Chapter 3

Oxidative stress in zinc-induced dopaminergic neurodegeneration: Implications of superoxide dismutase and heme oxygenase-1
3.1 INTRODUCTION

Parkinson's disease (PD) is the second most common progressive neurodegenerative disorder after Alzheimer disease, characterized by a progressive loss of dopaminergic neurons in the substantia nigra pars compacta. 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 (Dauer et al., 2003). However, ageing, genetic factors and environmental exposure to pesticides and heavy metals have emerged as the putative risk factors (Patt et al., 1991).

Epidemiological and experimental data have established pesticides and heavy metals exposure as the major environmental risk factors for PD (Gorell et al., 1997; Singh et al., 2007). Clinical studies with postmortem brain tissues of PD patients have shown an increased accumulation of zinc (Zn) in the SN region indicating its role in PD pathogenesis (Dexter et al., 1992; Jenner et al., 1992). Zn is an essential transition element, which forms structural or functional component of several proteins including enzymes necessary for replication, transcription and translation thereby facilitating normal physiological processes, cell division and differentiation (Takeda, 2000). Zn possesses both antioxidant and prooxidant properties therefore; may be neuroprotective or neurotoxic depending upon the concentration. Excess Zn levels are associated with neurodegenerative diseases including Alzheimer disease (AD) and PD (Dexter et al., 1991; Takeda 2000., Lin et al., 2003).

Oxidative stress is implicated as the primary event in idiopathic and chemically-induced PD. Increased free radical production; mitochondrial dysfunction and impaired antioxidant defense system are the key players contributing to oxidative stress in PD pathogenesis. Although Zn is redox inactive, the role of oxidative stress in Zn-induced dopaminergic neurodegeneration is documented, which is supported by the protective effect of vitamin D3 against Zn-induced neurodegeneration in rats in vivo (Lin et al., 2003; Kumar et al., 2010). Zn is reported to potentiate the neurodegeneration induced by 1-methyl 4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), dopamine and iron (Fe) via increased oxidative stress (Lin et al., 2001; Hussain et al., 2002; Lo et al., 2004). Role of superoxide free radical generated by nicotinamide
adenine dinucleotide phosphate oxidase (NADPH oxidase) in sporadic and chemically-induced PD has been implicated (Wu et al., 2003; Cristovao et al., 2009). Protective effect of superoxide dismutase (SOD)/catalase mimetics against PD has confirmed the deleterious role of free radical-induced oxidative stress in PD pathogenesis (Pong et al., 2002; Mollace et al., 2003). In vitro studies with cultured cortical cells have shown Zn-induced superoxide radical formation (Noh et al., 2000). SOD is the enzyme responsible for removal of superoxide radicals via conversion into hydrogen peroxide, which is then neutralized by peroxidases such as catalase, glutathione peroxidase (GPx), etc. SOD exists mainly in three isoforms-cytosolic/Cu,Zn-SOD/SOD1, mitochondrial/Mn-SOD/SOD2 and extracellular SOD/SOD3. Several studies have reported altered SOD activity in the substantia nigra of brain of PD patients and chemically-induced PD phenotypes (Jenner et al., 1992; Saggu et al., 1989; Yu et al., 2010). Hemeoxygenase-1 (HO-1), an enzyme involved in heme metabolism, is a cellular stress protein, which protects against oxidative stress-induced damage (Schipper et al., 2004; Minelli et al., 2009). Postmortem studies showing increased HO-1 expression in substantia nigra of brain and serum of PD patients implicated its role in PD pathology (Schipper et al., 1995; Mateo et al., 2010). HO-1 is found to protect against 1-methyl-4-phenylpyridinium (MPP+) and 6-OHDA-induced dopaminergic neurodegeneration (Huang et al., 2010; Yamamoto et al., 2010). Recent genetic studies have also reported 4-fold higher risk for PD in individuals possessing single nucleotide polymorphism (SNP) in HO-1 and glycogen synthase kinase-3β (GSK3β) genes (Schipper et al., 1998). HO-1 gene is regulated by Nrf-2 transcription factor via mitogen activated protein kinase (MAPK) and p38 pathways, which increase anti-inflammatory changes thereby protecting against inflammation (Jazwa et al., 2010). Glutathione (GSH) plays a protective role against oxidative stress-induced damage via neutralization of free radicals. GSH synthesis from amino acids is catalyzed by the enzymes- γ-glutamyl cysteine synthetase (γ-GCS) and glutathione synthase (GS). Reduced GSH levels are reported in both sporadic PD and animal models of PD (Sian et al., 1994; Kang et al., 2009; Tsai et al., 2010). Intra-nigral Zn infusion is reported to attenuate GSH levels but its effect on GCS is not yet known. Although systemic Zn exposure attenuates spontaneous locomotor activity (SLA), striatal dopamine content, tyrosine hydroxylase (TH) positive cells and increased lipid peroxidation in exposed rats (Kumar et al., 2010), the effect of Zn on cytosolic and mitochondrial superoxide dismutases (SOD), heme-
oxygenase-1 (HO-1) and glutathione synthesizing enzyme- $\gamma$-glutamyl cysteine synthetase ($\gamma$-GCS) is not yet investigated. The present study was therefore undertaken to investigate the effect of Zn on activities and/or expressions of total, cytosolic and mitochondrial SODs along with HO-1 and $\gamma$-GCS and to correlate the changes with the Zn-induced changes in the motor activities, striatal dopamine content and changes in TH protein expression in Zn-exposed animals to further explore the mechanism of oxidative stress in Zn-induced neurodegeneration in rats.

