Chapter III

Materials & Methods

3.1 Chemicals

Cadmium, quercetin, atropine sulphate, haloperidol, Triton-X-100, DCFH-DA, JC1 dye, MDC (monodansylcadervarine), DMSO, 4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI) and cresyl violet were purchased from Sigma-Aldrich, USA. The primary monoclonal antibodies - anti-rabbit choline acetyltransferase (ChAT), anti-rabbit tyrosine hydroxylase (TH), anti-rabbit Bcl2, anti-rabbit Bax, anti-rabbit Caspase-3, anti-rabbit Dopamine transporter (DAT), anti-rabbit vesicular monoamine transporter (VMAT-2), anti-rabbit protein kinase A (PKA), anti-rabbit DARPP32, anti-rabbit PP1α and anti-rabbit β- actin were purchased from Cell Signaling Technology, USA. Polyclonal antibodies – anti-rabbit dopamine D1 receptor and anti-rabbit dopamine D2 receptor were procured from Abcam, U.K while anti-rabbit PKCβ1 was purchased from Cell Signaling Technology, USA. Secondary antibodies – (goat anti-rabbit IgG- HRP, goat anti-mouse IgG-HRP) were also procured from Cell Signaling Technology, USA. Radioligands - ^{3}H-Quinuclidinyl benzilate (specific activity - 42 Ci/mmol) and ^{3}H-Spiperone (specific activity - 18.5 Ci/mmol) were purchased from PerkinElmer, USA. Rapamycin (Tocris Biosciences), 3-methyle adenine (Tocris Biosciences), LysoTracker®

Red DND-99 (Invitrogen), DAPI with antifade (Invitrogen), and GFP-LC-III expression kit (Invitrogen) was also procured. Other chemicals used in the study were of analytical grade and arranged from local commercial sources.

3.2 Instruments

Actimot (TSE, Germany), Grip strength meter (TSE, Germany), Y-maze, (TSE, Germany), Rotamex (Columbus Instruments, USA), Shuttle Box (Techno, India), Homogenizer (Kika Labortechnik, Germany), Sorvall RC-5B high speed refrigerated centrifuge (DuPont, USA), Vortex - Spinix (Tarson, India), Hot plate – Spinot (Tarson, India), Balance (Afcoset, India), pH meter - Cyberscan - 510 (Merck, Germany), UV-visible spectrophotometer (Cary 300 Bio, Varian, USA), Multiwell plate reader (Biotek Synergy HT, USA), NanoDrop ND-1000 (Thermo Scientific, Wilmington, DE, USA), Hydride system 60 atomic absorption spectrophotometer (HSAAS, Zeenit 700), High pressure liquid chromatograph (Waters, USA), Robotic liquid handling system – Multi*Probe* II_{EX} (Perkin Elmer, USA), Top Count *NXT* (Perkin Elmer, USA), and β-liquid scintillation counter (Perkin Elmer, USA), High resolution binocular microscope - Eclipse, E600 (Nikon, Japan) with computerized image analysis system - Leica Qwin 500 image analysis software (London, UK), Digital gel image analysis system – Image quant LAS, Cryotome – Microm (HM 520, USA), 7900HT Fast Real Time PCR System (Applied Biosystem, USA) were the major instruments used in the study.

3.3 *In vivo* Studies

3.3.1 Experimental Animals and Housing Conditions

Adult male rats (180 ± 20g) of Wistar strain obtained from the central animal breeding colony of CSIR-Indian Institute of Toxicology Research (CSIR-IITR), Lucknow were housed in a temperature controlled experimental room (25 ± 2ºC) with a 12-h light/dark cycle under standard hygiene conditions. The animals had free access to pellet diet procured from the national suppliers and water *ad libitum*. The animals were acclimatized for 7 days before starting the experiment. The study was approved by the institutional

animal ethics committee of CSIR-IITR, Lucknow (IITR/IAEC/50/13) and all experiments were carried out in accordance with the guidelines approved by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forests (Government of India), New Delhi, India.

3.3.2 Treatment Procedure and Sample Preparation

To assess the protective efficacy of quercetin in cadmium induced neurotoxicity, rats were randomly divided into four treatment groups and treated as per following schedule.

