MATERIALS AND METHODS
**Experimental design**

Male albino rats weighing 150 ± 20 gm from Central Animal House, JN Medical College were used throughout the study. Animals were housed in air-conditioned room and had free access to pellet diet Hindustan Lever Ltd., Mumbai, India and water *ad libitum*.

For various sets of biochemical studies different groups comprising six animals each were used. Animals from Group I served as control while, animals of Group II, III and IV were used as experimental sets. Group II animals were given 1 mg/kg body weight Methyl mercury Chloride (MeHgCl). Animals of Group III received 35 mg/kg body weight Alpha Lipoic Acid. Group IV animals were given 1 mg/kg body weight MeHgCl and 35-mg/kg body weight Alpha Lipoic Acid. All groups were treated once a day each for seven days intraperitonially. On eighth day the animals were tested in Y-maze, photoactometer and Rota rod. The animals were sacrificed later on the day by cervical dislocation and immediately the brains were taken out on ice and separated into cerebrum, cerebellum and brain stem. The brain parts were measured to the nearest mg on Shimadzu A200 digital balance and later processed for the assay of lipid peroxidation, total and free sulphydryl groups, nucleic acids, protein, superoxide dismutase,
glutathione s-transferase, glutathione reductase, and monoamine oxidase. Beckman DU 640B spectrophotometer was used for taking optical density. Blood was taken out for estimation of bilirubin, creatinine and alanine amino transferase.

**Chemicals**

Methyl mercury chloride, alpha lipoic acid and BSA (bovine serum albumin) used in the study were purchased from SIGMA Chemical Co., USA. NADPH, DTNB (Dithio bisnitro benzoic acid), TBA (Thiobarbuturic acid), DNA (Deoxyribonucleic acid), RNA (Ribonucleic acid), TCA (Trichloroacetic acid), GSH used in the study were obtained from SRL, India. Rest of the chemicals were of analytical grade. Kits manufactured by Techno Pharmchem was used for the estimation of creatinine and bilirubin.

**ESTIMATION OF RATE OF LIPID PEROXIDATION**

The method of Utley et al., (1967) was used for the estimation of the amount of malonaldehyde formed per 30 min. during lipid peroxidation.

**Principle**

TBA reacts with lipid peroxide, hydroperoxides and oxygen labile double bonds to form colour products.

**Chemicals and reagents**

1) 0.15 M KCl: 2.2368 g. KCl dissolved in 200ml. DDW
2) 10% (w/v) Trichloroacetic acid (TCA): 10gm TCA dissolved in 100ml DDW

3) 0.67% 2-Thiobarbituric acid (TBA): This was prepared by dissolving 0.67gm of TBA in 25-50 ml. DDW by adding two pellets of NaOH. The pH of the solution was adjusted to 7.2 with the help of 1 N HCl and the volume was made upto 100ml. with DDW.

Procedure

Brain parts- cerebellum, cerebrum and brain stem were separated and homogenized (10%w/v) in chilled 0.15 M KCl. 1ml. of each homogenate was taken in a 25ml. conical flask and incubated at 37±1°C in a metabolic shaker (120 strokes/min., amplitude 1 cm.) for 1 hour. Then, 1.0 ml. of the same homogenate was pipetted in centrifuge tube, and protein was precipitated by adding 1.0 ml. of 10% TCA. After incubating 1.0 ml. of 10% TCA was added to each sample and both incubated as well as non-incubated samples were centrifuged at 3000 r.p.m. for 10 min. 1ml. of the clear supernatant was mixed with 1.0 ml. of 0.67% TBA and 1.0ml. DDW and the tubes were placed in a boiling water bath for 10 min., cooled and absorbance was measured at 535 nm.

Calculation

The following formula was used to estimate Lipid peroxidation
where,

\[ X = \text{nanomoles of malondialdehyde formed per 30 mins.} \]

\[ OD = \text{change of optical density at zero hour and 2 hour incubation of the sample.} \]

**Isolation of Nucleic Acids**

The method of Searchy and Macinnis (1970) was used for the isolation of nucleic acids. Different brain regions were weighed and homogenized in 5.0 ml of 0.5 N perchloric acid. The homogenates was heated at 90°C in boiling water bath for 10 min., cooled and centrifuged at 3,000 rpm for 10 min. Supernatants were taken in graduated test tubes and the volume was maintained upto 5.0 ml. with 0.5 N perchloric acid. This extract was used in the estimation of DNA and RNA.

