MATERIALS AND METHODS
4.MATERIALS AND METHODS

In this investigation, both experimental animals as well as primary cell culture were used. The primary hippocampal cell culture technique was standardized in the laboratory. Considering the advantages of in vitro studies to delineate the mechanisms of memory dysfunction during hypoxic conditions the materials and methods section first describes the culture conditions used, culture material, oxidative stress parameters estimation etc. After knowing the mechanisms in vitro the rats were used for in vivo studies, which consisted of memory testing in Morris water maze, morphological studies, oxidative stress and antioxidant parameters estimation in hippocampus. All the biochemical experiments were done using spectrofluorimeter (Fig 10) and spectrophotometer supplied by Varian, USA. The kits used in the present study were supplied by RANDOX, Germany. The chemicals used in the present study are all of Sigma analytical grade.

The cell culture was done using hippocampus tissue of the newborn rat pups. The cultured cells consisting of mainly neurons and glia (80: 20) were subjected to 3h hypoxia. Free radical production, nitric oxide levels, glutathione content, glutathione peroxidase and glutathione reductase activities, intracellular calcium levels, cytotoxicity, mitochondrial membrane potential and DNA fragmentation experiments were carried out .In another set of experiments N- acetyl cysetine was added to the cells during hypoxic conditions and the changes were assessed and compared with the cells without the supplementation of NAC to find out the protective effect of NAC against oxidative stress. The experimental design for in vitro studies is given as a diagrammatic representation in Fig 11.
FIG 10: SPECTROFLUORIMETER
FIG 11: EXPERIMENTAL DESIGN: IN VITRO STUDIES

PRIMARY HIPPOCAMPAL CELL CULTURE FROM 0 DAY OLD RAT PUPS

INCUBATION AT 37°C FOR 8 DAYS

EXPOSURE TO 3 h HYPOXIA ON 8TH DAY

1 h RECOVERY UNDER NORMOXIA

ESTIMATION OF FR, NO, GSH, GPx, GR, MMP, Ca²⁺, LDH, DNA FRAGMENTATION, GLUTAMATE PRODUCTION
Morris Water Maze technique was standardized in the laboratory for assessment of spatial working memory in rats. The experimental design for *in vivo* studies is given in Fig 12. The changes in latency and path length to reach the target were assessed. Morphological studies were carried out in CA1 and CA3 area of hippocampus during hypoxic conditions. Besides this the free radical production, antioxidant status and lipid peroxidation studies were carried out.

Both in vitro and in vivo studies consisted of the following four groups:

- **Group I** - Normoxia
- **Group II** - Hypoxia exposed
- **Group III** - Hypoxia + NAC
- **Group IV** - Normoxia + NAC

The details of the protocols followed for the studies are discussed in the ensuing paragraphs.

### 4.1. CONDITIONS FOR IN VITRO STUDIES

1. Horizontal Laminar flow (Fig 13).

A laminar flow (Hicon instruments, Delhi, India) was used which maintained a sterile work environment. It provided unidirectional oriented flow of filtered clean air at graded velocities designed to flush contaminating particles. The ultraviolet lamp was switched on for an hour prior to the use of flow cabinet. The entire work place within the laminar flow cabinet was sterilized by 70% alcohol, at the end of each experiment.

2. Glassware, storage vials, pipette tips, dissection instruments and filters were sterilized by steam autoclaving (15 psi for 45 minutes)

3. All solutions were filter sterilized, using 0.22µ low protein-binding filters.
FIG 12: EXPERIMENTAL DESIGN: IN VIVO STUDIES

