Chapter 2

Materials & Methods
**MATERIALS AND METHODS**

### 2.1. Chemicals

Acetic acid, disodium hydrogen phosphate, dibutyl phthalate xylene (DPX), 5, 5'-dithiobis 2-nitrobenzoic acid (DTNB), glutathione reduced (GSH), heptane sulfonic acid, nicotinamide adenine dinucleotide reduced form (NADH), potassium chloride and sodium dihydrogen phosphate were procured from Sisco Research Laboratories (SRL, Mumbai, India). Folin Ciocalteau reagent, hydrogen peroxide (H$_2$O$_2$), nitroblue tetrazolium (NBT), phenazine methosulphate (PMS), potassium cyanide and potassium dichromate were purchased from Merck (Darmstadt, Germany). Acrylamide, biotinylated anti-mouse secondary antibody, bisacrylamide, bovine serum albumin (BSA), bromophenol blue, β-mercaptoethanol, 1-chloro 2,4-dinitrobenzene (CDNB), dextran, ethylenediaminetetraacetic acid (EDTA), ethylene glycol tetraacetic acid (EGTA), 7-Nitroindazole (7-NI), phenylmethanesulphonyl fluoride (PMSF), protease inhibitor cocktail, sodium deoxycholate, Sodium nitroprusside (SNP), sodium dodecyl sulphate (SDS), sodium pyrophosphate, sodium orthovanadate, sodium pyrophosphate, N,N,N',N'-tetramethylethylene diamine (TEMED), thiobarbituric acid (TBA), tris-base, triton-X 100, tween-20, and zinc sulphate (ZnSO$_4$), were procured from Sigma-Aldrich (St. Louis, MO, USA). Neg-50 was purchased from Richard Allen Scientific (Kalamazoo, MI). Perchloric acid was purchased from Ranbaxy Private Limited (New Delhi, India). cDNA synthesis kits, dNTPs, MgCl$_2$, Taq buffer and Taq DNA polymerase were purchased from MBI Fermentas, USA. Gene specific primers were synthesized from Metabion GmbH, Germany. Immobiline pH gradient strips, immobiline pH gradient buffer, and dry strip cover fluid were purchased from GE Healthcare (Chalfont St Giles, UK). Formaldehyde, glycerol, potassium dihydrogen orthophosphate, methanol, silver nitrate, and sodium carbonate were procured from Merck Limited (Mumbai, India). Sodium chloride, magnesium chloride, thiourea, and xylene were procured from Sisco Research Laboratory (Mumbai, India). S-nitroso-N-acetyl-D, L-penicillamine (SNAP) was procured from Cayman chemicals, USA. Mouse monoclonal anti-β-actin antibody, mouse monoclonal anti-cytochrome c antibody, bovine anti-mouse alkaline phosphatase (AP) conjugated, bovine anti-rabbit (AP) and rabbit anti-goat (AP) conjugated antibodies, mouse monoclonal anti-caspase-3, mouse monoclonal anti-nNOS and mouse monoclonal anti HO-1 were procured from Santacruz.
Biotechnology (Santa Cruz, CA, USA). Rabbit anti-Peroxiredoxin2, rabbit anti-Profilin2 and mouse monoclonal anti-TH antibody were procured from Sigma-Aldrich (St. Louis, MO, USA). Mouse anti-NeuN antibody was purchased from Chemicon (Temecula, CA). DAB system, normal goat serum and Streptavidin-peroxidase were procured from Bangalore Genei India Pvt. Ltd. (Bangalore, India). Polyvinylidene difloride (PVDF) membrane was procured from Millipore Corporation (MA, USA). Other chemicals were purchased locally.

2.2. Animal treatment

Male Wistar rats weighing 150-180 g were obtained from the animal facility centre of Indian Institute of Toxicology Research (IITR), Lucknow, India. The study was approved by the Institutional Animal Ethical Committee. Animals were maintained on a 12 h light/dark cycle and given proper pellet diet and water ad libitum. The animals were divided into control and zinc sulfate treated groups. Animals were administered zinc sulfate at 10, 15 and 20 mg/kg body weight, intraperitoneally twice a week for 2, 4, 8 and 12 weeks along with a vehicle which received normal saline. Since 20 mg/kg dose for 12 weeks showed significant neurodegeneration along with PD-like neurobehavioral features therefore this dose was used in further experiments performed for elucidating the role of nitric oxide. Animals were treated with Zn in presence and absence of NO donors' twice weekly along with respective controls for 12 weeks. The donors' (SNP/SNAP- 1mg/kg body weight) were administered intraperitoneally one hour prior to Zn treatment.