3.2 Objective

- To investigate the effect of Zn exposure on neurobehavioral parameters (SLA and rotarod), striatal dopamine and its metabolite content, TH-immunoreactivity and its protein expression in nigrostriatal region of control and exposed animals.
- To investigate the effect of Zn on activities and/or expressions of total, cytosolic and mitochondrial SODs along with HO-1 and $\gamma$-GCS and to correlate the changes with zinc-induced neurodegenerative indices to further explore the role of oxidative stress in Zn-induced neurodegeneration in rats.

3.3 Results

3.3.1. Neurobehavioral analyses

A significant dose and time dependent decrease was observed in SLA of treated groups following zinc exposure from 4 weeks onwards and the maximum attenuation was observed after 12 weeks of exposure at 20 mg/kg zinc sulfate dose (Fig. 1A).

Zinc treatment caused a significant decrease in the time of stay on rotarod in the Zn-exposed animals and the reduction was in a dose and time of exposure dependent manner. Animals treated with 15 and 20 mg/kg dose showed reduction after 4 weeks of treatment while the dose dependent effect was observed for all three doses after 8 and 12 weeks of exposures. Rats treated with 20 mg/kg dose showed maximum reduction in the stay time at the rotating rod following 12 weeks of exposure (Fig. 1B).
Figure 1: Effect of Zn exposure on the SLA (A) and rotarod performance of rats (B) following 2, 4, 8 and 12 weeks exposure at different doses. The data expressed are mean ± SEM (n = 3 independent set of experiments and each set included 5 animals per group). (w = week, *** = p<0.001, ** = p<0.01, and * = p<0.05 as compared with control; ### = p<0.001 as compared with Zn 10 mg/kg treated group, as compared with Zn 15 mg/kg treated group).
3.3.2. Striatal dopamine and its metabolites content

Zn exposure resulted in significant attenuation of striatal dopamine content along with its metabolites i.e., DOPAC and HVA in a dose and time of exposure dependent manner resulting in the maximum decrease following Zn exposure at 20 mg/kg dose after 12 weeks of treatment (Fig. 2A, 2B & 2C).