PROCEDURE

HABITUATION IN MORRIS WATER MAZE

ACQUISITION IN MORRIS WATER MAZE FOR SPATIAL MEMORY

SPATIAL MEMORY TESTING

EXPOSURE TO HYPOBARIC HYPOXIA (6100 m) FOR 3 DAYS

SPATIAL MEMORY TESTING

MORPHOLOGY & ESTIMATION OF FR, GSH, GPx, GR, LP IN HIPPOCAMPUS

DAYS

01

2-8

9

10-12

12

12
FIG 13: LAMINAR FLOW
4.2. PREPARATION OF CULTURE MEDIUM

The medium was prepared by dissolving Dulbecco's Modified Eagles Medium (DMEM) powder (SIGMA) in 1 litre of distilled water (13.4 g/l). To this medium penicillin G (50mg/l) and streptomycin (50mg/l) were added along with sodium bicarbonate (1.1g/l). The pH of the medium was adjusted to 7.3. This medium was then sterilized by passing through sterile filter assembly, fitted with 0.22μ membrane filter using vacuum pump. Later the medium was stored in sterile polypropylene bottles, at 4°C, for further use. For the preparation of complete medium, Fetal Bovine Serum (FBS) was added to incomplete medium to give a final concentration of 10%.

4.3. PREPARATION OF PRIMARY HIPPOCAMPAL CELL CULTURE

Newborn (0 day old) Wistar rat pups were used for primary culture as described by the method of Sunanda et al., 1998. Pups were decapitated and the heads were immersed in 70% ethanol. The hippocampi were removed quickly and placed in a sterile petridish with the culture medium. Hippocampi from all the pups were pooled in 2ml of the culture medium in a sterile petridish (or penicillin vial). The tissue was then mechanically triturated through an 18-guage needle attached to a 10 ml syringe.

A multi well culture plate (24 wells) was coated with poly-l-lysine (0.1mg/ml) and incubated for one hour. After incubation poly-l-lysine was removed and again the tray was incubated with 500 μl of culture medium for half an hour, thereon 300 μl of cell suspension was added bringing the final volume to 800 μl. Cells were cultured (or grown) in a 5% CO₂ incubator at 100% humidity for 8-10 days and checked periodically for any contamination. The medium was replaced every alternative day. After 8 days in vitro,
successfully cultured neurons (identified by a phase contrast microscope) were selected for further studies (Fig 14).

4.4. HYPOXIC EXPOSURE OF CELLS

The hippocampal cell cultures were exposed to hypoxia for 3 h by transferring the culture plates to a humidified incubation chamber (Fig 15) maintained at 37°C and flushed with a gas mixture consisting of 95% N₂, 5% CO₂. Later, cultures were allowed to recover at 37°C under normoxic conditions for 1 h. The controls were maintained under normoxic conditions throughout. The Po₂ level was checked in the extra cellular medium collected just prior to the beginning and immediately after 3 h of hypoxic insult using IL-1312 Gas Analyzer (IL, SPA, USA). The Po₂ level in the culture medium under normoxic condition was found to be 165-167 mmHg and after 3 h of exposure to 95% N₂ and 5% CO₂ the reduction of the Po₂ was 83-85 mmHg indicating cells were exposed to reduced partial pressure of oxygen in the culture medium by about 50±3% as compared to the control.

4.5. SUPPLEMENTATION OF NAC

Earlier studies using different concentrations of NAC ranging from 10-100 μM concentrations has shown the optimum dose of NAC as 50 μM. NAC solution was prepared freshly in filter sterilized PBS before adding to the media. Since the optimum concentration of NAC was found to be 50 μM, all the experiments have been conducted in this concentration.
FIG 14: PHASE CONTRAST MICROGRAPH OF PRIMARY HIPPOCAMPAL CELL CULTURE ON 8\textsuperscript{TH} DAY
FIG 15: CO₂ INCUBATOR
4.6. MEASUREMENT OF FREE RADICAL PRODUCTION

Free radicals were estimated as described by the method of Cathcart et al., 1983.

**PRINCIPLE**

Dichlorofluoresceindiacetate (DCFH-DA) is a nonfluorescent diester, which can be taken up by the cells. In the cells, the esterases in the cytoplasm hydrolyse DCFHDA to DCFH by releasing acetic acid molecules. The free radicals produced in the cells now react with DCFH to release highly fluorescent fluorescein, which is proportional to the concentration of free radicals.

**PROCEDURE**

To the cell samples, 10 µl of 100 µM DCFH-DA was added and further incubated for 40 min. The medium was removed and 200µl of lysis buffer was added to the cells and after 10 min, 150 µl of the solution was transferred to 3 ml phosphate saline buffer and the absorbance was measured using spectrofluorimeter at 485nm excitation and 530 nm emission. Values were expressed as fluorescent units formed as a measure of free radical formation.

