyses of the nigrostriatal tissues have been performed in various PD models, the proteomic profiling of Zn-induced neurotoxicity in the nigrostriatal tissues of rat has not yet been deciphered. It was worthwhile to perform the proteome profiling in the nigrostriatal tissues of Zn-exposed rats, owing to its uniqueness, significance in agricultural fields and environmental relevance. Etiological insights of Zn-induced nigrostriatal dopaminergic neurodegeneration could be worth for designing the preventive and therapeutic strategies to encounter PD. The present study was undertaken to investigate the effect of Zn on the differential expression of proteins in the nigrostriatal tissues of Zn-exposed rats.

5.2. OBJECTIVES

➢ To identify the differentially expressed proteins in the nigrostriatal tissues of Zn-exposed rats.
➢ To validate the expression pattern of a few differentially expressed proteins by western blotting and their comparison with other rat models of PD.
5.3. RESULTS

5.3.3. Proteome profiling of the nigrostriatal tissue

5.3.3.1. 2-D PAGE and MS

Although a total of 30 proteins was differentially expressed in the nigrostriatal tissue of Zn exposed rat at some or the other time or dose of Zn exposure as compared with respective controls. 8 differentially expressed proteins, which showed consistent expression pattern were selected and identified by mass spectrometry. The data obtained from mass spectrometry were also matched with the molecular weight and isoelectric point of the respective protein spots in the 2-D gel. The effect of Zn on the differential expression pattern of nigrostriatal proteins was observed maximum at 12 weeks of exposure.

MALDI-TOF and LC/MS analyses identified 8 excised proteins as peroxiredoxin-2 (PRDX 2), profilin-2 (PFN 2), chain-1 refined X-ray structure of rat parvalbumin (PVALB), thioredoxin-dependent peroxide reductase (PRDX 3), RBL-NDP kinase 18 kDa subunit (p18), dihydrolipoamide acetyltransferase (E2), malate dehydrogenase (MDH) and hypoxanthine-guanine phospho ribosyl transferase (HGPRT) proteins and their locations in the gels are shown (Fig. 1). Among 8 identified proteins 7 were up regulated in 2-D PAGE while PVALB was down regulated. Other proteins could not be identified either because of quantitative limitations or owing to some other experimental reasons.

MALDI mass spectra and probability plot showed m/z-values obtained for each protein that were used to search proteins in available protein database. For proteins ions score was \(-10\log (P)\), observed match was random and protein scores more than 46 were considered significant \((p<0.05)\). Individual ion scores indicated identity or extensive homology and protein scores were derived from ion scores as a non-probabilistic basis for ranking protein hits. The probability based MOWSE score obtained for peroxiredoxin-2 was 52 \((p<0.05)\) with 2 query and maximum 20 hits. The probability based MOWSE score obtained for chain-1 refined X-ray structure of rat parvalbumin was 96 \((p<0.05)\) with 1 query and maximum 20 hits. The probability based MOWSE score obtained for malate dehydrogenase was 46 \((p<0.05)\) with 1 query and maximum 20 hits. The probability based MOWSE score obtained for
profilin-2 was \(101\) (\(p<0.05\)) with \(3\) query and maximum \(20\) hits. The probability based MOWSE score obtained for thioredoxin-dependent peroxide reductase was \(60\) (\(p<0.05\)) with \(1\) query and maximum \(20\) hits. The probability based MOWSE score obtained for HGPRT was \(93\) (\(p<0.05\)) with \(1\) query and maximum \(20\) hits. The probability based MOWSE score obtained for dihydrolipoamide acetyltransferase was \(220\) (\(p<0.05\)) with \(6\) query and maximum \(20\) hits. The probability based MOWSE score obtained for RBL-NDP kinase 18 kDa subunit (p18) was \(61\) (\(p<0.05\)) with \(3\) query and maximum \(20\) hits.