Fig. 2A:

![Graph showing striatal dopamine content with different doses of Zn exposure at various time points.](image)

Fig. 2B:

![Graph showing DOPAC content with different doses of Zn exposure at various time points.](image)
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Figure 2: Effect of Zn on striatal dopamine level (A), DOPAC (B) and HVA levels (C) in rats following 2, 4, 8 and 12 weeks of exposure. The results are expressed as mean ± SEM (n = 4-5). (w = week, *** = p<0.001, ** = p<0.01, and * = p<0.05 as compared with control; ### = p<0.001, ## = p<0.01, # = p<0.05 as compared with Zn 10 mg/kg treated group; $$$ = p<0.001, $$ = p<0.001 and $ = p<0.05 as compared with Zn 15 mg/kg treated group).

3.3.3. Immunohistochemistry and TH protein expression

Immunohistochemical analysis of TH-positive neurons was performed in control and Zn-treated (20 mg/kg) animals at different exposure time periods to investigate the effect of Zn exposure on dopaminergic neurodegeneration. A significant and time dependent decrease was observed in the number of TH-positive neurons in the SN region of the brain of exposed groups as compared to control groups from 4 weeks onwards indicating that Zn induced dopaminergic neuronal loss characteristic of PD (Fig 3A). Zn treatment resulted in time dependent decrease in the TH protein levels in the nigrostriatal tissues of exposed groups from 4 weeks onwards (Fig. 3B). Maximum reduction was obtained after 12 weeks of Zn exposure.
Fig. 3A:

![Images showing time of exposure (2w, 4w, 8w, 12w) for control (C) and zinc (Z) groups.]

Fig. 3B:

![Bar graph showing TH protein expression (TH/β-actin band density ratio) at 2w, 4w, 8w, and 12w.]

- TH (55 kDa)
- β-actin (43 kDa)
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3.3.4. Oxidative stress Indices

3.3.4.1. LPO, catalase and GST

A significant augmentation was observed in LPO levels in the nigrostriatal tissues of brain of Zn-treated animals in a dose and time of exposure dependent manner with maximum increase obtained after 12 weeks of exposure (Fig. 4A). On the contrary, Zn exposure resulted in attenuation of both catalase and CDNB related total GST activities in the nigrostriatal tissues of treated groups in a dose and time of exposure dependent manner (Fig. 4B & 4C).

Figure 3: (A) Immunohistochemical analysis of TH-positive dopaminergic neurons in the SN of the control and Zn (20 mg/kg) treated animals. The upper panel shows the representative pictures of TH-immunoreactivity and lower panel depicts the results in bar diagram showing % change in a number of TH-positive neurons from control groups (n = 4). (B) Effect of Zn (20 mg/kg) on TH protein expression in control and treated rats after 2, 4, 8 and 12 weeks of exposure (n = 4). The results are expressed as mean ± SEM. (w = week, C = control; Z = Zn treated; (*** = p<0.001, ** = p<0.01, and * = p<0.05 as compared with control group).
Fig. 4A:

![Graph showing MDA levels over time for different zinc doses](image)

Fig. 4B:

![Graph showing Catalase activity over time for different zinc doses](image)
3.3.4.2. SOD activity

The total SOD activity was increased significantly in treated groups after 2, 4, 8, and 12 weeks in a dose and time of exposure dependent manner (Fig. 5A). Furthermore, in order to investigate whether the effect was due to cytosolic (SOD1/CuZn-SOD) or mitochondrial SOD (SOD2/ Mn-SOD), activities of SOD1 and SOD2 were also estimated. Zn exposure significantly augmented the activities of both SOD1 and SOD2 in a dose and time of exposure dependent manner. Increase in SOD1 activity was observed from 2 weeks onwards while that in SOD2 activity was observed from 4 weeks exposure onwards (Fig. 5B & 5C). Augmentation in SOD1 activity was greater than that obtained in SOD2 and preceded that observed in SOD2 activity.
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Fig. 5A:

![Graph showing Total SOD Activity over Time](image)

Fig. 5B:

![Graph showing Cu-Zn SOD activity over Time](image)
Figure 5: Effect of Zn treatment on the activity of total SOD along with its cytosolic (Cu,Zn-SOD/SOD1) and mitochondrial (Mn-SOD/SOD2) isoforms in the nigrostriatal tissue of rat brain following 2, 4, 8 and 12 weeks of exposure. (A) Shows total SOD activity at different doses of Zn. (B) and (C) show effect of Zn exposure on the activity of SOD1 and SOD2 respectively. The results are expressed as mean ± SEM (n = 4-5). (w = week, *** = p<0.001, ** = p<0.01, and * = p<0.05 as compared with control; ### = p<0.001, ## = p<0.01, # = p<0.05 as compared with Zn 10 mg/kg treated group; and $$$ = p<0.001, $$ = p<0.01, as compared with Zn 15 mg/kg treated group).

3.3.5. Protein/gene expression of SOD1 and SOD2

Since SOD1 and SOD2 activity were induced following Zn exposure therefore, effect of zinc exposure was also analyzed on protein/mRNA expression of SOD1 (Cu,Zn-SOD) and SOD2 (Mn-SOD) following 2, 4, 8 and 12 weeks of exposure at the highest dose i.e., 20 mg/kg body weight. A significant increase was observed in both the protein and mRNA expression of SOD1 following Zn treatment after 4 weeks in a time of exposure dependent manner while protein/mRNA expression of SOD2 remained unaltered following Zn treatment even after 12 weeks of exposure (Fig. 6A & 6B).
Fig. 6A:

![Image of gel electrophoresis with bands for SOD1 and β-actin](image)

**Graph 1:**
- X-axis: Time of exposure (2w, 4w, 8w, 12w)
- Y-axis: Band density ratio (SOD1/β-actin)
- Data points with error bars for each time point

**Graph 2:**
- X-axis: Time of exposure (2w, 4w, 8w, 12w)
- Y-axis: SOD1 mRNA expression (SOD1/β-actin band density ratio)
- Data points with error bars for each time point

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Fig. 6B:

**Band density ratio (SOD2/β-actin)**

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**SOD2 (483bp)**

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**β-Actin (155 bp)**

**SOD2 mRNA expression (SOD2/β-actin band density ratio)**

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Figure 6: (A) Effect of Zn treatment on SOD1 protein and mRNA expression in nigrostriatal tissue of control and Zn (20 mg/kg) treated groups following 2, 4, 8 and 12 weeks of exposure. Upper panel shows representative picture of SOD1 protein expression along with its densitometric analysis. Lower panel shows mRNA expression of SOD1 in control and treated rats at different exposure time periods along with densitometric analysis of the same. (B) Effect of Zn treatment on SOD2 protein and mRNA expression in the nigrostriatal tissue of the control and Zn (20 mg/kg) treated groups following 2, 4, 8 and 12 weeks of exposure. Upper panel shows representative picture of SOD2 protein expression in the control and Zn-treated groups and densitometric analysis of the same. Lower panel shows representative gel picture of mRNA expression of SOD2 in the control and treated rats at different exposure time periods and its densitometric analysis. The results are expressed as mean ± SEM (n = 4). (w = week, C = control and Z = Zn-treated; *** = p<0.001, ** = p<0.01 and * = p<0.05 as compared with control groups).

3.3.6. Gene/Protein expression on HO-1 and γ-GCS

Effect of Zn on oxidative stress marker gene HO-1 and γ-GCS were also analyzed. Protein/mRNA expression analysis of HO-1 performed in control and Zn-treated groups at the highest dose and varying time periods of exposure revealed significant augmentation in HO-1 expression in Zn exposed groups in a time of exposure dependent manner indicating the oxidative stress induction following Zn exposure while no significant change was observed in the γ-GCS expression after Zn treatment (Fig. 7A & 7B).
Fig. 7A:

**HO-1 protein expression (HO-1/β-actin band density ratio)**

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<td>Z</td>
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<td>1.4</td>
<td>1.6</td>
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**HO-1 mRNA expression (HO-1/β-actin band density ratio)**

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Figure 7: Effect of Zn exposure on protein/gene expression of HO-1 and γ -GCS in nigrostriatal tissue of rat brain following 2, 4, 8 and 12 weeks of treatment at 20 mg/kg dose. (A) Upper panel shows representative image of the protein/gene expression of HO-1 in control and Zn exposed groups and lower panel shows the densitometric analysis of the same. (B) Upper panel shows representative image of the gene expression of γ -GCS in control and Zn exposed groups and lower panel shows the densitometric analysis of the same. The results are expressed as mean ± SEM (n = 4). (w = week, C = control; Z = Zn-treated; *** = p<0.001, ** = p<0.01, and * = p<0.05, as compared with control group).