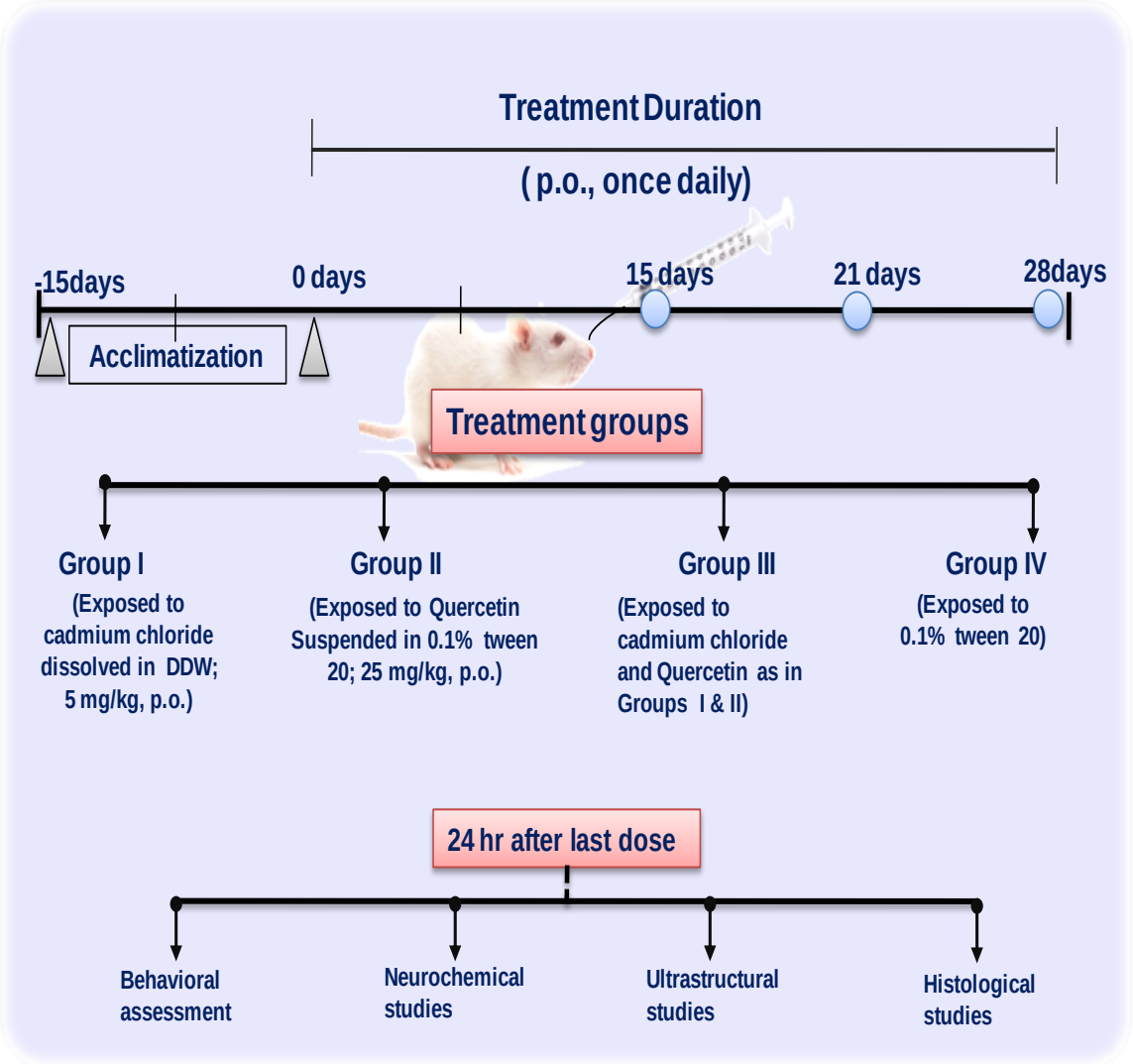


Figure – 3.1. Treatment Schedule

Rats in group I were treated with cadmium as cadmium chloride (5 mg/kg body weight, p.o., once daily for 28 days). In Group II, rats received quercetin (suspended in 0.1% tween 20, 25 mg/kg body weight, p.o., once daily for 28 days). Rats in group III were treated with cadmium and quercetin simultaneously as in treatment groups I and II respectively while rats in group IV received vehicle and served as controls (Figure – 3.1).

24 hours after the last dose of treatment with cadmium and quercetin, a separate set of rats was used for behavioral studies. For neurochemical studies, rats were quickly decapitated and brains were taken out quickly and washed in ice cold saline and dissected into different regions (frontal cortex, hippocampus, corpus striatum) following the standard procedure as described by (Glowinski and Iversen, 1966). For qRT-PCR studies, the brain regions were immediately immersed in Trizol reagent (Qiagen, Germany) and stored at –80˚C until used. For histological studies, a set of rats was perfused in paraformaldehyde (4%) and brains were stored in paraformaldehyde (10%). For ultrastructural studies, rats were perfused with paraformaldehyde (4%) and glutaraldehyde (0.1%).

3.3.3 Behavioral Studies

3.3.3.1 Spontaneous Motor Activity

Alterations in motor activity if any on cadmium exposure in rats was assessed by computerized Actimot (TSE, Germany) following the standard protocol as described by (Yadav et al., 2009). The actimot is fully automated with high density of infrared beams (32 × 32) and measures the activity based on light beam principle. Before starting the experiment, rats were acclimatized for 30min. Rats were placed in the centre of the cage and allowed to freely move for 5 min. The sensor becomes activated and the movement of the animals is tracked automatically. The details of the parameters assessed are as follows,

3.3.3.1.1 Total distance traveled (cm): Distance traveled is the basic feature of animal activity and used in open field studies.

3.3.3.1.2 Resting time (sec): The period during which no movement in the animal occurs and it remains at a fixed place is considered to be resting period.

During this time, no infrared beams are interrupted.

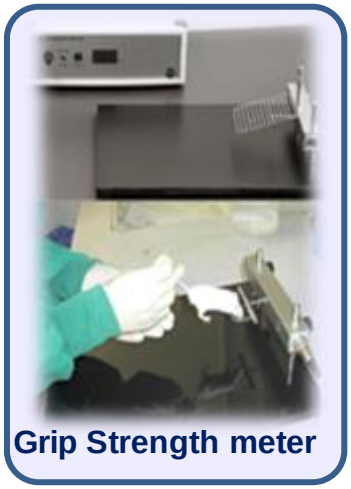

Actimot

3.3.3.1.3 Time moving (sec): The period during which the animal moves in the cage is designated as time moving.

3.3.3.1.4 Number of rearing: The period during which rat stands on its hind limbs away from the wall and with its forelimbs unsupported is referred as rearing.

3.3.3.1.5 Stereotypic count: Scratching, grooming and head swings during which the animal remains in the same location but interrupts light beams is considered as stereotypic movement. The number of these activities is recorded as stereotypic count.

3.3.3.2 Grip Strength


Grip Strength meter

Computerized grip strength meter (TSE, Germany) was used to measure the forelimb grip strength following the method as described earlier (Shukla et al., 2016). Briefly, holding the rat from nape and the base of the tail, the forelimbs were placed on the tension bar. The rat was pulled back gently until it released the bar. Reading was recorded automatically on the computer. Five successive pulls for each animal in the study group were tried by a person unaware of their treatment status. The mean of all the values was taken and processed for statistical analysis. Values are expressed in Pound.

3.3.3.3 Rota-Rod Performance

The motor co-ordination in rats was assessed using Rotamex (Columbus Instruments, USA) following the procedure as described earlier (Yadav et al., 2009). A group of rats from each treatment group received training on the rotating rod of Rotamex (at a constant speed 8 rpm). The rotational speed gradually increased from 4 to 40 rpm for a period of

300 s during the final trial. Further, the final trial was carried out by a person unaware of the treatment status. The time of fall from the rotating rod was scored.