Fig. 1
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Box 1. Dihdrolipoamide acetyltransferase (E2)

Box 2. Malate dehydrogenase (MDH)
Hypoxanthine-guanine phospho ribosyl transferase (HGPRT)

Box 3. Thioredoxin-dependent peroxide Reductase (PRDX 3)

Box 4. Peroxiredoxin-2 (PRDX 2)

Box 5. RBL-NDP Kinase 18 KDa subunit (p18)
Profilin-2 (PFN 2)

Box 6. Parvalbumin (PVALB)
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Figure 1: MALDI-TOF and LC/MS identified protein spots of 2-D gels in the nigrostriatal tissues of control and Zn treated rats. The location of differentially expressed proteins in the gel, which were established following mass spectrometry of the spots and database search for homology, are also shown. MALDI-TOF and LC/MS identified proteins as (box. 1) dihydrolipoamide acetyltransferase (E2), (box. 2) malate dehydrogenase (MDH) and hypoxanthine-guanine phospho ribosyl transferase (HGPRT), (box. 3) thioredoxin-dependent peroxide reductase (PRDX 3), (box. 4) peroxiredoxin-2 (PRDX 2), (box. 5) RBL-NDP kinase18 KDa subunit (p18) and profilin-2 (PFN 2), (box. 6) chain-1 refined X-ray structure of rat parvalbumin (PVALB). C = control and Z = zinc treated.

5.3.3.2. Western blot analysis of Peroxiredoxin 2 and Profilin 2

The increased expression of PRDX 2 and PFN 2 in nigrostriatal tissues of Zn-treated rats as compared with controls were observed in western blot as also obtained in the 2-D PAGE. The expression of PRDX 2 (Fig. 2A) and PFN 2 (Fig. 2B) were augmented in Zn-treated rats in a time of exposure dependent manner as compared to controls.

Fig. 2A
Figure 2: (A) Effect of Zn treatment on the expression of peroxiredoxin 2 and (B) profilin 2 protein in nigrostriatal tissue of control and Zn (20 mg/kg) treated rats after 2, 4, 8 and 12 weeks of exposure. Upper panels of blots show representative figure of peroxiredoxin 2 and profilin 2 protein, while lower panels show respective densitometric analyses. The results are expressed as mean ± SEM (n = 4). (w = week, C = control and Z = Zn-treated; *** = p<0.001 and * = p<0.05 as compared with control groups).

5.4. DISCUSSION

Zn induces reactive oxygen species production by promoting mitochondrial and extra-mitochondrial pathway. Our findings are consistent with the view of oxidative stress involvement in PD pathogenesis, as suggested by over expression of mitochondrial and ROS-scavenging proteins. Increased expression of PRDX 2 and PFN 2 from the proteomics confirmed that Zn induces oxidative stress and show PD like phenotype.
PRDX 2, an endogenous antioxidant plays very important role in human PD pathogenesis as well as in toxins-induced models of PD. The increased expression of PRDX 2 in the Zn-exposed rats validated the role of PRDX 2 in oxidative stress as well as in PD (Basso et al., 2004; Orth et al., 2002; Przedborski et al., 2000). PRDX 2 provides protection against oxidative stress and regulates apoptosis by eliminating peroxides generated during metabolism (Yim et al., 1994; Netto et al., 1996; Kim et al., 2000; Saratlan et al., 1999). PRDX 3 is also an antioxidant enzyme which regulates the oxidative stress generated by redox reactions in the cell. It protects radical-sensitive enzymes from oxidative damage by a radical-generating system. It also acts with MAP3K13 to regulate the activation of NF-kappa-B in the cytosol (Masaki, et al., 2003). Acidic shift of PRDX 3 has been observed in PC12 cells following 6-OHDA treatment (Saito et al., 2007), which indicate oxidative modification of PRDX 3 and its close involvement in the regulation of 6-OHDA-induced apoptotic signaling in DA neuronal death. Thus, PRDX 3 may be one of the key enzymes that control the intracellular concentration of hydrogen peroxide in mitochondria, which are toxic to cells. An increased expression of PRDX 2 and PRDX 3 in Zn-treated rats is in accordance with a study which has shown an increased expression of PRDX 2 and PRDX 3 in MB and PQ exposed neuroblastoma cells (Roede et al., 2011). The increased expression of PRDX 2 and PRDX 3 could be an adaptive mechanism to resist against oxidative stress induced by Zn.

An increased expression of PFN 2 in Zn-treated rats is in accordance with a study which has shown an increased expression of profilin in PD patients (Basso et al., 2004). PFN 2, a cytoskeleton interacting protein binds to actin monomers resulting in cytoskeletal changes. PFN 2 is required for actin polymerization in the synapse, which controls neurotransmitter release, neuronal excitability and novelty-seeking behavior in mice (Boyl et al., 2007).

\[ \text{Ca}^{2+} \] buffering in SNpc region is essential to protect the dopaminergic cells. A good \[ \text{Ca}^{2+} \] buffering capacity is necessary, if toxicity through oxidative stress or excitotoxicity is to be avoided (Surmeier et al., 2011). Decreased level of PVALB in Zn-induced PD phenotype might be a reason to enhance the susceptibility of Zn-exposure, since \[ \text{Ca}^{2+} \] buffering become hampered, consequently cells become more prone to excitotoxic death or degeneration by other means. \[ \text{Ca}^{2+} \] entry through L-type channels in SNpc dopaminergic neurons occur throughout pace making cycle,
contrasting them with neighboring dopaminergic neurons in ventral tegmental area (VTA), which is less affected region in PD (Guzman et al., 2010; Heizmann, 1992).