4.7. MEASUREMENT OF NITRIC OXIDE PRODUCTION

Nitric oxide (NO) was assayed by the method of Green et al., 1982.

**PRINCIPLE**

On stimulation, cells release nitric oxide (NO) in the medium, which is readily converted into nitrite (-NO₂). The Greiss reagent (40µg/ml) contains α-napthalamine and sulphanillic acid, which reacts with the nitrite (NO₂) to give a pink colour, which is quantified by measuring the absorbance at 540nm using spectrophotometer.
**PROCEDURE**

After the exposure of cells to hypoxia for 3 hrs 50μl of supernatant was taken followed by the addition of equal amount of Griess reagent. The contents were further incubated for 15min and the absorbance was measured at 540nm using Elisa reader in spectrophotometer. The results were expressed as OD at 540 nm.

**4.8. LACTATE DEHYDROGENASE ESTIMATION**

The cytotoxicity was determined by measuring the LDH release in the medium spectrophotometrically as described by Koh and Choi (1987).

**PRINCIPLE**

\[
\text{Pyruvate} + \text{NADH} + \text{H}^+ \rightarrow \text{Lactate} + \text{NAD}^+ 
\]

**REAGENTS**

- Sodium pyruvate 7.5 mM
- NADH 1mM
- Phosphate Buffer 0.1 M

**PROCEDURE**

LDH activity was assessed spectrophotometrically by kinetic determination: Briefly 100μl of sample, 10μl of sodium pyruvate (7.5mM), and 80μl of phosphate buffer (pH 7.2) were added. Then 10μl of NADH (1mM) was added and the NADH consumption was followed for 2 min at 340 nm and expressed as NADH/min/mg protein.

**4.9. MITOCHONDRIAL MEMBRANE POTENTIAL**

**PRINCIPLE**

Rhodamin 123, a cationic fluorescent dye binds in the mitochondria membrane and the fluorescence intensity of the dye decreases quantitatively...
in response to dissipation of the mitochondrial membrane potential. In the present study, Rhoamin123 was used to evaluate perturbations in mitochondrial membrane potential as described by the method of Russel et al., 1999.

**PROCEDURE**

Cells were incubated with 5μl of Rhodamin 123 for 30 min. Then 200μl of lysis buffer was added to the cells and after 10 min, 150μl of the solution was transferred and diluted prior to fluorescence measurement at 485nm excitation and 530 nm emission in spectrofluorimeter. The Rhodamin 123 tracing was done in cells immediately at the end of hypoxia, 30 min and 1h of recovery under normoxic conditions, as well as in the NAC treated group. The fluorescent units are expressed as percent variation from controls.

**4.10. GLUTATHIONE ESTIMATION**

Glutathione was estimated by the method of Bruchill et al., (1978).

**REAGENTS**

Metaphosphoric acid

o-pthaldehyde (OPT)

**PRINCIPLE**

Glutathione (GSH) is an intracellular molecule and is stable in acidic medium. Therefore the cells are lysed using metaphosphoric acid to release GSH into the medium. Phosphate buffer is added to maintain the pH. The thiol group of GSH reacts with o-pthaldehyde (OPT) to form a complex, which gives fluorescence with an excitation at 350nm and emission at 420nm.
PROCEDURE

After incubation, the medium was removed and the cells were washed with saline thrice. The cells were lysed by adding 200µl of lysis buffer and incubated for 10 min. To this, 200µl of metaphosphoric acid was added. The contents were centrifuged for 10min at 10,000 rpm. Later, 150µl of the supernatant was collected and 150µl of o-pthaldehyde (OPT) was added. To this 2.7 ml of Phosphate buffered saline was added and the volume was made upto 3ml. After 15min of incubation absorbance was measured at 350nm excitation and 420nm emission. The results are expressed as ng / mg protein.

4.11. GLUTATHIONE PEROXIDASE ESTIMATION

This method is based on that of Paglia and Valentine (1967) by using kit. GPx catalyses the oxidation of Glutathione (GSH) by cumene hydroperoxide. In the presence of glutathione reductase (GR) and NADPH the oxidized glutathione (GSSG) is immediately converted to the reduced form with a concomitant oxidation of NADPH to NADP⁺. The decrease in absorbance at 340 nm is measured.