**ESTIMATION OF DNA**

DNA was estimated following the method of Burton (1956).

**Principle:** Deoxyribose is converted into highly reactive hydroxy vulaldehyde, which react with diphenylamine (DPA) to give a blue coloured complex.
Deoxyribose sugar + DNA $\rightarrow$ hydroxy vinaldehyde.

**Chemicals and reagents**

Diphenylamine reagent: 1.5 gm diphenylamine was dissolved in about 50-60 ml glacial acetic acid. 1.5 ml conc. H₂SO₄ was added to it and the final volume was made upto 100 ml. with glacial acetic acid.

**Procedure**

2.0 ml of the perchloric acid supernatant of nucleic acid extract was taken in a test tube. To this 4.0 ml. diphenylamine (15% in CH₃COOH-H₂SO₄) reagent was added and the tubes were heated on boiling water bath for 15 min. After cooling, the colour intensity was measured at 600nm. against a blank sample (2.0 ml DDW in place of supernatant). A standard curve was prepared by using standard solution in 0.5 NHClO₄ (100-600µg) according to the procedure described. The values were plotted by the least square method.

**Calculation**

\[
\text{DNA} = \frac{C \times V}{V_t \times W_t}
\]

Where,

\[
C = \text{Conc. In mg. (in 20 ml. extract)}
\]

\[
V = \text{Total volume of the extract (4.0 ml)}
\]
\[ V_t = \text{Volume taken for the estimation} \]
\[ W_t = \text{Fresh weight of the brain in mg.} \]

DNA in the reaction product was calculated using the standard curve of DNA that was run simultaneously with the test sample. Results were expressed as mg. DNA/ gm fresh tissue weight.

**ESTIMATION OF RNA**

RNA was estimated by the method of Dische (1955).

**Principle**

Pentose sugars are converted to furfural derivatives by heating with conc. HCl. In the presence of FeCl3 solution the furfural derivatives react with orcinol and procedure a green coloured complex.

**Chemicals and reagents**

Orcinol reagent: 33.0 mg. Ferric chloride was dissolved in about 50 ml conc. HCl, 3.5 ml. 6% orcinol (dissolved in absolute alcohol) was mixed with it and the volume was made upto 100 ml. with HCl.

**Procedure**

2.0 ml. of the supernatant of nucleic acid was taken in test tubes. 4.0 ml. of the orcinol reagent was added to it. Test tubes were heated in boiling water bath for 15 min., cooled and the absorbance was read at 660nm against a reagent blank.

**Calculation**
RNA = $C \times \frac{V}{V_t} \times W_t$

Where,

$C$ = Conc. in mg. (in 20 ml. extract)

$V$ = Total volume of the extract (4.0 ml)

$V_t$ = Volume taken for the estimation

$W_t$ = Fresh weight of the brain in mg.

RNA content in the samples was calculated using the standard curve. The results were expressed as mg. RNA/gm fresh tissue weight.

**ESTIMATION OF PROTEIN**

Protein estimation was done by the method of Lowry et al., (1951).

**Principle**

This method is based on the colour reactions of amino acids - tryptophen and tyrosine with Folin phenol reagent. Due to the reaction of these amino acids with phosphomolybdic acid and phosphotungstic acid (present in Folin's reagent) a blue is formed. The colour is the result of reduction of phosphomolybdic acid and biuret reaction of proteins with Cu ions in alkaline medium. O. D. was read at 625 nm.
**Chemicals and reagents**

1. Standard solution: A standard solution of 1.0 mg.
BSA/ml. was prepared. Stock standard was diluted
ten times to get the working standard of 100μg/ml.

2. Copper reagent
Reagent A: 4.0% Sodium Carbonate in DDW.
Reagent B: 2.0% Copper sulphate in DDW.
Reagent C: 4.0% (w/v) Sodium potassium tartarate in
DDW.
Alkaline Copper Reagent: 1.0 ml. of reagent B + 1.0 ml. of
reagent C + 48 ml of reagent A were mixed in
the same sequence.