Rotamex

3.3.3.4 Learning and Memory

Effect on learning and memory in rats treated with cadmium or quercetin alone or in combination was assessed by passive avoidance test using shuttle box. However, spatial memory and learning and continuous alteration test were assessed by Y- maze.

3.3.3.4.1 Y-Maze

Spatial memory was assessed through novelty seeking behavior using Y-maze and following the procedure as described by (Wang et al., 2009). Briefly, the test consists of two trials separated by an inter-trial interval of 4hr to assess spatial recognition memory. Out of three arms of Y-maze, one arm was blocked and termed as the novel arm. During the first trial, rats were placed in the start arm and allowed to explore the start arm and other arm for 15 minutes as the third arm (novel arm) was blocked. After an inter-trial interval of 4 hours, rats were placed in the same arm (start arm) and allowed to explore all arms for a period of 5 min. During this time, rats were free to visit all the three arms. Number of entries and time spent in the novel arm versus other arm was recorded on computer. The results are expressed as % time spent and % entries in the novel arm versus other arm.


Y- maze

Further, continuous alternation in rats was assessed using Y- maze as described by (Yamada et al., 1996). Briefly, rats were placed at the end of one arm of Y-maze and allowed to move freely for 5 minutes. The series and sequence of entry in to each arm of the Y-maze was recorded automatically.

the alternation percentage was calculated as the ratio of actual to possible alternation multiplied by 100.

3.3.3.4.2 Passive Avoidance Response

Passive Avoidance Response

The passive avoidance response was monitored using the shuttle box (Techno, India) following the procedure as described earlier (Yadav et al., 2011). Briefly, the shuttle box consists of two chambers – a lighted and a dark chamber separated by a guillotine door. Rats were placed in the lighted chamber of the shuttle box and after acclimatization for 30 sec, the guillotine door was opened. As the rats crossed in to the dark chamber, the guillitone door was closed and a low-intensity foot shock (0.5mA; 10 sec) was given. The 1st trial was for the acquisition and retention was assessed in subsequent trials carried out 24 hr after the 1st trial. The transfer of rat from light to dark compartment was recorded as transfer latency time (TLT) in seconds. The criterion for improved cognitive activity was considered as increase in the TLT on retention trial (2nd trial and more) as compared to the acquisition trial (1st trial). The shock was not given to the rats in the retention trials to avoid reacquisition.

Multiprobe IIEx

3.3.4 Neurochemical Studies

3.3.4.1 Assay of Neurotransmitter Receptors

Radioligand receptor binding technique was employed to assay the muscarinic-cholinergic receptors in frontal cortex and hippocampus and dopamine receptors in corpus striatum following the standard procedure as described earlier by (Khanna et al.,

1994). The method for preparation of crude synaptic membrane and binding assay is briefly described.

3.3.4.1.1 Preparation of Crude Synaptic Membrane

The isolated brain region (frontal cortex, hippocampus, corpus striatum) was homogenized in 19 volumes of Tris-HCl buffer (5mM, pH 7.4). The homogenate was centrifuged (40,000x g) for 15 min at 4ºC. The sedimented pellet was suspended in homogenization buffer (5 mM Tris-HCl, pH 7.4) and recentrifuged (40,000x g) for 15 min at 4ºC. The pellet thus obtained was finally suspended in Tris-HCl buffer (40 mM, pH 7.4) and stored at -20ºC.

3.3.4.1.2 Radioligand Binding Assay

Briefly, the reaction mixture in a final volume of 1 ml containing Tris-HCl buffer (40mM, pH 7.4) together with appropriate radioligand and membrane protein (~300-400 µg) was incubated (15min, 37^{o} c). To determine the extent of nonspecific binding, a set of tubes containing unlabelled competing agent, specific in each case, were run in parallel. Briefly, the assay of DA-D2 receptor was performed using ^{3}H-Spiperone (18.5 Ci/mmol, 1 X 10^{-9} M) as the radioligand and haloperidol (1 X 10^{-6} M) as competitor. For the assay of cholinergic - muscarinic receptors, ^{3}H-Quinuclidinyl benzilate (42 Ci/mmol, 1 X 10^{-9} M) was used as a radioligand and atropine sulphate (1 x 10^{-6} M) as competitor (Table – 3.1).

At the end of the incubation, the samples were rapidly filtered on glass fiber discs (25 mm diameter, 0.3 µm pore size, Whatman (GF/B) and washed twice with 5 ml cold Tris-HCl buffer (40mM, pH 7.4). Filter discs were dried and counted in 5 ml of scintillation mixture containing 2,5-diphenyloxazole, 1,4-bis(5-phenyloxazolezyl) benzene, naphthalene, toluene, 1,4-dioxan and methanol. To determine the membrane bound radioactivity, samples were counted using β-scintillation counter (Packard, USA) at an efficiency of 30-40% for ^{3}H. Specific binding has been calculated by subtracting the nonspecific binding (in the presence of competitor) from the total binding (in the absence of competitor)

Table – 3.1. Radioligands and competitors used for the assay of neurotransmitter receptors

Receptor	Brain Regions	Radioligand (Concentration)	Competitor (Concentration)
Cholinergic - muscarinic	Frontal cortex Hippocampus	3H-Quinuclidinyl benzilate ($1x10^{-9}$ M)	Atropine sulphate ($1X10^{-6}$ M)
DA – D_2	Corpus striatum	3H-Spiperone ($1x10^{-9}$ M)	Haloperidol ($1X10^{-6}$ M)

Concentration of radioligands and competitors used indicated in the parentheses

and results have been expressed as pmoles ligand bound/g protein. Scatchard analysis was carried out using different concentrations of radioligands (normally 1/10 to 10 times of the affinity of radioligand) to determine whether change in the binding is due to alteration in the affinity (Kd) or number of receptor binding sites (Bmax). The method used was essentially similar to other filtration binding method (Khanna et al., 1994). Saturability, specificity, reversibility and regional distribution of receptors were standardized prior to the experiments. The amount of membrane protein was determined following the standard procedure (Lowry et al., 1951) using bovine serum albumin as a standard.