2.3. Behavioral studies

For monitoring the motor activity of animals, the two neurobehavioral test i.e. spontaneous locomotor activity (SLA) and rotarod were performed. SLA was assessed in control and treated groups using infrared beam-activated movement monitoring chamber (OptoVarimax-Mini A; Columbus Instruments, Columbus, OH). The animals were placed in the chamber for 1 min before initiating the recording of the activity and locomotor activity was measured for the next 5 min (Kumar et al., 2010). For SLA, at least, 5 animals were included in each group for one experiment and minimum of 3 independent sets of experiments were performed. Results are expressed in terms of total distance travelled in cms/5 min. The rotarod test was also performed in control and treated animals for assessing motor coordination using Omni rotor (Omnitech Electronics Inc., Columbus, OH, USA) consisting of a rod rotating at
a constant speed (5 rpm) with 5 min cut-off time. The animals were trained for three consecutive days before the treatment and the final readings were taken by measuring the time spent on the rotating rod by the animals of control and treated groups (Singh et al., 2010). Similar to SLA, minimum of 3 independent sets of experiments were performed and each set included 5 animals per group. Results were expressed as time of stay in seconds on rotarod.

2.4. Decapitation and dissection of brain

The animals were sacrificed three days after the administration of the last dose by cervical dislocation, decapitated and brain was removed quickly. The brain was dissected under ice cold conditions to isolate the striatum and SN as described earlier (Cruz et al., 1996; Chiu et al., 2007). In brief, the cerebral hemispheres were separated and a cut passing through corpus callosum was made. The cortex was peeled off carefully to expose the striatum, which was taken out. In order to isolate the SN, the mesencephalon was cut into two parts from the ventral side, perpendicular to its long axis at the caudal border exposing SN, which was carefully dissected out. The striatum and SN were pooled and used as nigrostriatal tissues in all experiments except in the measurement of dopamine and its metabolites and TH-immunoreactivity.

2.5. Measurements of striatal dopamine, DOPAC, HVA and Serotonin by HPLC

Dopamine is the neurotransmitter responsible for normal motor functions and disturbance in its metabolism leads to motor dysfunction, characteristic to PD. Therefore, striatal dopamine content and its metabolites i.e., 3,4-dihydroxyphenyl acetic acid (DOPAC) and homovanillic acid (HVA) were estimated through standard HPLC method described elsewhere (De vito and Wagner, 1989). In brief, striatum from brain of experimental rats was suspended in 0.1 M perchloric acid (10% w/v), homogenized, sonicated for 30 sec with 2 intermittent pulses each of 15 seconds and centrifuged at 21,000 \( \times g \) for 30 min at 4\(^\circ\)C. The supernatant was filtered with 0.22 \( \mu \)m syringe filters and levels of dopamine and its metabolites were detected in filtrate by HPLC coupled with electrochemical detector. Mobile phase consisted of 1 mM heptane sulphonic acid, 0.1 M potassium phosphate and 10% methanol (pH 4.0). Filtrate was manually injected into reverse phase C-18 high performance liquid chromatography column and separation of analytes to generate a chromatograph was
obtained at flow rate of 1 ml/min. The dopamine content, DOPAC, HVA and serotonin were calculated using known amount of standards for respective metabolites. The values are expressed as ng/mg tissue.