3. Folin – Ciocalteau Phenol reagent: 2 N solution obtained
commercially was diluted 1:1 with DDW before use.

**Procedure**

The supernatant was taken for nuclei acid estimation and
the residue left in the test tubes were dissolved in 5.0 ml. of DDW.
1.0 ml. of the aliquot was taken in the test tubes from this solution
and the volume was increased upto 1.0 ml. with DDW. To this, 5.0
ml. of Copper reagent was added and shaken thoroughly on a
cyclo-mixer. After 10 min., 1.0 ml. of Folin-Ciocalteau reagent was
added. Blue colour was developed. O.D. was read at 625 nm
exactly after 30 minutes. Standard protein solution (BSA 20 – 100μg) and blank were run simultaneously.

**Calculation**

Protein in the samples was calculated using the standard curve of BSA and the results were expressed as mg/g. weight of wet tissue.

**ESTIMATION OF TOTAL SULFHYDRL GROUPS (TSH)**

Total sulfhydryl group was estimated according to the method of Sedlack and Lindsay (1968).

**Principle**

5-5’ dithioibis – 2 nitrobenzoic acid (DTNB) is reduced by – SH groups of glutathione (GSH) in alkaline medium to produce 1 mol. of 2 nitro - 5 mercaptobenzoic acid per mole of – SH group. The anion (2 nitro – 5 mercaptobenzoic acid) has an intense yellow colour which is used to measure –SH group at 412 nm.

**Chemicals and Reagents**

Standard Solution: A standard solution of $2 \times 10^{-3}$M of GSH was prepared by dissolving 6.146 mg. GSH in 10 ml. of 0.02 M. EDTA.

1. 0.15 M. KCL.
2. 0.2 M tris buffer in 0.02 M EDTA, pH 8.2.

3. 0.01 M DTNB: 0.01 M solution of DTNB was prepared by dissolving 99 mg DTNB in 25 ml of absolute methanol.

4. Absolute methanol.

**Procedure**

Different parts of the brain were homogenized in chilled 0.15 M KCL and the volume was adjusted to give a 10% w/v homogenate. 1.5 ml 0.2 m tris EDTA buffer and 0.1 ml DTNB were added. The mixture was shaken and made to 10 ml with 8.3 ml of absolute methanol. The reaction mixture was centrifuged at 6000 rpm for 5 min in cold. The absorbance of the clear supernatant was read in cold. The absorbance was read at 412 nm. A calibration curve with different concentrations of GSH (61.46 - 491.68) was obtained according to the same procedure as described above.

**Calculation**

Total -SH group in the samples were calculated using the calibration curve and the results were expressed as μ moles/g tissue.
ESTIMATION OF FREE SULFHYDRYL GROUPS (GSH)

Free sulfhydryl group was estimated by the method of Ellman (1959) modified by Sedlak and Lindsay (1968).

Procedure

Same as for total sulfhydryl group estimation.

Chemicals and Reagents

1. Standard solution: A standard solution of $2 \times 10^{-3}$ M of GSH was prepared by dissolving 6.146 mg. of GSH in 10 ml. of 0.02 M EDTA

2. 0.015 M EDTA

3. 10% TCA

4. 0.4 M tris buffer in 0.2 M EDTA, pH – 8.9

5. 0.01 M DTNB.

Procedure

Brain parts were homogenised (10% w/v) in chilled 0.015 M KCL. 1 ml. brain (10%) homogenate was deproteinized by adding 1 ml. of 10% TCA and centrifuged at 6000 rpm for 5 min. 0.5 ml.
aliquot from clear supernatant was mixed with 0.5 ml DDW. Then, 2 ml., 0.4 M tris buffer and 0.1 ml. DTNB were added to it with proper stirring. The absorbance was read within 5 mins of the addition of DTNB at 412 nm. A calibration curve with different concentrations of GSH (61.46 – 491.68 µg) was drawn by the procedure described above.

**Calculation**

Free -SH (GSH reduced) in the samples were calculated using the standard curve of GSH and the results were expressed as µ moles/g. tissue.

**ESTIMATION OF GLUTATHIONE REDUCTASE (GR)**

Glutathione reductase was assayed by the method of Hazelton and Lang (1985).

**Principles**

Glutathione reductase (GR) catalyzes the reduction of oxidized glutathione (GSSG) by NADPH to reduce glutathione (GSH) according to the following equation

\[
\text{GR} \quad \text{GSSG} + \text{NADPH} + \text{H}^+ \rightarrow \text{NADP}^+ + \text{GSH}
\]
The activity of the enzyme was measured by following the decrease in optical density per minute at 340 nm during oxidation of NADPH.