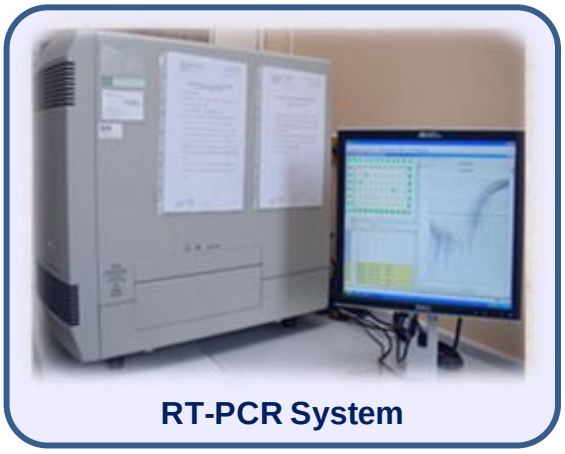
3.3.4.2 Expression of Neurotransmitter Receptor Gene

Quantitative real-time polymerase chain reaction (qRT-PCR) was carried out to assess the expression of various genes for receptors and enzymes following the standard procedure.

3.3.4.2.1 RNA Extraction and cDNA Synthesis

The selected brain regions (frontal cortex, corpus striatum and hippocampus) were homogenized in 1 ml of ice-cold Trizol (Invitrogen, Carlsbad, CA, USA) followed by chloroform extraction and isopropyl alcohol precipitation. The isolated RNA was dissolved in RNAse free water (50 µl). RNA (0.5 µg) was loaded onto agarose gel (1%)

subjected to electrophoresis, stained with ethidium bromide and visualized by UV transillumination. The degradation of RNA was not observed as indicated by intact ribosomal bands 28S and 18S in all samples. The amount of RNA samples was assessed spectrophotometrically by NanoDrop ND-1000 (Thermo Scientific, Wilmington, DE, USA) at OD 260/280. The ratio of the OD 260 / 280 of all extracted RNA samples was between 1.8 and 2.0. RNA was transcribed into single strand cDNA using the high capacity cDNA reverse transcription kit (Applied Biosystems, USA).



RT-PCR System

The 2X transcription master mix was prepared and for 10µl reaction, 10X RT buffer, 25X DNTP mix (100mM), 10X RT random primer, multiscribe reverse transcriptase and nuclease free water were added and kept in ice. 2 X RT master mix (10µl) was pipetted into each individual tube. 10µl of RNA sample was added into each tube and the tubes were sealed. The tube was placed into thermal cycler using following conditions,

Step I - 25°C for 10 min; Step II - 37°C for 120 min; Step III - 85°C for 5 min;
Step IV - ∞

Table – 3.2. Primer sequences used for real time polymerase chain reaction (RT-PCR)

	CHRM1
	Forward 5'-3 CTGGTTTCCTTCGTTCTCTG
	Reverse 5'-3' GCTGCCTTCTTCTCCTTGAC
	CHRM2
	Forward 5'-3' GGCAAGCAAGAGTAGAATAAA
	Reverse 5'-3' GCCAACAGGATAGCCAAGTG
	CHRM3
	Forward 5'-3 GTGGTGTGATGGATTGGTCTG

	Reverse 5'-3'	TCTGCCGAGGAGTTGGTGTC
CHRM4		
	Forward 5'-3	AGTGCTTCATCCAGTTCTTGTCCA
	Reverse 5'-3'	CACATTCATTGCCTGTCTGCTTTG
CHRM5		
	Forward 5'-3	CTCATCATTGGCATCTTCTCCA
	Reverse 5'-3'	GGTCCTTGGTTCGCTTCTCTGT
ChAT		
	Forward 5'-3	CGGGATCCTGCCTCATCTTCTCTGGTGT
	Reverse 5'-3'	GGCGGAATTCAATCACAACATC
AChE		
	Forward 5'-3	GCTCACGTAGATTTATGCCACCAGA
	Reverse 5'-3'	TTGATCCAGCAGGCCTACATTG
DA-D1		
	Forward 5'-3	GTGGACCTCATGGCCTACAT
	Reverse 5'-3'	TGTGAGGGAGATGCTCAGTG
DA-D2		
	Forward 5'-3	TGGATCCACTGAACCTGTC
	Reverse 5'-3'	TCTCCTCCGACACCTACCCCGA
DA-D3		
	Forward 5'-3	TTAGCCCACATTGCTGTCTG
	Reverse 5'-3'	GGAGTTGAGGTGGGTGCTTA
DA-D4		
	Forward 5'-3	ATGGCCCCTGACTGCAAATC
	Reverse 5'-3'	AGTCCGGTGCCAGTACCTAA
DA-D5		
	Forward 5'-3	AGCATGCTCAGAGTTGCCGG
	Reverse 5'-3'	ACAAGGGAAGCCAGTCTTTGG
TH		
	Forward 5'-3	CCACGGTGTACTGGTTCACT
	Reverse 5'-3'	GGCATAGTTCCTGAGCTTGT

	β-actin	
	Forward 5'-3	CGTGGGCCGCCCTAGGCACCA
	Reverse 5'-3'	GGGGGGACTTGGGATTCCGGTT

3.3.4.2.2 Quantitative RT-PCR Analysis

For quantitative RT-PCR, the PCR reaction mixture in 20 µl contained 1XTaq Man Universal PCR Master Mix (Applied Biosystems), 10pM of each gene primers, 2 µl cDNA and nuclease-free water using the cDNA synthesized by High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) was done. The sequence of primers used has been described in Table – 3.2. RT-PCR assay was performed in triplicate on cDNA samples in 96-well optical plates on an ABI 7900HT Fast Real-Time PCR System (Applied Biosystems, USA). PCR conditions were set – 50°C for 2 min, 90°C for 10 min, 95°C for 0.15 s and 60°C for 1 min. The results have been analyzed by the $2^{-\Delta\Delta CT}$ method using β-actin as a reference gene (Singh et al., 2015).