2.6. Tyrosine hydroxylase (TH)-immunoreactivity

Dopamine synthesis is catalyzed by tyrosine hydroxylase (TH) located inside dopaminergic neurons in the SN of the brain therefore, immunohistochemical studies were performed to analyze the effect of Zn-exposure on TH positive/dopaminergic neurons as described previously (Kumar et al., 2010). In brief, rats were anaesthetized and brain was perfused with chilled normal saline followed by 4% paraformaldehyde in phosphate buffered saline (PBS). The brain was removed, post fixed in paraformaldehyde solution overnight and cryoprotected in sucrose (10%, 20%, and 30% in PBS) solution. The coronal brain sections (20 μm) were cut serially and incubated in H2O2 solution (0.5%) in methanol for 15 minutes to block endogenous peroxidase activity. The sections were then kept in blocking buffer (PBS containing 5% normal goat serum, 1% BSA and 0.1% Triton X-100) followed by incubation in anti-TH antibody (1:2000) as described earlier (Kumar et al., 2010). The sections were rinsed with PBS, incubated in biotinylated anti-mouse secondary antibody (1:300) and followed by strepavidin peroxidase complex for 30 min. The color was developed using 3, 3 diaminobenzidine (DAB) system and sections were mounted permanently using dibutyl phthalate xylene (DPX). The mounted sections were snapped with a bright field microscope (Leica Mikroskopie-GMBH; Wetzlar, Germany) at 10X magnification. Bilateral counting was done using computerized analysis software (QWin Pro, Leica, Germany) to calculate the number of TH-positive neurons in minimum of 3 animals in each group by a researcher, unknown to experimental protocol to ensure unbiased counting. The results are expressed as percent of control.

2.7. Neuronal nuclei/tyrosine hydroxylase immunoreactivity (NeuN/TH-immunohistochemistry)

NeuN/TH-immunohistochemical staining were performed for more accurate analysis of TH-positive neurons in the substantia nigra of the brain sections. The NeuN/TH staining was done as described previously (Singh et al., 2012). In brief, the blocking of non-specific binding and endoperoxidase activity was carried out as in TH-
The brain sections were then incubated in cocktail of monoclonal anti-NeuN (1:500) and anti-TH (1:500) primary antibodies for 48 h at 4°C followed by washing and incubation in secondary antibody. Visualization was done using DAB system and images were captured as in TH-immunostaining described above. NeuN/TH-positive cells were counted using computerized Leica QWin pro software. A minimum of three animals were used per group for NeuN/TH-immunoreactivity and data are expressed as percent of control.

2.8. Super oxide dismutase (SOD)

SOD activity was estimated using standard spectrophotometric method developed by (Nishikimi et al., 1972) with slight modifications as described earlier (Tsai et al., 2010). Briefly, standard assay mixture containing 1.2 ml sodium pyrophosphate buffer (pH 8.3, 0.052 M), 0.1ml PMS (186 μM), 0.3 ml NBT (300 μM), 0.2 ml NADH (780 μM) and 0.1 ml tissue homogenate was made up to 3 ml with distilled water and incubated at 30°C for 90 seconds. To estimate the activity of SOD2/Mn-SOD the reaction was carried out in presence of 4 mM potassium cyanide (KCN), which inhibits SOD1/CuZn-SOD. The reaction was stopped by the addition of 1.0 ml of glacial acetic acid. The reaction mixture was stirred and mixed vigorously after addition of 4 ml of n-butanol and allowed to stand for 10 minutes at room temperature. The samples were centrifuged and butanol layer was separated. The intensity of the chromogen in butanol layer was measured at 560 nm. CuZn-SOD/SOD1 activity was calculated as the difference between total SOD and Mn-SOD/SOD2 activity. The results are expressed as units/ml/min.

2.9. Catalase

Catalase was estimated according to method described by Sinha (1972). In brief, 5.0 ml assay mixture containing 0.01 M phosphate buffer (pH 7.0), 0.2 M hydrogen peroxide and 0.1 ml sample was incubated at 37°C for 1 min. Reaction was stopped by the addition of potassium dichromate and glacial acetic acid and remaining hydrogen peroxide was estimated by measuring chromium acetate formed by incubating the samples in boiling water bath for 15 min. Samples were then cooled and absorbance was taken at 570 nm against control devoid of hydrogen peroxide. The enzymatic activity was calculated in μmoles/min/mg protein.
2.10. Lipid peroxidation (LPO)

Malondialdehyde (MDA), an indicator of the level of LPO, was determined using the method described by (Ohkawa and coworkers, 1979). In brief, 0.1 ml of tissue homogenate was mixed with 1.6 ml water followed by addition of 600 µl of 10% SDS solution and incubated for 5 min at room temperature. 600 µl of 20% glacial acetic acid was added to the reaction mixture and incubated for 2-5 min at room temperature. Finally, 600 µl of 0.8% thiobarbituric acid (TBA) solution was added to the reaction mixture followed by incubation for 1 hr in a boiling water bath. The reaction mixture was cooled, centrifuged and the absorbance of the supernatant was read at 532 nm against control devoid of homogenate. LPO levels are expressed as nmoles MDA/mg tissue.