E2, an enzyme component of the multi-enzyme pyruvate dehydrogenase complex, is responsible for the pyruvate decarboxylation. This protein was found to be up regulated in Zn-exposed animals. Although E2 is not directly involved in PD pathogenesis, its expression could be due to Zn-mediated dysfunction of the carbohydrate metabolism. Furthermore, MDH and HGPRT are the enzymes involved in the carbohydrate metabolism and nucleotide biosynthesis pathways. The alteration in the expressions of these proteins in the Zn-exposed rats further supports assumption that Zn induces impairment of various metabolic pathways of PD, as observed in other neurological diseases (Shelinen et al., 2010; Sensi et al., 2000).

Thus, the present study insights the Zn-induced alteration of metabolic, mitochondrial and oxidative stress pathway which causes degeneration of dopaminergic neurons resulted in PD. The proteins obtained from Zn-exposed rats showed the resemblance with other pesticide-induced PD model confirming that involvement of Zn in dopaminergic neurodegeneration, however, identification of more proteins is needed for better insight into the molecular mechanism of Zn-induced Parkinsonism.
Parkinson's disease (PD) is the second most common chronic neurodegenerative disease. It is characterized by selective and progressive degeneration of dopaminergic neurons in the substantia nigra pars compacta (SNpc) leading to substantial deficit in dopamine, responsible for motor function regulation. Despite first scientific description of the disease in 1817 by James Parkinson, complete molecular pathogenesis, exact contributory factors and permanent cure are unknown till date. Age, genetic factors and environmental factors mainly contribute to the multifactorial etiology of PD. Among environmental factors, heavy metals exposure has shown strong association with increased risk for onset and progression of PD. Postmortem studies have shown increased accumulation of Zinc (Zn) in brain of PD patients indicating its role in PD.

Zn is the second most abundant transition metal inside brain. It is redox inert and has structural, catalytic, and regulatory roles in cellular biology. ZnSO$_4$ is globally used in the agricultural field by farmers to improve the quality and quantity of food grains. Besides, people involved in paint manufacturing, electrometallurgy and mine and smelting industries are also exposed to excessive level of Zn. The intranigral Zn infusion and systemic exposure of Zn is also reported to cause neurodegeneration in rats. Although previous studies have documented oxidative stress-mediated neurodegeneration by Zn but the entire molecular mechanism underlying Zn-induced dopaminergic neurodegeneration has yet not been completely deciphered.

In first part of study (Chapter 3), we investigated the dose and time response of Zn exposure on motor functions in exposed animals. Furthermore, Zn-induced neurodegenerative changes were correlated with oxidative stress and antioxidant enzymes in the nigrostriatal tissues of brain of exposed rats. For this male Wistar rats were exposed with Zn for 2-12 weeks and neurobehavioral parameters, level of dopamine, its metabolites and oxidative stress indices were assessed. The tyrosine hydroxylase (TH) immunoreactivity was performed in the SNpc to assess dopaminergic neurodegeneration in the Zn-treated animals. A significant reduction was observed in neurobehavioral parameters (SLA and rotarod), levels of striatal dopamine and its metabolites. Differential modulation of cytosolic Cu,Zn-SOD/SOD1 and mitochondrial Mn-SOD/SOD2 was observed following Zn exposure at activity
and protein/gene level, thereby suggesting the role of increased superoxide radical formation via both cytosolic and mitochondrial pathways in Zn-induced oxidative stress in rat nigrostriatal brain tissue. Zn-induced time dependent increase in HO-1 expression and LPO level further supported the role of oxidative stress in Zn-induced neurodegeneration. The results obtained thus suggest that Zn-induced oxidative stress exhibited by increased LPO, SOD, HO-1 and decreased catalase and GST activities could be responsible for Zn-induced neurobehavioral, biochemical and molecular changes characteristic of PD phenotype.