3.3.4.3 Nitric Oxide Levels

Levels of nitric oxide in frontal cortex, corpus striatum and hippocampus were estimated using the assay kit procured commercially (Calbiochem, USA). The assay principle involves the conversion of nitrate to nitrite by the enzymatic action of nitrate reductase. Addition of 2,3 - diaminonaphthalene (DAN) and sodium hydroxide converts nitrite to a fluorescent compound 1(H)-naphthotriazole which is measured spectroflurometrically (excitation 430 nm / emission 450 nm) using multiwell plate reader (Misko et al., 1993). The results are expressed as fold changes.

3.3.4.4 Multiplex Bead Assay

Levels of pro and anti-inflammatory cytokines in brain regions were assessed involving specific Magnetic Bead Based Multiplex assay (Hulse et al., 2004).

3.3.4.4.1 Preparation of Membranes from Brain Tissues

The isolated brain regions were homogenized in cell lysis buffer containing PMSF and centrifuged at (12,500xg) 4^{o} C for 15min. The Supernatant was collected and used for further assay. The protein concentration was determined following the method as described earlier (Lowry et al., 1951).


Multiplex Reader

3.3.4.4.2 Bead Assay

To assess the expression of cytokines, a bead based Multiplex assay platform (Bio-Plex MAGPIX Multiplex Reader, Bio-Rad Laboratories, Hercules, CA) and Rat Cytokine /Chemokine Magnetic Bead Panel (RECYTMAG-65K, Millipore) was used. Bio-Plex MAGPIX system is based on the principle of sandwich ELISA which uses magnetic bead coupled antibodies in suspension phase kinetics. Levels of cytokines in samples were calculated after plotting the standard curves and expressed as pg/ml.

3.3.4.5 Western Blotting

Immunoexpression of selected proteins was assessed by Western Blotting following the procedure as described by (Jamal et al., 2007). Briefly, frontal cortex / hippocampus was lysed and equal amount of protein (30 µg protein / lane) was electrophoresed on SDS–PAGE (12%) and transferred on to nitrocellulose membrane followed by blocking with buffer containing BSA (5%). The membrane was incubated overnight at 4^{o}C with either of the monoclonal antibody [AChE (1:1000), ChAT (1:1000), TH (1:1000), DAT (1:1000), VMAT-2 (1: 1000), DA-D1 and DA-D2 receptor (1: 1000), PKA (1:1000), phospho PKA


Western Blotting

(1:1000), phospho DARPP32 (1 : 1000) (thr34), phospho PP1-α (1:1000) (thr320), Akt (1:1000), phospho Akt (1:1000), GSK-3β (1:1000), phospho GSK-3β (1:1000), CREB (1:1000), phospho CREB (1:1000)(ser133), PKC β-1(Sigma, 1:1000), GFAP (1:1000), Iba1 (1:1000), nNOS (1:1000), iNOS (1:1000), COX-2 (1 : 1000), STAT 3 (1 : 1000), CamkIIα (1:1000), MT3 (1:1000 dilution), Bcl-2 (1 : 1000), Bax (1 : 1000), Caspase-3 (1 : 1000), pJNK1/2, phospho JNK3 (1: 1000), phospho p38 (1:1000), AP1 (1:1000), Cyt C (1:1000), ERK1/2 (1:1000), phospho ERK1/2 (1:1000) (thr202/tyr204), LC3-II (1:1000), P62 (1:1000), Beclin1 (1:1000), Atg 3, 5, 7, 12, 16 (1:1000), Lamp 2a (1:1000) and β- Actin (CST, 1:1000) followed by incubation with horseradish peroxidase-linked secondary antibody (anti-mouse IgG, 1:4000; anti-rabbit IgG 1:4000) at room temperature for 60 min and detected by chemiluminescent method. Densitometric measurements of bands in the immunoblots were carried out using digital gel image analysis system (Image Quant LAS 500) and normalized by β- actin to correct variations, if any in protein loading.

3.3.4.6 Assessment of Mitochondrial Integrity

3.3.4.6.1 Assay of Mitochondrial Complexes

To assess the complex I (NADH-ferricyanide reductase) activity, ferricyanide was used as electron acceptor and method of (Hatefi, 1978) was followed. Briefly, the reaction mixture (final volume 1 ml) contained phosphate buffer (50 mM, pH 7.4), NADH (0.17 mM), ferricyanide (0.6 mM), and Triton X-100 (0.1% v/v). Mitochondrial suspension (~ 10 - 30 µg protein) was added to the reaction mixture in the cuvette to start the reaction at 30 °C. The rate of oxidation of NADH was assessed using a spectrophotometer at 340 nm. The activity of complex I has been expressed as nmoles of NADH oxidized/min/mg protein.

For the assay of complex II-III (succinate - cytochrome c reductase) activity, reduction of ferricytochrome c to ferrocytochrome c was monitored in the presence of succinate following the method as described by (Clark et al., 1997). The assay mixture in a total volume of 1 ml contained phosphate buffer (100 mM), succinate (2 mM), KCN (1 mM), EDTA (0.3 mM) and cytochrome c (1.2 mg/ml). Mitochondrial suspension (~ 10 - 30

µg) was added to initiate the reaction and reduction of ferricytochrome c was monitored at 550 nm. The activity of complex II-III has been expressed as nmoles oxidized cytochrome c reduced/min/mg protein.

The activity of complex IV (cytochrome c-oxidase) was assessed by monitoring the oxidation of reduced cytochrome c (ferrocytochrome c) following the standard procedure (Wharton, 1967). Briefly, ferricyanide (1 mM) in phosphate buffer (10 mM, pH 7.4) was added to oxidized ferrocytochrome c in a final volume of 1 ml at room temperature. The reaction was initiated by adding mitochondrial suspension (~ 10 - 30 µg protein) and rate of oxidation was recorded at 550 nm. The activity of complex IV has been expressed as nmoles reduced cytochrome c oxidized/min/mg protein.