2.11. Glutathione S-transferase (GST)

GST activity was measured using spectrophotometric method (Pabst et. al, 1974). In brief, tissue homogenate was mixed with 2.9 ml of 0.2 M phosphate buffer (pH 6.5). The reaction was initiated by addition of l-chloro-2, 4-dinitrobenzene and optical density was read at 340 nm for 3 min at the intervals of 30 sec. Enzyme activity was calculated in nM/min/mg protein.

2.12. Nitrite content

Nitrite content was estimated in nigrostriatal tissue of control and treated animals using Griess reagent (Granger et. al, 1996). Tissue homogenate was mixed with 0.7 M ammonium chloride solution to final volume of 500 µl. Equal amount of Griess reagent [0.1% N (1-naphthyl) ethylene diamine dihydrochloride and 1% sulfanilamide in 2.5% orthophosphoric acid] was added to the reaction mixture followed by incubation for 10 min at 37°C. The absorbance was read at 548 nm against control. Nitrite content was calculated using standard curve of sodium nitrite and the results are expressed in µM/mg tissue.

2.13. Nitric oxide synthase (NOS) activity

Brain tissue was weighed and homogenized in ice-cold 50 mmol/L Tris-Cl buffer (pH 7.2) containing sucrose (300 mM), DTT (1mM), phenylmethyl sulfonyl fluoride (1 mM) and protease inhibitor cocktail. The brain lysate was subjected to centrifugation
at 10,000 × g, at 4°C for 10 minutes and the supernatant was used for measuring nitric oxide synthase (NOS) activity. NOS activity was measured according to (Chen et al., 1999). The total NOS activity in the supernatant was measured by adding 30 µL of the supernatant to 70 µL of incubation buffer containing 50 mmol/L Bis-Tris propane (pH 7.2), 2 mmol/L L-arginine, 3 mmol/L DTT, 2 mmol/L NADPH, 4 µmol/L tetrahydro-L-biopterin, and 1 mmol/L CaCl₂. Samples were incubated at 37°C for 90 minutes before termination of the reaction by removing NADPH. The NADPH was removed by incubation with 10 units of lactate dehydrogenase and 5 mmol/L sodium pyruvate at 37°C for 10 minutes. Nitrite levels in the reaction mixtures were spectrophotometrically determined using Griess reagent as mentioned above. Ca²⁺ dependent L-NAME inhibitable NOS activity gave the measure of total NOS activity while Ca²⁺ independent L-NAME inhibitable activity denoted iNOS activity. Difference between total and iNOS gave cNOS activity. cNOS activity in presence of 7-NI denoted eNOS activity and 7-NI inhibited activity denoted nNOS activity. The results are expressed as % of controls.

2.14. Gene expression analyses using reverse transcriptase polymerase chain reaction

Total RNA was isolated from nigrostriatal tissue of control and treated rats using Trizol reagent by standard procedure. cDNA was synthesized using total RNA (5 µg) by RT-Mul M reverse transcriptase kit as per manufacturer’s protocol. Amplification of SOD-1, SOD-2, γ-GCS, HO-1, and β-actin genes was carried out as reported elsewhere (Sugino et al., 1998; Chen et al., 2005; Luceri et al., 2002; Garcon et al., 2004; Ahmed et al., 2008). The primers used are given below: SOD1: forward primer: 5’TTC GAG CAG AAG GCA AGC GGT GAA-3’, reverse primer: 5’-AAT CCC AAT CAC ACC ACA ACA CAA-3’; SOD2: forward primer: 5’- TCA ATC CCC AGC AGT GGA ATA AGG C-3’, reverse primer: 5’-TCA ATC CCC AGC AGC AGT GGA ATA AGG C-3’; HO-1: forward primer: 5’-ACT TTC AGA AGG GTC AGG TGT CC-3’, reverse primer: 5’-TTG AGC AGG AAG GCG GTC TTA G-3’; γ-GCS forward primer, 5’-GCT GCA TCG CCA TTT TAC CGA G-3’, reverse primer, 5’-TGG CAA CAG TCA TTA GTT CTC CA-3’; β-actin: forward primer: 5’- CCT CTA TGC CAA CAC AGT-3’, reverse primer: 5’-AGC CAC CAA TCC ACA CAG-3’. nNOS gene primers were designed using DNA star software, forward primer: 5’- AAC GAT CGG CCC TTG GTA GAC-3’, reverse primer: 5’-GGG CGG AGC TTT
GTG CGA TTT G-3'. β-actin was amplified concurrently with the respective genes. The PCR products were visualized through agarose gel electrophoresis and densitometry was performed using Alpha Imager software taking β-actin as reference gene.