3.4. DISCUSSION

Zinc, an essential transition element, is required in trace amounts for maintenance of normal physiological processes. Epidemiological studies have shown strong positive association of heavy metals exposure with PD (Gorell et al., 1997; Singh et al., 2007). Clinical studies have demonstrated an increase in accumulation of Zn in the SN pars compacta of brain of PD patients implicating its role in PD pathogenesis (Jenner et al., 1992; 1989). The 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) however; the entire mechanism of Zn-induced dopaminergic neurodegeneration is not yet completely established. In the present study, Zn exposure resulted in dose and time dependent decrease in SLA and rotarod performance, which indicated that Zn induces neurobehavioral changes characteristic to PD in accordance to earlier reports (Lin *et al.*, 2003; Kumar *et al.*, 2010).

The significant attenuation of striatal dopamine content along with reduction of its metabolites (DOPAC and HVA) suggested that dopamine metabolism was affected by Zn exposure as previously depicted in sporadic and chemically-induced PD phenotypes, including Zn-induced dopaminergic neurodegeneration (Lin *et al.*, 2003; Kumar *et al.*, 2010; Patel *et al.*, 2006; Thiruchelvam *et al.*, 2003). Since TH is the enzyme responsible for dopamine synthesis, its reduction in time of exposure dependent manner further supported the HPLC data interpretation that dopamine synthesis is impaired in the nigrostriatal tissue of brain of Zn-treated animals suggesting Zn-induced dopaminergic neurodegeneration as reported in earlier studies showing decreased TH-immunoreactivity and TH protein expression following intra-nigral Zn infusion and systemic Zn exposure in rats (Lin *et al.*, 2001; Kumar *et al.*, 2010). We have earlier reported that systemic Zn exposure caused reduced SLA, striatal dopamine content, TH protein expression and number of TH-positive dopaminergic neurons along with increased LPO levels (Kumar *et al.*, 2010) and these parameters were assessed in this study as well to correlate with Zn-induced alterations in SOD1, SOD2, HO-1 and GCS and Zn-induced dopaminergic neuronal degeneration.