3.3.4.6.2 Estimation of Reactive Oxygen Species

The generation of reactive oxygen species (ROS) in brain regions was assessed following the method of (Rush et al., 2007). Briefly, mitochondrial suspension was incubated with DCFH-DA dye (10µl, 100µM final concentration) at room temperature for 30 min. The DCFH-DA is oxidized to fluorescent DCF by intracellular ROS during the course of reaction. The generation of ROS was measured using fluorescence reader at excitation 485 nm / emission 520 nm and results have been expressed as % of control.

3.3.4.6.3 Assessment of Mitochondrial Membrane Potential

The mitochondrial membrane potential (MMP) in selected brain regions was estimated using JC-1 dye as it is a sensitive indicator to assess the change in fluorescence from red to green following the standard procedure (Kane et al., 2008). Briefly, the mitochondrial cell suspension was incubated with JC-1 dye (10 µM) at 37^{o}C for 15 min. and washed with PBS. The cells were finally suspended in 0.5ml PBS (10^{6} cells/ml) and analyzed by flow cytometer (FACS CantoTM II, BD Bio- Sciences, San Jose, CA, USA).

3.3.4.7 Estimation of Cadmium Levels

Estimation of cadmium levels in brain regions - frontal cortex, hippocampus and corpus

striatum was carried out using atomic absorption spectrometer (AAS). Briefly, samples were prepared by digestion of samples in the acid mixture following the procedure as described in Bulat et al., 2012. In short, brain samples were exposed to acid mineralizing process with concentrated HNO3 and HClO4 in a ratio 4:1 at 130°C for 3 hr or overnight. The solution was digested over a sand bath until it became yellow in color. If the color of the digest tissue was brown, more acid mixture added and the process of oxidation repeated. After mineralization process, samples were diluted with 0.1mol/L HNO3 and used for the estimation of cadmium levels in different brain regions using atomic absorption spectrometer. Detection limit of the instrument was 1 ppb.

atomic absorption Spectrometer

3.3.4.8 Estimation of Neurotransmitter Levels and Their Metabolites in Brain Regions

Levels of dopamine (DA), norepinephrine (NE), epinephrine (EPN), serotonin (5HT) and metabolites of dopamine - 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) were estimated in brain regions (frontal cortex, corpus striatum and hippocampus) of rats treated with cadmium or quercetin alone or on their simultaneous treatment for 28 days using reversed phase high performance liquid chromatography with electrochemical detector (HPLC-ECD) following the standard procedure of (Kim et al., 1987) with minor modifications.

High Performance Liquid Chromatography

Briefly, brain regions were homogenized in perchloric acid (0.1M) containing 3,4 - dihydroxybenzylamine, an internal standard at a final concentration of 25 ng/ml followed by centrifugation at 36,000×g for 10 min at 4°C. The supernatant obtained was then filtered through 0.25 µm nylon filters and used for the determination of neurotransmitter levels. Sample (20 µl) was injected in the injector port. The mobile phase (pH 4.2) containing sodium dihydrogen phosphate (0.15 M), ethylenediaminetetra acetic acid (0.25 mM), sodium octyl sulphate (1.75 mM) and methanol (4%) was used to separate peaks in the samples at a flow rate of 1.5 ml/min. Electrochemical detector with glassy carbon and silver nitrate electrode was operated at a potential of +0.800V with sensitivity range 2nA at ambient temperature. Data were recorded and analyzed with the help of Empower2 software and results are expressed as ng/g tissue weight.

3.3.5 Histological Studies

Histological studies were carried out using Nissl staining as described by (Veena et al., 2011). Briefly, thin sections from the brain were cut using Cryotome (Microm, HM520 USA.). The sections were stained with cresyl violet (0.1%) and dehydrated through graded series of alcohol. Finally, the sections were cover slipped with DPX mounting media and the intensity of Nissl stained neurons was determined using a computerized image analysis system (Image J).

3.3.6 Ultrastructural studies

For ultrastructural studies, a separate set of rats was used. Rats were anesthetized with ketamine (30 mg/kg) and perfused with paraformaldehyde (4%) and glutaraldehyde (0.1%). The perfused brain was dissected into frontal cortex and hippocampus and cut into fine pieces (2mm approx). Primary fixation of sections was carried out for 2 hr in glutaraldehyde (2.5%) prepared in sodium cacodylate buffer (0.1 M, pH 7.2). Subsequently, post fixation was carried out in osmium tetraoxide (1%) for 1 – 2 h followed by dehydration and embedding in araldite and DDSA medium. The tissues were baked at 65 °C for 48 h and cut into thin sections (60–90 nm) using ultramicrotome (Leica EM UC 67). The thin sections on copper mesh grids were stained with uranyl

acetate and lead citrate (2%) for contrast. Examination of the brain sections was carried out through transmission electron microscope (Tecnai G2 spirit transmission electron microscope equipped with Gatan CCD/Orius camera at 60 KV).

3.4 *In vitro* Studies

3.4.1 Characterization and Preparation of PLGA Quercetin-NPs

Poly (D, L-lactide-co-glycolide) (PLGA) quercetin-NPs were prepared following the emulsion solvent evaporation method with minor modifications. Briefly, 100 mg of quercetin was dissolved in 3.5 mL of dimethyl sulfoxide in dark. PLGA (1.0 g) solution prepared in 10 mL of DCM was added and the solution was stirred at 1,600 rpm for 15 min. Following this, 1% polyvinyl alcohol (PVA) solution (50 mL) was added slowly into organic solution and stirred for 2 h at 25ºC. The reaction mixture was kept in vented position at 25ºC for 12 h with stirring at 1,600 rpm to remove the organic solvent. The yellow colored emulsion was centrifuged at 12,000 rpm for 30 min at 4ºC. The yellow pellet was re-suspended in water and centrifuged and the process was repeated thrice. The yellow colored nanoparticles received were freeze dried to obtain dry powder. Nanoparticles were stored at 4ºC under anhydrous conditions in dark till use.