2.15. Protein estimation

Protein content was estimated in control and treated groups using Lowry’s method (Lowry, 1951). Protein concentration was calculated using the standard curve of bovine serum albumin (BSA).

2.16. Two-dimensional polyacrylamide gel electrophoresis (2-D PAGE)

The nigrostriatal tissue of controls and treated animals were dissected out and protein samples were prepared in lysis buffer as described (Patel et al., 2007; Sinha et al., 2009; Singh et al., 2011b). The homogenized content was sonicated and centrifuged at 10,000 g for 20 min at 4°C. The supernatant was taken and used for further experiments. Protein content was measured and 2-D PAGE was carried out employing previously reported protocols (Patel et al., 2007; Sinha et al., 2009; Singh et al., 2011b). In brief, IPG strip (pH 4–7) was rehydrated with 150 μg of protein and 150 μl commercial isoelectric focusing rehydration buffer containing 8 M urea, 2% CHAPS, 0.5% IPG buffer, 0.002% bromophenol blue and 15 mM DTT. The rehydration reaction was performed overnight at room temperature and isoelectric focusing was performed in a Protean IEF Cell (Bio Rad) for 14,000-volt hours (500 V for 30 min, 1000 V for 10 min, 2000 V for 10 min, 5000 V for 10 min, 8000 V for remaining period). After isoelectric focusing, IPG strips were equilibrated for 20 min in equilibration buffer containing 6 M urea, 30% glycerol, 2% SDS, 0.05 M Tris–HCl (pH 8.8) and 0.002% bromophenol blue. Second dimension electrophoresis was performed using 12.5% resolving polyacrylamide gel.

The resultant gels were stained with brilliant blue R-250/silver nitrate. Both staining strategies were initially used to check the reproducibility in the expression level of spots and also to avoid experimental variations which was the outcome staining. The images of 2-D gels were compared using Image Master 2D platinum software. The size and colour intensity of gels were normalized and the spot volume/intensity calibration, spot detection, background subtraction and matching, etc., were
performed with the software. Equal loading and similar staining procedures were used for the gels considered for the study to minimize the biological variations arising due to sample loading and run-to-run variability (Patel et al., 2007; Sinha et al., 2009; Singh et al., 2011b). Only those differentially expressed proteins were selected for mass spectrometry that showed more than 40% alteration in terms of % volume as compared with controls. The determination of differentially expressed proteins was made not only on the basis of spot volume but also on spot density following normalization to minimize the variation obtained from protein loading, staining procedure and run-to-run variability in 2-D PAGE. Intensity of each spot was quantified by calculation of spot volume after normalization of the image using the total spot volume normalization method multiplied by the total area of all the spots. Spot densities were determined and normalized to the total spot volume of the protein spots on each gel.

2.17. Matrix assisted laser desorption/ionization time of flight (MALDI-TOF) and liquid chromatography mass spectrometry (LC-MS) analyses

The protein spots were cut from the gels, trypsinized and the digested peptides were dissolved in α-cyano-4-hydroxy cinnamic acid (Patel et al., 2007). In brief, differentially expressed silver stained spots were excised, cut into pieces and washed with 500 μl water with agitation on a vortex mixer. Excised spots were incubated with 250 μl of 50 mM sodium thiosulphate and 15 mM potassium ferricyanide for 5 min to remove silver stains and washed twice with 500 μl water to remove reducing agents. In a tube, excised spot and 500 μl of 100 mM NH₄HCO₃ and 45 mM DTT was added and incubated at 60°C for 30 min. Following cooling, 500 μl of 100 mM iodoacetamide was added and further incubated for 30 min in dark at room temperature. Excised spots were sliced and equilibrated with 500 μl of 50 mM ammonium bicarbonate in 50% acetonitrile, dehydrated with 500 μl of 100% acetonitrile for 20 min and airdried. Sliced spots were rehydrated in 30 μl of a solution containing 0.02 μg/μl trypsin and 50 mM ammonium bicarbonate at 4°C for 60 min. The supernatant was discarded and 30–50 μl of 50 mM ammonium bicarbonate was added in excised spots, incubated at 37°C for 16–18 h and supernatant was taken. In the pellet, 25–50 μl of 1% TFA in 60% acetonitrile was added and sonicated for 10 min. The supernatant was taken following centrifugation.
Chapter 2 Material and Methods