REACTION PRINCIPLE

\[
\begin{align*}
2\text{GSH} + \text{ROOH} &\xrightarrow{\text{GPx}} \text{ROH} + \text{GSSG} + \text{H}_2\text{O} \\
2\text{GSSG} + \text{NADPH} + \text{H}^+ &\xrightarrow{\text{GR}} \text{NADP}^+ + 2\text{GSH}
\end{align*}
\]
**REAGENTS COMPOSITION**

1. Reagent
   - Glutathione 4 mmol/l
   - Glutathione reductase ≥ 0.5 U/l
   - NADPH 0.34 mmol/l

2. Buffer
   - Phosphate buffer 0.05 mmol/l; pH 7.2
   - EDTA 4.3 mmol/l
   - Cumene Hydroperoxide 0.18 mmol/l

**PREPARATION OF SOLUTIONS**

1. Reagent
   One vial of reagent 1 was reconstituted with 10 ml of Buffer 2.

2. Cumene Hydroperoxide
   10 μl of cumene hydroperoxide was diluted with 10 ml of redistilled water and mixed thoroughly by shaking vigorously as the cumene is difficult to dissolve. It was prepared fresh daily.

**PROCEDURE**

To 250 μl of buffer, 20 μl of cell lysate and 10 μl of cumene hydroperoxide were added and immediately absorbance was measured. Temperature was maintained at 37°C. For blank, instead of cells distilled water was used. Absorbance was measured after 1 min (A₁), 2 min (A₂) and 3 min (A₃) interval.

**CALCULATION**

\[ \Delta A/\text{min (change per minute)} = \frac{A₃ - A₁}{3} \]
ΔA/min value of blank was subtracted from each sample.

ΔA/min sample - ΔA/min blank = ΔA/min sample

ΔA/min sample x 8412 = U/mg protein.

The GPX activity was expressed as U/mg protein.

4.12. GLUTATHIONE REDUCTASE ESTIMATION

REACTION PRINCIPLE

Glutathione reductase catalyses the reduction of glutathione (GSSG) in the presence of NADPH, which is oxidized to NADP⁺. The decrease in absorbance at 340nm is measured.

NADPH + H⁺ + GSSG GR
NADP⁺ + 2 GSH
(GSH=Reduced Glutathione)

REAGENT COMPOSITION

1. Buffer
Phosphate Buffer 250mmol/l pH 7.3
EDTA 0.5mmol/l

2. Substrate
GSSG 2.2mmol/l

3. NADPH
0.17mmol/l

PREPARATION OF REAGENTS

1. Substrate
The contents of one vial of substrate 2 were reconstituted with 5ml of Buffer1.

2. NADPH
One vial of NADPH 3 was reconstituted with 3ml of redistilled water.
PROCEDURE

To 200μl of substrate, 8 μl of cell lysate and 40μl of NADPH were added and immediately absorbance was measured. Absorbance was measured after 1min(A1), 2min(A2) and 3min(A3) interval.

CALCULATION

ΔA/min (change per minute) value was calculated by

\[ \frac{A3-A1}{3} = \Delta A/\text{min} \]

ΔA/min sample x 4983 = U/mg protein.

The GR activity is expressed as U/mg protein

4.13. DETERMINATION OF INTRACELLULAR Ca^{2+} LEVELS

The cultured cells were exposed to hypoxia for 3 h and Ca^{2+} was determined by using 2μM of Fura 2AM, for 1 h at 37°C as described by Phillip et al., 1999. The cells were washed twice with analysis buffer (KH2PO4 5.3mM, NaCl 137 mM, CaCl2 4.2mM, BSA, 0.1%) pH 7.4, incubated with Fura 2AM for 1h at 37 °C. Later the fluorescence was measured at excitation wavelengths of 340-380 nm, with an emission wavelength at 510 nm using spectrofluorimeter. The ratio between the fluorescent intensities of Fura 2-Ca^{2+} complex and the unchealated Fura 2 (F340-/F380) reflects the cytosolic Ca^{2+} concentration. The Fura 2 tracing was done in cells immediately at the end of hypoxia, 30 min and 1 h of recovery under normoxic conditions in hypoxic as well as in the NAC treated group. The results are expressed as fluorescence units 340/380 ratio.
4.14. DNA FRAGMENTATION