Oxidative stress is established as a major player in the onset and progression of PD. Increased LPO levels are reported in the SN of PD patients and neurotoxin-induced animal PD models (Jenner *et al.*, 1992; Kumar *et al.*, 2010; Dexter *et al.*, 1989; Singh *et al.*, 2008; Gupta *et al.*, 2010). The present study exhibited dose and time dependent augmentation in LPO levels of the nigro striatal brain tissues of Zn-treated animals similar to previous studies showing an increased LPO levels by intra-nigral Zn infusion and systemic Zn exposure in rats (Lin *et al.*, 2001; Kumar *et al.*, 2010). Involvement of free radical generating enzymes viz., NADPH oxidase, xanthine oxidase, nitric oxide synthase and mitochondrial dysfunction is reported in ROS
production in sporadic and chemically-induced PD (Wu et al., 2003; Miller et al., 2007). SOD facilitates dismutation of superoxide radicals to form H$_2$O$_2$, thereby protecting against free radical-mediated damage. Significant augmentation observed in the total SOD activity might result in accumulation of end product H$_2$O$_2$, which could lead to inhibition of catalase activity due to feedback inhibition obtained in the present study and reported in our previous study also (Kumar et al., 2010). Differential modulation of cytosolic and mitochondrial SOD is reported in PD patients. A study has shown reduction of Cu,Zn-SOD in the SN of PD patients’ brain while others have reported increased particulate SOD/Mn-SOD levels in the SN of brain of sporadic PD patients (Jenner et al., 1992; Saggu et al., 1989; Kunikowska et al., 2003). Protective effect of SOD/catalase mimetics against chemically-induced experimental models of PD along with decreased dopaminergic neuronal toxicity in animals overexpressing Cu,Zn-SOD have established the protective role of SOD against oxidative stress-mediated PD pathogenesis (Hung et al., 1998; Cadet et al., 1994; Thiruchelvam et al., 2005; Peng et al., 2005). Zn-induced augmentation of total SOD activity in exposed animals observed in this study is similar to previous reports showing increased SOD levels in PD patients’ brain and toxin-induced animal PD phenotypes (Saggu et al., 1989; Lessner et al., 2010). The increased SOD activity might be the defense mechanism against increased oxidative stress, which is supported by previous study showing protective action of SOD/catalase mimic EUK-139 against Zn-induced ROS generation in cortical neuronal cultures (Pong et al., 2002). The present study exhibited differential modulation of cytosolic Cu,Zn-SOD/SOD1 and mitochondrial Mn-SOD/SOD2 following Zn exposure, which suggested the role of increased superoxide radical formation via both cytosolic and mitochondrial pathways in Zn-induced oxidative stress in rat nigrostriatal brain tissue. This is in accordance with earlier reports demonstrating the involvement of NADPH oxidase (comprised of cytosolic and plasma membrane bound subunits) and mitochondrial dysfunction in Zn-induced increased free radical formation in vitro in cortical and microglial cell cultures (Noh et al., 2000; Kauppinen et al., 2008). SOD1 levels were elevated earlier and to a greater extent than the increase observed in SOD2 activity and protein/gene expression of only SOD1 was augmented suggesting that cytosolic pathway might be the major contributor in free radical production and the preceding event in the free radical generation cascade induced by Zn exposure in the rat brain however, further investigation is needed to establish the actual sequence
of the events. 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). Increased HO-1 expression is reported in brain of PD patients and 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). Zn-induced time dependent increase in HO-1 protein/mRNA expression further supported the role of oxidative stress in Zn-induced neurodegeneration and is in sync with the previous reports documenting increased HO-1 activity and expression by oxidative stress-inducing agents (Barlow et al., 2005; Yang et al., 2005; Noriega et al., 2002).

Protective role of GSTs, a family of phase II toxicant metabolizing enzymes is well documented in idiopathic PD and chemically-induced PD phenotypes (Kumar et al., 2010; Patel et al., 2006; Smeyn et al., 2007; Shi et al., 2009). Altered GST activity/expression is reported in the experimental PD models (Patel et al., 2006; Singh et al., 2008; Smeyn et al., 2007; Shi et al., 2009). GSTs are known to facilitate the detoxification of reactive metabolites of toxins formed during phase I reactions by conjugation with GSH therefore dose and time dependent attenuation of CDNB related GST activity observed in Zn-treated groups implies that either Zn might be directly inhibiting the GST activity or GSH depletion might be responsible for reduction in GST activity. Since GSTs play a protective role against neurodegeneration therefore, decreased GST activity might contribute to increased neuronal loss following Zn treatment. GSH is the main non-enzymatic antioxidant, which 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 is reported in literature but direct effect of Zn on GCS, a rate limiting enzyme in GSH synthesis is not yet investigated (Lin et al., 2001). The present study revealed no significant alterations in the mRNA expression of GCS in Zn-exposed groups suggesting that Zn does not interfere with GSH synthesis at gene level and GSH depletion could be due to an increased consumption for neutralizing free radicals generated by Zn exposure.
or because of direct chelation of GSH by Zn as suggested earlier in study with neonatal cortical neurons in vitro (Chen et al., 2003).

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. Differential modulation of SOD1 and SOD2 isozymes by Zn suggests that both cytosolic and mitochondrial pathways might be involved in Zn-induced oxidative stress leading to dopaminergic neurodegeneration.