at 12,000 \times g for 30 s. Both the supernatants were mixed, freeze-dried and concentrated by centrifugal evaporation to dryness.

MALDI-TOF and LC-MS analyses of the samples were performed and protein identity was established, as described elsewhere (Patel et al., 2007; Sinha et al., 2009; Singh et al., 2011b). In brief, MALDI-TOF/TOF and 2-D Nano LC-ESI-Trap were used for mass spectrometric identification. Peaks of auto-digested trypsin were used as an internal standard to ensure mass accuracy. The MASCOT search engine based on NCBI and Swiss Prot protein databases was used to identify peptide mass fingerprints. Bio-tools version 2.2, flex control and flex analysis software were used for acquisition and analyses. Peptide mass tolerance ±2 Da was the allowed error for matching the peptide values. Probability based MOWSE score was estimated as ion scores \(-10^4 \log_{10} \text{ (probability)}\). Identification of proteins was confirmed with their molecular weight and isoelectric points in the gel (Patel et al., 2007; Sinha et al., 2009; Singh et al., 2011b).

2.18. Western Blotting

The protein sample was prepared according to the nature of protein, their occurrence and as per requirement. The cytosolic and mitochondrial fractions were separated using standard procedure (Fujimura et al., 1999). In brief, the nigrostriatal tissue was homogenized in 7 volumes of cold suspension buffer [HEPES-KOH (20mM; pH 7.5), sucrose (250mM), KCl (10mM), MgCl2 (1.5mM), EDTA (1mM), EGTA (1mM), DTT (1mM), PMSF (0.1mM) and protease inhibitors cocktail]. The nigrostriatal tissue homogenate was centrifuged at 750\times g at 4°C for 10min. The supernatant was centrifuged at 8000\times g for 20 min at 4°C. The pellet was washed and used as the mitochondrial fraction while supernatant used as cytosolic fraction.

Effect of Zn (20 mg/kg) exposure on SOD1, SOD2, nNOS, TH, HO-1, caspase-3, cytochrome c release, peroxiredoxin-2 and profilin-2 was analyzed in nigrostriatal tissue of control and treated groups by western blotting. Cytochrome c release from mitochondria was assessed by testing their relative protein levels in mitochondrial and cytosolic fractions. In brief, denatured proteins were resolved by SDS-polyacrylamide gel electrophoresis and electroblotted onto PVDF membrane (Millipore). Blots were blocked overnight with Tris buffered saline containing 0.05 % Tween-20 (TBS-T) and 5% non fat dry milk, washed, subsequently incubated with primary antibodies against
Chapter 2 Material and Methods

SOD1 (1:3000), SOD2 (1:4000), nNOS (1:3000), TH(1:5000), HO-1(1:3000), caspase-3(1:1000), cytochrome c (1:5000), peroxiredoxin-2 (1:5000), profilin-2 (1:5000) and β-actin(1:4000) for 2 hours. Blots were washed to remove excess antibodies and followed by incubation with alkaline phosphatase (AP) conjugated with anti-mouse, anti-goat and anti-rabbit secondary antibodies in TBS-T buffer and finally blots were visualized using BCIP/NBT. Relative band density was calculated with β-actin as the reference using Alpha Imager software and data are expressed as mean ± SE of band density ratio of at least four experiments.

2.19. Statistical analysis

Results are expressed as mean ± standard error of means (SEM) for separate groups. One/Two-way analysis of variance (ANOVA) was used for statistical analysis. Newmann Keuls/Bonferroni post-test was used for multiple comparisons and the differences were considered statistically significant when p value was less than 0.05.