The comet assay was used to assess DNA strand breaks as described by Tice et al. 1990. Microscope slides were precoated with agarose (0.1%). The cells were exposed to hypoxia for 3 h. The cells were then washed in PBS and 100μl of cell suspension (3.5x10⁴ cells) was mixed in warm low gelling agarose (0.75%) and pipetted onto the slides and put immediately on ice to allow the agarose to solidify. The slides were placed in cold lysis solution (SDS 2.5%; sodium sarcosinate 1% and EDTA 25 mM, pH 9.5) for 15 min at room temperature. After washing the slides in distilled water, the slides were placed in horizontal electrophoresis tank (BIORAD) containing electrophoresis buffer (Tris 90mM, Boric acid 90mM, EDTA 25mM, pH 8.3) and electrophoresed for 5 min at 2V/cm. The slides were washed in distilled water, stained with propidium iodide (25 µg/ml) and cells were then visualized using Hunds Fluorescent microscope. One hundred nuclei on each slide were scored and given a score from 0 (Undamaged nuclei) to 4 (severely damage nuclei) and scored randomly as per the method described by Collins et al., 1995. Results were expressed as arbitrary units.

4.15. GLUTAMATE PRODUCTION

Cells were cultured on 24 well plates and were exposed to acute hypoxia. Later cells were given 1h reoxygenation and were fixed with 3% gluteraldehyde overnight at 20° C. Next day cells were permeablized in PBS containing 0.1% Triton X 100. This was followed by exposure to the anti glutamate mouse monoclonal primary antibody for 2h. After washing the cells in PBS, the secondary antibody labeled with horsh radish peroxidase (1:200 concentration) was added for 2 h at room temperature. The cells were then
washed with PBS thrice, after which diaminobenzidine was added and kept in dark for 15 min. Then again cells were washed with PBS and observed under microscope for glutamate production qualitatively.

**IN VIVO STUDIES**

**4.16. HYPOBARIC HYPOXIA FOR IN VIVO STUDIES**

For simulating high altitude conditions, a specially designed environmental decompression chamber was fabricated, where pressure, temperature and humidity could be controlled at a desirable level (Fig 16). There is a camera fixed inside the chamber for recording the behavior of the animal during hypobaric exposure, which could be viewed on the computer monitor. At one point of time, 12 rat cages can be accommodated in the decompression chamber. Hypoxic group and hypoxia + NAC treated group were exposed to hypobaric hypoxic chamber operated under a 12 Oh light and dark cycle. Rats were exposed to hypobaric hypoxia equivalent to 6,100 m for 3 days continuously except for 30-40 min when they were brought to sea level for replenishment of food and water and for supplementation of NAC. The temperature inside the chamber was kept at 30°C ± 0.5°C, pressure at 380 ± 2 mmHg, and humidity was maintained at 60± 2 % and the fresh air was allowed to flow into the chamber at the rate of 5.5 l/m.

**4.17. ANIMAL PREPARATION**

Male Wistar rats (210 to 230 mg) were obtained from the animal house of Defence Institute of Physiology and Allied Sciences. They were maintained in a standard environment with 12 light hours and a constant temperature of 25°C. They had free access of food and water *ad libitum*. Animals were
divided into four groups viz. Control (N=5), Hypoxia (N=5), Hypoxia+NAC treated groups (N=5) and control+NAC treated group (N=5). They were exposed to chronic hypoxia for 3 days in hypobaric hypoxic chamber (20,000ft). NAC (750 mg/kg) was given as an oral supplementation to the rats daily for 3 days. Control rats received vehicle only.

4.18. MORRIS WATER MAZE TEST OF SPATIAL MEMORY

The mammalian hippocampus plays a central role in spatial as well as declarative /relational and episodic memory in rats. In human and non-human primates, selective deficits in declarative or explicit memory are associated with neuronal loss/damage in the CA1 region of hippocampus (Squire 1992).

