Chapter 3

Materials and Methods
Material and Method

Drugs and chemicals
Antibiotic-antimycotic, F-12 Hams media, fetal bovine serum, horse serum, nerve growth factor (NGF), phosphate buffer saline (PBS) were purchased from Invitrogen, USA, acrylic acid (AA), 5,5’-dithio-bis-2-nitrobenzoic acid (DTNB), amyloid beta 25-35 fragment (Aβ25-35), antibodies (NOS-2, NFkB, Caspase3, Bak, Bcl-2, Bax, Hsp-70), bovine serum albumin (BSA), cineole (Cin), diaminobenzidine (DAB), dimethylsulfoxide (DMSO), ethylene-diamine tetraacetic acid (EDTA), ferrous ammonium sulphate (FAS), glutathione reduced (GSH) and oxidized (GSSG), glutathione reductase (GR), horse reddish peroxidase (HRP) labeled anti-mouse IgG (H+L) and anti-rabbit IgG (H+L), JC-1[5,50,6,60-tetrachloro-1,10,3,30-tetraethylbenzimidazolylcarbocyanine iodide, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), N,N’-methylene bis acrylamide (MBA), nicotinamide adenine dinucleotide phosphate reduced (NADPH), N-isopropylacrylamide (NIPAAAM), Neutral Red Uptake (NRU), N-vinyl-2-pyrrolidone (VP), ouabain, Poly-L-Lysine (PLL), streptozotocin (STZ), thiobarbituric acid (TBA), thymoquinone (TQ), tricyclodecan-9-yl-xanthogenate (D609), trypan blue, trypsin-EDTA, were purchased from Sigma-Aldrich Chemical Pvt. Ltd., India. Caspase-9 and COX-2 antibodies were imported from M/s Santa Cruz Biotechnology Inc. California, USA. ChAT antibody was purchased from Abcam, Cambridge. PC-12 cell line was procured from NCCS, Pune. In vitro Toxicology Assay kit Lactate dehydrogenase based from Biovision. All other chemicals and reagents were of analytical grade and purchased locally.

Formulation of nanoD609 drug

Formulation of polymer:
A co-polymer of N-isopropylacrylamide (NIPAAAM) with N-vinyl-2-pyrrolidone (VP) and acrylic acid (AA) was synthesized through free radical polymerization. NIPAAAM was recrystallized using hexane; VP and AA were freshly distilled before use. Thereafter, NIPAAAM, VP and AA were dissolved in water in 90:5:5 molar ratios. The polymerization was initiated using ammonium persulphate (APS) as an initiator in a nitrogen (N₂) atmosphere. Ferrous ammonium
sulphate FAS (0.5 % w/v), which acts as promoter, was added to activate the polymerization reaction, and also to ensure complete polymerization of the monomers. In a typical experimental protocol, 90 mg NIPAAM, 5µl freshly distilled AA and 5µl freshly distilled VP were mixed and dissolved in 10 ml distilled water. To cross-link the polymer chains, 30 µl of N,N'-methylene bis acrylamide (MBA) (0.049 g/ml) was added to the aqueous solution of monomers. The dissolved oxygen was removed by passing nitrogen gas for 45 minutes. Thereafter, 20 µl of FAS (0.5 % w/v) and 30 µl of APS were added to initiate the polymerization reaction. The polymerization was performed at 30 °C for 24 h in a N₂ atmosphere. After the polymerization was completed, the total aqueous solution of polymer was dialyzed overnight with 2-3 times change of water using a Spectrapore® membrane dialysis bag (12 kD cut off) to remove any residual monomers. The dialyzed solution was then lyophilized immediately to obtain a dry powder for subsequent use, which was easily re-dispersible in aqueous media. The yield of the polymeric nanoparticles was typically ~ 90 % (Bisht et al., 2007).