3.4.2 Characterization of PLGA-Quercetin-NPs

3.4.2.1 Percent Yield

After achieving the constant weight, yield (%) of NPs was calculated by following formula:

$$\text{Yield (\%)} = \frac{\text{Weight of nanoparticle}}{\text{Weight of (drug + polymer)}} \times 100$$

3.4.2.2. Particle Size Measurement

The mean particle size and the polydispersity index (PDI) of PLGA-quercetin-NPs were determined by dynamic light scattering (DLS) technique employing a nominal 5mW He-Ne laser operating at 633 nm wavelength. The freeze dried nanoparticles were dispersed

in aqueous buffer and the size was measured. The measurement was carried out at 25 ºC with the following settings: 10 measurements per sample; refractive indices of water, 1.33; viscosity of water, 0.89 cP. The particle size was measured in triplicate.

3.4.2.3 Drug Loading and Entrapment Efficiency

The drug loading and encapsulation efficiency were determined by analyzing the NPs spectrophotometrically using Lambda Bio 20 UV/VIS Spectrophotometer (Perkin Elmer, USA). The amount of quercetin present in the nanoparticles was estimated as per following procedure.

Known amount of NPs (1.0 mg, dry powder) was dispersed in 1 mL double distilled water by stirring the sample vigorously and the absorbance of the solution was measured at 373 nm and the amount of drug present was calculated from a previously drawn calibration curve of concentration vs. absorbance with different concentrations of known drugs. All the measurements were performed in triplicate. The percent drug loading (%DL) and entrapment efficiency (%EE) were calculated using following formula.

$$\%DL = \frac{\text{Weight of drug in NPs x100}}{\text{Weight of NPs}}$$

$$\%EE = \frac{\text{Amount of drug present in the polymeric NPs x100}}{\text{Amount of drug used}}$$

3.4.2.4 Nanoparticle Surface Morphology

The surface morphology of the NPs was characterized using transmission electron microscopy (TEM) and scanning electron microscopy (SEM).

3.4.2.4.1 Transmission Electron Microscopy (TEM)

Briefly, a drop of aqueous solution of lyophilized powder (1 mg/mL) was placed on a TEM grid surface and a drop of 1% uranyl acetate was added to the surface of the

Formvar-coated grid. After 1 min of incubation, excess fluid was removed and the grid surface was air dried at 25 ± 2 ºC before being loaded into the microscope. The NPs were visualized under the transmission electron microscope (FEI Company, OR, USA) operated at 80 kV, attached to a Gatan Digital Micrograph (PA, USA).

Transmission Electron Microscopy

3.4.2.4.2 Scanning Electron Microscopy (SEM)

The NPs were characterized for their shape, surface morphology, and particle size distribution by high-resolution field emission SEM (Quanta FEG 450, FEI, Netherlands). The sample was placed on a double-stick conducting carbon tape over an aluminum stub and coated with gold under an argon atmosphere by means of a sputter coater (SC 7620, mini sputter coater, Quorum Technology Ltd., UK). Samples were analyzed at an accelerating voltage of 10 kV and a working distance of 10 mm in a high-vacuum mode.

SEM

3.4.2.4.3 Evaluation of Quercetin Released From Nps

To determine the release profile of PLGA-quercetin-NPs, known quantity of the particles (~5 mg) was dispersed in 1 mL of phosphate buffered saline (PBS), pH 7.4, and kept in the dialysis tube, which was suspended in 20 mL of PBS in a glass vial and the solution was stirred at 1600 rpm at 37 ºC. At pre-determined intervals of time, samples were collected (ca. 200 mL) from the glass vial followed by spectroscopic analysis at 373 nm using UV/VIS spectrophotometer. The same amount of fresh buffer was added to the

glass vial and the release study was continued. The quantity of the released drug was then calculated using a previously drawn standard curve of the pure drugs in PBS.

3.4.3 Cell Culture and NGF Induced Neuronal Differentiation

PC12, a cell line derived from the pheochromocytoma of the rat adrenal medulla and SHSY5Y, a human neuroblastoma originally procured from National Centre for Cell Science (NCCS), Pune has been maintained at *In vitro* Toxicology Laboratory at CSIR-IITR and used for the present study. In brief, PC12 cells were cultured in standard conditions in RPMI cell culture medium supplemented with FBS (5%), HS (10%), sodium bicarbonate (0.2%) and antibiotic/antimycotic cocktail (1%) under CO2 (5%), and high atmospheric humidity at 37°C. SH-SY5Y cells were cultured in the following conditions:5 % CO2, 95 % atmosphere of high humidity at 37 °C in DMEM/F-12 Ham's cell culture medium supplemented with 10 % FBS, 0.2 % sodium bicarbonate and antibiotic/ antimycotic cocktail (1×). For all studies, cells at passage 6–12 were used. Viability of cells was measured by trypan blue dye exclusion and batches of cells having more than 95% viability were used in the study.

After passage # 6, cells were plated on poly-L Lysine (PLL) coated flasks. For inducing neuronal differentiation, cells were incubated in medium containing NGF (100 ng/ml). Differentiation medium was changed on alternate day. For experimental purpose, confluent growing cells were sub-cultured in PLL pre-coated six-well culture plate and 75-Cm^2 culture flask.

3.4.4 Exposure Schedule

After identification of noncytotoxic and cytoprotective doses of cadmium, quercetin and nanoquercetin respectively at time point 24, 48, 72 and 96 hr using standard endpoints - tetrazolium bromide MTT assay as described by Agrawal et al. 2012, differentiated cells were exposed to Cadmium (10 μM) and quercetin (100 μM) and coexposed with cadmium and quercetin for 24 hr. To assess the comparative profiling of bulk vs nano quercetin, another set was also run where the differentiated cells were exposed to

cadmium (10 μM for 24 hr), quercetin (100 μM for 48hr) and nanoquercetin (100 μM for 48 hr) individually and in combination with cadmium, quercetin and nanoquercetin. Combined exposure was further devised in three major groups - pre-exposure, post-exposure and co-exposure for quercetin and nanoquercetin both. Unexposed sets were also run under identical conditions and served as basal control. Following respective exposures, cell viability was assessed. Further, markers associated with dopamine receptor mediated signaling, autophagy and apoptosis were also assessed.