Spatial learning and memory was assessed in Morris water maze (180cm in diameter and 153cm height, filled to a depth of 50cm) with 27°C water, clouded by the addition of non toxic white paint (150ml) as described previously (Row et al., 2002; Gozal et al., 2001). Positions were designated from middle of the N, S, E, and W quadrants (Fig 17). The tank has an escape platform centered in one of the four maze quadrants and submerged 2cm below the maze surface. The room was dimly illuminated. Tapes of all trials were recorded in data sheets and analyzed by computer assisted tracking system, videomax V, Columbus instruments; USA. An overhead videocamera was mounted above the water tank. The latency (time taken by the rat to find out the platform) and distance (traveling of rat in the maze in cms) to find the platform was measured using the Morris Water Maze software.
FIG 16: ANIMAL DECOMPRESSION CHAMBER
FIG 17: MORRIS WATER MAZE WITH VIDEO TRACKING SYSTEM
4.19. WORKING MEMORY TRIALS ACQUISITION

Rats were daily submitted to four trials per day for 8 days to find out the submerged platform. The task consisted of two sessions per day for 8 days. The platform position changed between each session. The session began by placing the rat on the platform for 30 sec. The rat was then placed into an entry point facing the wall of the tank, and allowed to find the platform for a maximum of 60 sec. They remained on the platform for 30 sec before being removed to their cage. The entry points used within a session were equidistant from the platform and were randomly assigned for each trial. The distance traveled by the rat to find the hidden platform was measured and expressed as pathlength (cms) and the time taken by the rat to find the hidden platform is measured and expressed as latency (sec).

4.20. HISTOLOGY STUDIES

PRINCIPLES OF TISSUE PROCESSING

The aim of the tissue processing is to embed the tissue in a solid medium firm enough to support the tissue and give it sufficient rigidity to enable thin sections to be cut. The most satisfactory embedding material for routine histology is paraffin wax. Most fixatives are aqueous based and these are not miscible with paraffin wax. To enable impregnation with this medium, the tissue must be processed. The stages involved are

1. Perfusion
2. Fixation
3. Dehydration
4. Clearing
5. Embedding tissue in paraffin wax
6. Block making

**PERFUSION**

Animals were perfused intracardially with 0.001M Phosphate buffered saline (PBS) and 10% buffered formalin in PBS.

**FIXATION**

Brain tissue was removed and fixed in 10% formalin till the processing was done. Fixation of the tissue was done to prevent autolysis and for preserving various chemical constituents of the cell. It protects the tissue against alteration during embedding and sectioning. It also prepares the tissue for subsequent treatments such as staining.

**DEHYDRATION**

Tissue contains about 80% of water, which must be replaced with suitable organic solvent before infiltration. This is the first stage of processing. Dehydration was done using different grades of ethanol. This is a clear colorless flammable liquid. It is hydrophilic and therefore miscible with water and with many organic solvents. The water was removed by passing the tissue through series of solutions of ascending concentration of ethanol as indicated below.

1. 30% alcohol 2hrs
2. 70% alcohol 2hrs
3. 90% alcohol 3hrs
4. 100% alcohol 3hrs
CLEARING

Few dehydrating agents are miscible with wax. Fluids, which are miscible with both dehydrating agents and paraffin wax, are generally hydrocarbon solvents and most of them have refractive indices as protein. When the dehydrating agent has been entirely replaced by most of the solvents the tissue has a transparent appearance; hence the term clearing agent. Xylene was used as a clearing agent in the present study considering its criteria as a suitable clearing agent; 1. It removes the dehydrating agent rapidly 2. It can be easily removed by paraffin wax 3. It causes minimal tissue damage. 4. Low cost

After dehydration 2 changes of xylene was given as indicated below.