**D609 loading**

D609 was physically adsorbed at the nanoparticle surface. The process of drug loading is described as post polymerization loading. Briefly, 50 mg co-poly (NIPAAM-VP-AA) micellar lyophilized powder was dispersed in 10 ml of double distilled water. Then D609 (10 mg/ml) solution in DMSO was gradually added in the co-polymeric solution and stirred vigorously at room temperature with mild sonication (30 s at 25 °C). The drug loaded polymeric nanoparticles were lyophilized to obtain dried nanoparticles powder product to be used subsequently.

**Surface modification of polymeric nanoparticles with polysorbate 80**

Lyophilized powder 10 mg was dispersed in 1 ml distilled water. Aqueous solution of 0.1% v/v polysorbate 80 was prepared and calculated volume of it was added to nanoparticulate dispersion to give 0.1% coating. Polysorbate 80 is known to be the ‘lead substance’ for targeted delivery of nanoparticles across the blood-brain barrier (Fig. 3.1).

**Adsorption efficiency (E %)**

The adsorption efficiency (E %) of D609 loaded nanoparticle was
determined as follows: the nanoparticles were separated from the unentrapped free drug using (100 kD cut off) membrane filter and the amount of free drug in the filtrate was measured spectrophotometrically at 306 nm.

The E % was calculated as:

\[
E\% = \frac{(\text{total drug} - \text{free drug})}{\text{total drug}} \times 100
\]

Characterization

i. Dynamic light scattering (DLS)

DLS measurements for determining the average size and size distribution of the polymeric micelles nanoD609 were performed using a Nanosizer 90 ZS (Malvern Instruments, Southborough, MA). The intensity of scattered light was detected at 90° to an incident beam. The freeze-dried powder was dispersed in aqueous buffer (0.1 mg/ml - 1 mg/ml) and filtered (0.2 mm, Millipore). Thereafter the size was measured. All the data analysis was performed in automatic mode. Measured size was presented as the average value of 20 runs, with triplicate measurements within each run.

ii. Transmission electron microscopy (TEM)
Size and morphology of the nanoparticles was determined by JEOL JEM 2000 EX 200 model as follows: one drop of the aqueous dispersion (5mg/ml) of polymeric nanoparticles followed by one drop of 1% phosphotungstic acid were put on a formvar coated copper grid (1% solution of formvar was prepared in spectroscopic grade chloroform) and then air dried in a vacuum desiccator. The dried grid was examined under an electron microscope unit of All India Institute of Medical Sciences (AIIMS), New Delhi.

**In Vitro Materials and Methods**

**Cell Culture**

The PC12 cell line was obtained from National Facility for Animal Tissue and Cell Culture (NCCS), Pune, India and since then it has been maintained in our laboratory. Cells were cultivated in F-12 Hams supplemented with 2.5% fetal bovine serum (FBS), 15% horse serum (HS), 0.2% sodium bicarbonate and 1.5% (100 x solution) of antibiotics and antimycotic. Medium was replaced twice a week. A 100 µl of cell suspension was stained with trypan blue (0.2%) and viable cells were counted using a hemocytometer. All treatments were performed with 80% confluent cells. Before experiments, the cells were precultured for 8 days with 50 ng/ml nerve growth factor (NGF) to differentiate in neuronal cells.

**Treatment**

Cells were cultured in flasks or plates. After 8 days of differentiation, the medium was replaced and fresh medium containing drugs was added for 24 h. Thereafter, the fresh medium containing beta amyloid was added for 24 h replacing medium containing drugs.

**Cytotoxicity and cell viability**

The protective effect of drugs on PC12 cell line was determined by the MTT dye-uptake method as described earlier (Siddiqui et al., 2011). In brief, cells (1x10^4 per well) were seeded in poly-L-lysine pre-coated 96-well tissue culture plates and allowed to adhere for 24 h in CO2 incubator at 37°C. Cells were differentiated for the indicated time period. Thereafter, the medium was replaced with the fresh medium containing different doses of drug for a period up to 24 h. Tetrazolium bromide salt (5 mg/ml of stock in PBS) 10 µl/well was added in 100 ml of cell suspension and plate was incubated for 4 h. At the end of
incubation period, the reaction mixture was carefully taken out and 200 µl of DMSO was added to each well by pipetting up and down several times until the content gets homogenized. The plates were kept on rocker shaker for 10 min at room temperature and then read at 550 nm using Multiwell microplate reader (Biorad, USA).