3.4.5 Cell Viability Assay

Cell viability was ascertained by MTT assay following the standard protocol (Agrawal et al., 2012). In brief, cells (1x 10^4 cells/ml) were seeded in 96-well plates for 24 hr under high humid environment with CO2 (5%) and atmospheric air (95%) at 37^{o}C. The medium was aspirated and cells were exposed to variable concentrations of cadmium (0.1-1000 μM), quercetin (0.1-1000 μM) and naoquercetin (0.1-1000 μM) for 24 - 96 hr. Tetrazolium bromide salt (10 μl/well; 5 mg/ml of stock in PBS) was added 4 h prior to the completion of incubation in respective case. Plates were incubated at 37°C for 4 hr, MTT solution removed and cells were lysed using a culture grade DMSO by pipetting up and down several times until the content was homogenized. After 10 min incubation, the color was read at 550 nm using multi-well microplate reader (Synergy HT, Bio-Tek, USA). The unexposed sets were also run simultaneously under identical conditions which served as control.

3.4.6 Western Blotting (*In vitro*)

Western blotting was conducted following the protocol as described earlier (Kumar et al., 2015). After respective exposure with cadmium or quercetin alone or in combination, cells were scraped, pelleted, and lysed using CelLytic M Cell Lysis Reagent (Sigma) in the presence of protein inhibitor cocktail (Sigma). Equal amount (40 μg/ well) of denatured protein (determined by Bradford method) was loaded on SDS-PAGE gel (10-15%) and blotted onto a nitrocellulose membrane by wet transfer method. Nonspecific binding was blocked with BSA (5%) in TBST for 1 hr at 37°C. After blocking, the

membrane was incubated overnight at 4°C with primary antibodies specific for TH (1;1000), DAT (1:1000), VMAT2 (1 : 1000), DA-D1 and DA-D2 receptor (1 : 1000), PKA (1:000), phospho-PKA (1:1000), Akt antibody (1:1000), phospho Akt antibody (1:1000), phospho DARPP32 (1:1000), phospho PP1α (1:1000), GSK-3β (1:1000) CREB (1:000), phospho CREB (1:000), Bax (1:1000), Bcl2 (1:1000), caspase (1:1000) and β-actin (1;2000) in blocking buffer (pH 7.5). The membrane was incubated for 2 hr at room temperature with secondary anti-primary immunoglobulin G (IgG)–conjugated horseradish peroxidase. The blots were developed using Super Signal West Femto Chemiluminescent Substrate (ThermoFisher Scientific). The densitometry for protein-specific bands was conducted in Gel Documentation System (Alpha Innotech) with the help of Alpha Ease FC Stand Alone V.4.0 software. The marker proteins analyzed to study the altered expression were same as studied in the *in vivo* studies.

3.4.7 Pharmacological Inhibitor Studies

Role of DA-D2 and its downstream pathway molecules was confirmed using specific pharmacological inhibitors. The cells were seeded in PLL pre-coated 96 well culture plates and allowed to adhere for 24 h prior to the experimental exposure. Prior to exposure to cadmium (10μM) and quercetin (100μM) for 24 h, cells were exposed to pharmacological inhibitors of Akt (A6730: 5 μ M), PKA (H-89: 10 μM) for 1 hr respectively.

3.5 *In silico* Studies

To study the molecular level interaction of cadmium chloride (CdCl2), Quercetin with D1 and D2-Dopamine receptor, computational studies were done.

3.5.1 Homology Modeling of DA-D1 and DA-D2 Receptor

Protein sequence of the Dopamine D1 and D2 receptor in animal model *Rattus norvegicus* was retrieved from UniProt database (P18901, P61169). Homology based model for DA –D1 and DA- D2 receptor was built by homology modeling approach using Modeller version 9.15 (Martí-Renom et al., 2000). Templates for modeling protein

sequence were identified using blastP tool against Protein data Bank (Bernstein et al., 1977). Homolog having good structural similarity was used to build 200 protein models and top 20 models selected on basis of their lowest DOPE score were analyzed for structural stability using RAMACHANDRAN PLOT analysis feature of PROCHECK server (Laskowski et al., 1993).

3.5.2 Ligand Preparation

Structure of CdCl2, Quercetin and dopamine were built and minimized using MarvinSketch version 6.1.2 from ChemAxon and then clean in 3D using Steepest Descent method.

3.5.3 Molecular Docking

Ligand binding pockets on DA-D1 and DA- D2 protein were identified using binding site identification module of Discovery Studio 4.1(Wu et al., 2003). Top 3 sites identified in D2 receptor were docked with all 3 compounds (Quercetin, Dopamine and CdCl2) using CDOCKER module of DS version 4.1. UCSF Chimera was utilized for image generation (Pettersen et al., 2004).

3.5.4 DFT Studies

For predicting the structure of Cd-Quercetin complex, DFT calculations were performed with the Gaussian09 package (Frisch et al., 2009). DFT studies were carried out using Ground State, Default Spin and RB3LYP method. Basic set used was LanL2DZ. Guess method used was Default and solvation was performed without any constraint using the SMD model in water. At 298 K and1 atm Pressure, frequency analysis was carried out to confirm that each structure is a local minimum with no imaginary frequency, or either a transition state with only one imaginary frequency. The 3D images of the calculated structure were prepared using Gauss-View 5.0 (Dennington et al., 2009).

3.6 Statistical Analysis

Results are expressed as mean ± standard error of mean (SEM) for the values. One way analysis of variance (ANOVA) using the GraphPad prism3 software was used to analyze the data. To assess the levels of significance comparing all the pair of columns, Newman–Keuls test was employed and value up to $p < 0.05$ considered significant.