Increased generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS), play a critical role in idiopathic and chemically-induced PD. Among RNS, nitric oxide (NO) synthesized by nitric oxide synthase (NOS) plays a vital role in physiological and pathological conditions. The role of NOS (nNOS/iNOS) has been implicated in dopaminergic neurodegeneration in toxin based PD models as well as in sporadic PD. The second part of the study (Chapter 4) therefore deals with the role of NO/nNOS in Zn-induced dopaminergic neurodegeneration. A significant attenuation was observed in nitrite content and total NOS activity in time of exposure dependent manner in treated group, which was elucidated to be due to inhibition of nNOS at activity, protein and gene level. Physiological levels of NO/nNOS are vital for normal brain functions therefore; to investigate the association between altered NO/nNOS and neurodegeneration, the effect of NO donors (SNP/SNAP) was evaluated on Zn-induced neurodegeneration. Neurobehavioral analyses, striatal DA and its metabolites, immunohistochemical analyses of dopaminergic neurons and oxidative stress indices were assessed in control and Zn-treated in presence and absence of NO donors. In addition, effect of NO donors on apoptosis was also assessed by analyzing cytochrome c (cyt c) release and caspase-3 activation in control and treated groups. Both SNP and SNAP significantly restored Zn-induced modulations in neurodegenerative and oxidative stress indices. NO donors also protected against Zn-induced cyt c release and caspase-3 activation suggesting that decreased NO/nNOS facilitates dopaminergic neurodegeneration. The results thus suggested that NO plays a protective role against Zn-induced dopaminergic neuronal cell death in rats.

Proteins are the main effector molecules, which decide the fate of physiological and pathophysiological events. Study of a large number of proteins may be crucial for
understanding the Zn-induced PD. Proteomic studies have exposed many pathways that are linked with disease pathogenesis and it may lead to the development of potential therapeutic targets. The 3rd part of the study (Chapter 5) investigated the effect of Zn on proteome profile in the nigrostriatal region of brain of control and Zn exposed rats employing 2-D PAGE technique. 30 proteins were observed to be differentially expressed in treated groups at different time period (2, 4, 8, and 12 weeks) following Zn exposure. Eight out of differentially expressed proteins, which showed same pattern of expression at all time points, were randomly selected and identified by MALDI-TOF and LC/MS. The identified proteins were peroxiredoxin-2, profilin-2, parvalbumin, thioredoxin-dependent peroxide reductase also called peroxiredoxin-3, RBL-NDP kinase 18 kDa subunit (p18), dihydrolipoamide acetyltransferase, malate dehydrogenase and hypoxanthine-guanine phospho ribosyl transferase. Among identified proteins 7 were up-regulated while parvalbumin was down-regulated. Other proteins could not be identified either because of quantitative limitations or owing to some other experimental reasons. The increased expression of peroxiredoxin-2 profilin-2 was further validated by western blotting. Peroxiredoxin-2 and peroxiredoxin-3 act as ROS scavengers, dihydrolipoamide acetyltransferase is involved in pyruvate decarboxylation while malate dehydrogenase and hypoxanthine-guanine phospho ribosyl transferase are the enzymes involved in the carbohydrate metabolism and nucleotide biosynthesis pathways respectively. Profilin-2 is a synaptic protein, which helps in regulation of neurotransmitter release. Parvalbumin plays a role in calcium buffering. The results thus obtained suggest that proteins involved in oxidative stress regulation, energy production, metabolic pathways, neurotransmission and calcium homeostasis might contribute in Zn-induced Parkinsonism.

The results from the present study can be concluded as:

- Zn-induced oxidative stress as evident by increased LPO level and HO-1 expression, decreased catalase and GST activities, which caused dopaminergic neuronal loss in exposed rats.

- Differential modulation of cytosolic Cu,Zn-SOD/SOD1 and mitochondrial Mn-SOD/SOD2 suggested involvement of both cytosolic and mitochondrial
pathways in Zn-induced Parkinsonism similar to chemically-induced and idiopathic PD.

- Zn inhibited nNOS resulting in reduced nitrite content and increased oxidative stress, which in turn caused apoptosis of dopaminergic neurons. NO donors provided protection against Zn-induced neurodegeneration thereby suggesting protective role of nNOS in Zn-induced Parkinsonism.

- Analyses of Zn-induced differential protein pattern revealed proteins involved in oxidative stress regulation, energy production and neurotransmission might contribute in Zn-induced dopaminergic neurodegeneration.

Thus the present investigation provides an insight about behavioral, biochemical and molecular mechanisms of Zn-induced PD in rats and also brought forth the similarity and dissimilarity of Zn-induced Parkinsonism with the existing toxin-based PD models and sporadic PD.