**Neutral red uptake (NRU)**

PC12 cells were seeded in 96-well culture plate at the same density as for MTT and the cell viability was also assessed by NRU assay as described by Borenfreund and Puerner (1985). The medium was replaced with the fresh medium containing different doses of drugs for a period of 24 h. Briefly, 0.4% aqueous stock solution of NRU dye was prepared and an aliquot was added to the complete medium to make the final concentration of 50 μg/ml. The neutral red 200 µl was added in the wells medium and incubation for 3 h at 37ºC. The viable cells stained with the dye. Thereafter, the dye medium was taken out and cells were washed rapidly with 40% formaldehyde. Thereafter 200 µl of 1% acetic acid and 50% ethanol was added, followed by an incubation of 20 min at 37ºC. The plates were read at 540 nm using Multiplate Reader (BioRad Model 680). Results were expressed as percentage absorbance.

**LDH Assay**

Cells were seeded in 96-well plate at a density of 2 x 10^4 cells/well in culture medium. The LDH activity was measured in lysate and supernatant of the cells using *in vitro* toxicology assay kit (Biovision) in accordance with the manufacturer’s instructions. The absorbance was determined at 490 nm using plate reader.

**Preparation of cell lysate**

For each enzymatic and non enzymatic assay 1 x 10^5 cells were seeded in 25 cm^2 flasks and differentiated with NGF. Differentiated cells were exposed to drug and toxin. Thereafter, the cells of each flask were scrapped and centrifuged at 5000 x g for 5 min at 4ºC. The supernatant was discarded and pellet was washed twice with 1 M PBS. 100 µl of chilled lysis buffer (20 mM Tris HCl, 150 mM NaCl, 1 mM EDTA and 1% Triton X-100) containing 10 µl/ml protease inhibitor was added to each tube. After 30 min of incubation the cells were sonicated in SONICS Vibra Cells Sonicator.
Assay for Na⁺/K⁺-ATPase

Na⁺/K⁺-ATPase activity was measured by the method of Svoboda and Massinger (1981). The Na⁺/K⁺-ATPase activity was determined in two reaction media, A and B. The reaction mixture A consisted of 0.2 M KCl, 1.0 M NaCl, 0.1 M MgCl₂, 0.2 M Tris–HCl buffer (pH 7.4) and 0.1 ml of cell lysate in a total volume of 2.0 ml. The reaction mixture B consisted of 0.1 M MgCl₂, 10 mM ouabain, 1.0 M NaCl, 0.2 M Tris-HCl buffer (pH 7.4) and 0.1 ml of cell lysate in a total volume of 2.0 ml. The enzyme reaction was started by adding 0.2 ml of 25.0 mM ATP at 37°C and terminated after 15 min by adding 1.0 ml chilled 10% TCA. The mixture was centrifuged and supernatant (0.5 ml) was used for the estimation of inorganic phosphorous according to method of Fiske and Subborao (1925).

Measurement of intracellular ROS

ROS formation was detected using a nonfluorescent compound, DCFH₂-DA. Once inside the cell, the de-esterified product becomes the fluorescent compound, DCFH₂ on oxidation by ROS. The fluorescent signal given by DCFH₂ is proportional to ROS production (Koh et al., 1987). After the exposure with drug and Aβ₂₅₋₃₅ for the mentioned time period, the cells (2 x 10⁶/ml) were added to 800 µl PBS and incubated with 10 µM DCFH₂-DA at 37 °C for 20 min, in an atmosphere of 95% air and 5% CO₂. After loading with DCFH₂-DA, the cells were washed with the same buffer and fluorescence was measured at 365 nm excitation and 430 nm emission.