1. Pure xylene 3hrs
2. Xylene wax (1:1) 2hrs

EMBEDDING TISSUE IN PARAFFIN WAX

Paraffin wax continues to be the most popular embedding medium in histology for a number of reasons. It is cheap, easily handled and section production is easy. It has a wide range of melting points, ranging between 40-70 °C. Heating the wax to a high temperature alters the properties of paraffin wax by changing the crystalline structure and it improves the section cutting. Tissues are not rigid enough to be cut into thin sections without an additional support. This rigidity is provided by infiltering the wax into the tissue. It is done by gradually decreasing the concentration of clearing agent and proportionally increasing the concentration of the embedding medium i.e. wax. Embedding was done in pure wax using L shaped block embedding molds. Paper labels written with lead pencil were put inside the wax.
TRIMMING

Trimming was done to remove the extra wax from different sides of the tissues.

HISTOLOGY

Serial sections of the hippocampus were cut 5 µm thick at the level of −3.3mm bregma. All sections were mounted on the slides for morphological studies.

STAINING

Staining was done using Cresyl violet stain. Cresyl violet was prepared as follows. Briefly, 0.2 gm of cresyl violet was dissolved in 150 ml of distilled water. From this 10 ml was taken and dissolved in 100ml of 0.1 M acetic acid. The staining was done as described below

Xylene I 20min
Xylene II 20min
100% Alcohol 10min
90% Alcohol 5min
70% Alcohol 5min
50% alcohol 5min
30% Alcohol 5min
Tap water washing 2-3min

Then the slides were dipped in distilled water, followed by cresyl violet stain and the sections were kept at room temperature for 5 min. After 5 min slides were dipped in distilled water and kept for air drying for 35-40min. After complete drying distaining was done as described below

Xylene 2 min

The slides were mounted in DPX for morphological studies.
4.21. MORPHOMETRY

Morphometry and quantitative analysis included direct visual counting of the number of cells in CA1 and CA3 subfield of the hippocampus at a magnification of 400x. The cells in the CA1 and CA3 subsectors were counted at the level of dorsal hippocampus (Bregma AP-3.3 mm) over 100 μm length. Total number of cells were counted in both the hemispheres of six serial sections per rat. These six measurements were then averaged to derive a mean number of cells.

4.22. TISSUE PREPARATION FOR BIOCHEMICAL ASSAYS

At the end of exposure all the experimental groups were sacrificed by cervical dislocation and the brains were removed quickly and 10% homogenate was made in 0.154M KCl using a sonicator (spinning for 50 sec). Free radicals, glutathione and membrane lipid peroxidation was done on the very same day of sacrification. The glutathione peroxidase and reductase activity were estimated in the supernatant stored at -80°C.

4.23. FREE RADICAL PRODUCTION

Free radical production was estimated as described in the section 4.6.

4.24. ANTIOXIDANT STATUS

Glutathione content was determined in the tissue homogenates as described in the section 4.10. Glutahtione peroxidase and reductase were estimated in the supernatants as described in the section 4.11 and 4.12.

4.25. ESTIMATION OF MELANODIALDEHYDE

Malondialdeyde (MDA), one of the end products of lipid peroxidation was analyzed by the method of Dousset et al., 1983. Tetraethoxypropane
correlates quantitatively to MDA during reaction production. In this reaction tetraethoxypropane is combined with thiobarbituric acid (TBA), their hydrolysis by acid causes the formation of a pink colour compound, which is measured spectrophotometrically at 531 nm.

**REAGENTS REQUIRED**

- Tricarboxylic Acid (20%)
- Thiobarbituric acid (0.6%)
- 0.05M NaOH

**PROCEDURE**

Briefly, to 250µl of plasma, 750 µl of 20% TCA and 750 µl of 0.67% TBA was added. The samples were incubated in water bath at 85°C for 45 min. The samples were then kept at room temperature. The samples were then centrifuged at 2000rpm for 5 min. About 200µl of the supernatant was removed and the absorbance was taken at 531nm. The values were represented as nmols / mg protein.

4.26.**PROTEIN ESTIMATION**

Protein content was estimated for both *in vitro* and *in vivo* studies by the method of Lowry *et al.*, (1951) using Bovine serum albumin as standard.

4.27.**STATISTICAL ANALYSIS**

All biochemical experiments were carried out on three different occasions and the results are represented as mean ± SD of six individual observations and statistical difference for the comparison was evaluated using students 't' test. For morphological studies one way ANOVA was used. For memory testing one-way ANOVA with repeated measures, and 'F' test were used.