Mitochondrial membrane potential assessment

JC-1 (5, 5’, 6, 6’-tetrachloro-1, 1’, 3, 3’tetraethylbenzimidazolylcarbocyanine iodide) was used following the method of Smiley et al. (1991). Briefly, cells were seeded in 24-well plate at a density of 1×10⁶ cells/well and the treatment was given as mentioned above. The harvested cells were incubated in 0.5 ml JC-1 (10 µM) for 8 min at room temperature in dark. After centrifugation for 5 min at 500×g and washing twice by PBS (pH 7.4) to remove unincorporated dye, pellets were resuspended in 2 ml PBS (pH 7.4). Red fluorescence (excitation 570 nm, emission 610 nm) and green fluorescence (excitation 490 nm, emission 535 nm) were measured using an spectrofluorimeter (Spectrometer LS50B, Perkin Elmer).
Results were expressed as the ratio of green to red fluorescence.

**Nitric Oxide determination**

Nitric oxide was determined by measuring the nitrite content in culture medium (Ding et al., 1988). Briefly, the cells were seeded in 96-well plate at density of $2 \times 10^4$ cells/well and incubated overnight. Thereafter, media was discarded and cells were exposed to medium containing TQ with 3 doses and thereafter $\text{A}\beta_{25,35}$ treatment was given. After 24 h, media from each well was transferred to fresh tube and centrifuged at 500 x g for 5 min at 4°C. The supernatant 100 µl was transferred to fresh 96-well plate and mixed with an equal volume of Griess reagent (0.04 g/ml PBS, pH 7.4). After incubation at room temperature for 10 min, the absorbance of resultant color was measured at 540 nm using a microplate reader (Bio-Rad, U.S.A). Concentration of nitrite in medium of treated and untreated cells was calculated using a standard curve of sodium nitrite and expressed as percentage of control.

**Immunocytochemical Assay**

Immunocytochemical analysis was performed to determine the expression of NOS-2, NFκB, COX-2, Apaf-1, Bak, Bcl2, Caspase-3 and Caspase-9 in treated cells. Cells were seeded in Poly-L-Lysine coated 24 well plate at a density of $2 \times 10^4$ cells/well. Cells were differentiated with NGF for 8 days and subsequently exposed to drug with varying concentration. Thereafter, cells were exposed to beta amyloid (10 μM) for 24 h. Media was carefully removed and cells were washed twice with PBS (pH 7.4) and fixed with 4% paraformaldehyde for 30 min. Again cells were washed thrice with PBS and incubated with 0.5% H$_2$O$_2$ in methanol for 1 h followed by incubation in 0.02% Triton X-100 in 0.1% BSA for 15 min to block the non specific binding sites. Finally, cells were washed and incubated with primary antibody. Cells were incubated overnight at 4°C and then immediately incubated with anti mouse or anti rabbit secondary antibody (1:500) for 2 h at room temperature. Further cells were washed thrice with PBS and visualized by DAB (3,3’-Di-aminobenzidine tetrahydrochloride) under simple microscope.

**In vivo Material and Method**

**Animals**
Male Wistar rats (1 year old) weighing 475–490 g were obtained from the Central Animal House Facility of Hamdard University. They were kept in cages and maintained under standard housing conditions (room temperature of 25±2 °C and relative humidity 45–55%) with 12 h light/dark reverse cycles. The standard rodent pellet diet and water were available ad libitum. The food was withdrawn 12 h before the surgical procedure. Experiments were conducted in accordance with the Animal Ethics Committee of the University appointed by the Government of India.

**Intracerebroventricular injection of streptozotocin to rats**

Bilateral *intracerebroventricular* streptozotocin (ICV-STZ) injection was given to make lesion in rat’s brain. The animals were anesthetized with 400 mg/kg b.wt. chloral hydrate intraperitoneally, placed on a stereotaxic frame, which is a dual manipulator of which one arm is attached with drill machine and another with Hamilton syringe. The skull was exposed and the stereotaxic coordinates for the lateral ventricle (Paxinos and Watson, 1986) were measured accurately as anterioposterior −0.8 mm, lateral 1.5 mm and dorso–ventral −4.0 mm relative to bregma and ventral from dura with the tooth bar set at 0 mm. A hole of 0.5 mm was done on skull with the help of drill machine and through that hole, a 28-gauge Hamilton® syringe of 10 μl which was attached on another arm of the dual manipulator of stereotaxic apparatus used for injecting the STZ. The lesioned groups received ICV injection of STZ (1.5 mg/kg b.wt. in saline, 5 μl/injection site) into each lateral ventricle and left for 5 min (diffusion rate is 1 μl/min) as reported (Javed et al., 2011; Ishrat et al., 2006). The sham groups underwent the same surgical procedures, but the same volume of saline was injected instead of STZ.

After surgery, the animals were housed individually and had access to food and water *ad libitum.*

**Post-operative care**
After surgery, the animals took approximately 1–2 h to recover from anesthesia. The rats were kept in a well-ventilated room at 25 ± 2 °C in individual cages and had access to food and water ad libitum until they regained full consciousness and then were housed together two animals per cage. Food was placed inside the cage for 2–3 days so that the animals could easily access it without any physical trauma due to head surgery.

**Behavioural testing**

The behavioural tests were started 2 weeks after ICV-STZ infusion. The experiment was performed between 9.00 A.M. to 4.00 P.M. at standard laboratory conditions. Behavioural tests were performed and analyzed by a researcher blind to the experimental conditions.

**Morris water maze test (MWM)**

Spatial learning and memory of animals were tested in MWM (Morris, 1984). It consisted of a circular water tank (132 cm diameter, 60 cm height) filled 40 cm with water (25±2 °C). A non-toxic paint was used to render the water opaque. The pool was divided into four equal quadrants, labeled south-west (SW), south-east (SE), north-east (NE) and north-west (NW). An escape platform (10 cm x 5 cm) was hidden 2 cm below the surface of the water at a fixed location in one of the quadrants. The platform remained in the same quadrant during the entire experiment. Before the training started, rats were allowed to swim freely in the pool for 60 s without platform. They were given four trials (once from each starting position) per session for 5 days, each trial having a ceiling time of 60 s and a trial interval of approximately 30 s. After climbing onto the platform, the animal remained there for 30 s before the commencement of the next trial. If the rat failed to reach the escape platform within the maximum allowed time of 60 s, it was gently placed on the platform and allowed to remain there for the same interval of time. A computerized digital tracking system (Columbus Instruments’, Videomex-ONE, Ohio, USA) was used to record escape latencies and path length during each trial.

Twenty four hours after the acquisition phase, a probe test was conducted by removing the platform. Rats were allowed to swim freely in the pool for 60 s. The time spent in the target quadrant, which had previously
contained the hidden platform was recorded. The time spent in the target quadrant indicated the degree of memory consolidation which had taken place after learning.

**Biochemical analysis:**

**Tissue preparation for biochemical estimation:**

After 21 days of ICV-STZ infusion, the rats were sacrificed and their brains were taken out quickly to dissect the hippocampus and frontal cortex. To prevent auto-oxidation of the dissected brain parts, homogenization was carried out at 4 °C in 10 mM Tris-HCl buffer (pH 7.4) containing 10 μl/ml protease inhibitor to get 5% w/v homogenate. The homogenate was centrifuged at 800 × g for 5 min at 4 °C to separate the nuclear debris. The supernatant 1 (S1) was used for the estimation of TBARS and AChE. The remaining S1 was further centrifuged at 10,000× g for 30 min at 4 °C to get the post-mitochondrial supernatant (PMS), which was used for estimation of reduced glutathione and antioxidant enzymes.

**Histological examinations**

**Hematoxylin and eosin (H&E) stain**

The animals were anesthetized with chloral hydrate on 22nd day of lesioning and perfused transcardially through ascending aorta with 100 ml ice cold phosphate buffered saline (PBS 0.1 M pH 7.4) followed by 4% paraformaldehyde in cold PBS (0.1 M pH 7.4). Brains were removed quickly, post fixed in the paraformaldehyde solution for 48 h, and embedded with wax. Coronal sections of 5 μm thickness having hippocampus were dewaxed and stained with hematoxylin and eosin. Histological specimens were examined by light microscope (Olympus BX 50, Japan).

**Cresyl violet (CV) stain**

Adjacent sections were taken for the staining of CV to identify the pyramidal neurons degeneration in the CA1 region of hippocampus. CV is a dye which stains nuclei, Nissl bodies and neurons in histological tissue. Briefly, sections were dewaxed in two changes of xylene 10 minutes each and process in gradient ethyl alcohol 5 min each, and finally in distill water for 2 min. Sections were then placed for 2 min into the cresyl violet solution (0.5% cresyl violet solution: 2.5 g cresyl violet (Sigma®, USA) and 1.5 ml glacial acetic acid was added to 500
ml distilled water. Thereafter, the sections were placed into a basin with tap water in which a constant flow of fresh water replaced the cresyl violet solution over a period of 5 min. In order to dehydrate, the sections were subjected to increasing ethanol concentrations and placed for 3 minutes each into 70%, 95% and 100% ethanol and finally cleared with xylene. Sections were cover-slipped with DPX mounting medium (Merck, Germany).

**Immunohistochemistry**

Immunohistochemistry was performed to detect the expression of anti-ChAT polyclonal rabbit (dilution 1:100), anti-Cox-2 polyclonal rabbit (dilution 1:200), anti-NOS-2 monoclonal mouse (dilution 1:300), anti-NFkB monoclonal mouse (dilution 1:300), anti-Apaf-1 monoclonal mouse (dilution 1:250), anti-Bak polyclonal rabbit (dilution 1:250), anti-Bax polyclonal rabbit (dilution 1:250), anti-BCl2 monoclonal mouse (dilution 1:300), anti-Caspase-3 polyclonal rabbit (dilution 1:500), anti-Caspase-9 polyclonal rabbit (dilution 1:250) and anti-Hsp-70 monoclonal mouse (dilution 1:250). Coronal sections (5 μm thick) at the level of the hippocampus were dewaxed and processed for immunohistochemical staining. The sections were collected serially on PLL coated slides and placed in 3% H₂O₂ in methanol for 20 min at room temperature to eliminate the endogenous peroxidase activity. Slides were washed with PBS for three times and pre-incubated in 2% bovine serum albumin for 30 min at room temp; thereafter, the slides were incubated with primary antibody, at 4 °C for overnight. Then, sections were incubated with biotinylated goat anti-rabbit or goat anti-mouse IgG (Jackson ImmunoResearch USA, dilution 1:500) and the labeled sites were visualized with a solution of diaminobenzidine and hydrogen peroxide. Finally, the sections were dehydrated, cover-slipped and viewed under a microscope, and photomicrographs were taken under light microscope (Olympus BX50, Japan).

**TBARS content**

The method of Utley et al. (1967), as modified by Islam et al. (2002), was used to estimate the rate of lipid peroxidation (LPO). S1/cell lysate 0.25 ml was pipetted into 15×100 mm test tubes and incubated at 37 °C in a metabolic shaker bath for 1 h. An
equal volume of the same S1/cell lysate was pipetted into a centrifuge tube, placed at 0 °C incubation. After 1 h of incubation, 0.25 ml of 5% (w/v) chilled trichloroacetic acid (TCA) was added to each test tube, followed by 0.5 ml of 0.67% TBA (w/v) and centrifuged at 4000 × g for 10 min. Thereafter, the supernatant was transferred to other test tubes and placed in a boiling water bath for 10 min. The absorbance of pink color produced was measured at 535 nm in a spectrophotometer (Shimadzu-1601, Japan). The TBARS content was calculated by using a molar extinction coefficient of 1.56×10^5 M\(^{-1}\) cm\(^{-1}\) and expressed as nmol of TBARS formed/h/mg protein.

**Reduced glutathione (GSH) content**

GSH content was determined by the method of Jollow et al. (1974) with slight modification. PMS/cell lysate was mixed with 4.0% sulfosalislylic acid (w/v) in a 1:1 ratio (v/v). The samples were incubated at 4 °C for 1 h, and later centrifuged at 1200 × g for 15 min at 4 °C. The assay mixture contained 0.1 ml of supernatant, 1.0 mM DTNB and 0.1 M phosphate buffer (PB, pH 7.4) in a total volume of 1.0 ml. The yellow color developed was read immediately at 412 nm in spectrophotometer (Shimadzu-1601, Japan). The GSH content was calculated as μmol GSH/mg protein, using a molar extinction coefficient of 13.6×10^3 M\(^{-1}\) cm\(^{-1}\).

**Glutathione peroxidase (GPx)**

GPx activity was estimated according to the procedure described by Mohandas et al. (1984). The reaction mixture consisted of phosphate buffer (0.05 M, pH 7.0), EDTA (1 mM), sodium azide (1 mM), GR (1 EU/ml), glutathione (1 mM), NADPH (0.2 mM), hydrogen peroxide (0.25 mM) and 0.1 ml of PMS/cell lysate in the final volume of 1 ml. The disappearance of NADPH at 340 nm was recorded at room temperature and the enzyme activity was calculated as nmol NADPH oxidized/min/mg protein using molar extinction coefficient of 6.22×10^3 M\(^{-1}\) cm\(^{-1}\).

**Glutathione reductase (GR)**

GR activity was assayed by the method of Carlberg and Mannervik (1975) as modified by Mohandas et al. (1984). The assay mixture consisted of phosphate buffer (0.1 M, pH 7.6), NADPH (0.1 mM), EDTA (0.5 mM), GSSG (1 mM) and 0.05 ml of
PMS/cell lysate in total volume of 1 ml. The enzyme activity was quantified by measuring the disappearance of NADPH at 340 nm and was calculated as nmol NADPH oxidized/min/mg protein using molar extinction coefficient of 6.22 x 10³ M⁻¹ cm⁻¹.

Catalase (CAT)
CAT was assayed by the method of Clairborne (1985). In brief, the assay mixture consisted of 0.05 M phosphate buffer (pH 7.0), 0.019 M H₂O₂ and 0.05 ml PMS/Cell lysate in a total volume of 1.0 ml. The change in absorbance was recorded at 240 nm. CAT was calculated in terms of nmol H₂O₂ consumed/min/mg protein using molar extinction coefficient of 43.6×10³ M⁻¹ cm⁻¹.

Acetylcholinesterase (AChE) activity
AChE activity was determined by a modified method of Ellman et al. (1961). Briefly 2.6 ml of PB (0.1 M, pH 8.0), 40 μl of S1/cell lysate and 0.1 ml of buffered Ellman’s reagent (DTNB 10 mM, NaHCO₃ 15 mM) were preincubated for 5 min at room temperature. The reaction was started by adding 40 μl substrate (0.075 M acetylthiocholine iodide) and the optical density was measured at 412 nm within 5 min. AChE activity was expressed as nmol thiocholine formed min⁻¹ mg⁻¹ protein.

Measurement of Cytokines
Commercially available rat TNF-α, rat IL-1β and rat IL-6 kits (eBioscience, BD Bioscience, USA) with high sensitivity were used to quantify these cytokines according to the manufacturers’ instructions. PMS/Cell lysate were analyzed and each sample was in duplicate. Cytokine levels were quantified according to manufacturer’s guidelines. Activity was calculated as ng/mg protein or pg/ml.

Protein content
Protein content was determined by the method of Lowry et al. (1951) using bovine serum albumin (BSA) as a standard.

Statistical analysis
Results are expressed as mean±S.E.M. Statistical analysis of the data was done by applying the analysis of variance (ANOVA), followed by Tukey's test. Behavioural parameters were analysed by applying the two-way analysis of variance (ANOVA)
followed by Bonferroni test. The p-value < 0.05 was considered statistically significant.