**Chemical and reagents**

1. 0.1 M tris HCl buffer (pH 8.0)
2. 1.0 mM EDTA
3. 13.0 mM GSSG
4. 0.1 mM NADPH

**Procedure**

The reaction mixture consisted of 0.1 ml. of 1.0 mM NADPH, 0.2 ml. of 3.0 mM GSSG, 0.1 ml. of 1.0 mM EDTA, 2.5 ml of 0.1 M tris – HCl buffer (pH 8.0) and 0.1 ml tissue supernatant (10% w/v in tris HCl buffer, enzyme source) in a total volume of 3.0 ml. The reaction was initiated by the addition of tissue supernatant. Oxidation of NADPH was followed at 340 nm. Reference reaction was also run simultaneously. Protein content in enzyme source was also determined (Lowry et. al., 1951). Increase in optical density/minute was deduced.
Calculation

Enzyme activity was calculated using the molar extinction coefficient of NADPH \((6.22 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1})\) and results were expressed as \(\text{n mole of NADPH oxidized/min./mg protein.}\)

**ESTIMATION OF SUPEROXIDE DISMUTASE (SOD)**

SOD activity was measured by the method of Marklund and Marklund (1974).

**Principle**

SOD principle depends upon auto-oxidation of pyrogallol.

\[
\text{Auto-oxidation} \\
\text{Pyrogallol} + \text{O}_2 \rightarrow \text{oxidation product + O}_2 \quad (i) \\
\text{SOD} \\
2\text{O}_2^- + 2\text{H}^+ \rightarrow \text{O}_2 + \text{H}_2\text{O}_2 \quad (ii)
\]

**Procedure**

Different CNS parts (cleaned with normal saline) were homogenized in chilled 0.15 M KCl (10\% w/v). Homogenate was centrifuged in cold at 10,000 rpm for 15 min. 0.05 ml of clear supernatant was added to 2.85 ml of 0.05 M Tris-succinate buffer
(pH 8.2), mixed well and incubated at 25°C for 20 minutes. The reaction was started by adding 0.1 ml of 8 mM pyrogallol solution. The content were shaken well and change in OD/minute was immediately recorded for 3 minutes at 420 nm. A reference set consisting of 0.05 ml of DDW instead of the sample solution (clear supernatant) was also run similarly.

**Calculation**

\[
SOD = \frac{(A/\text{min. ref.} - A/\text{min. sample}) \times 30}{(A/\text{min. ref.}/2 \times 0.05 \times 1)}
\]

Where,

\[
A/\text{min. ref.} = \text{change of OD/min in ref. Set}
\]

\[
A/\text{min sample} = \text{change of OD/min in sample set.}
\]

**Activity unit**

One unit of the enzyme is defined as the amount of enzyme which causes a 50% inhibition of pyrogallol auto-oxidation under assay conditions.
ESTIMATION OF GLUTATHIONE S-TRANSFERASE (GST)

Glutathione S-transferase (GST) activity was assayed by the method of Habig et. al, 1974.

**Principle**

The enzyme activity was measured by following the increase of absorbance at 340 nm of CDNB – GSH conjugate generated as a result of GST catalysis between glutathione and 1-chloro-2, 4-dinitrobenzene (CDNB).

\[
\text{GST} \\
\text{CDNB} + \text{GSH} \rightarrow \text{CDNB} - \text{GSH} \text{ (conjugate product)}
\]

**Procedure**

In 0.1 ml of cytosol fraction (supernatant) (10%w/v in 0.15 M KCl), 2.7 ml of 1.0 mM glutathione solution (prepared in 0.2 M Phosphate buffer) and 0.2 ml CDNB (1.0 mM) substrate prepared in acetone were mixed. The change in absorbance at 340 nm was recorded at room temperature after 15 seconds each for 3 minutes against a blank containing 0.1 ml DDW in place of supernatant. Protein content in enzyme source was also determined.
Calculation

\[
\text{GST activity} = \frac{\text{OD} \times 625}{(\text{U/mg/min/protein}) \text{ conc. of protein}}
\]

The values were calculated on the basis of molar extinction coefficient of CDNB \(9.6 \times 10^3 \text{ M}^{-1} \times \text{cm}^{-1}\) and specific activity of enzyme was expressed in n mole of GSH-CDNB conjugate formed/min/mg protein.

**ESTIMATION OF MONOAMINE OXIDASE (MAO)**

MAO activity was determined by the method of Tabor et al., 1953.

**Principle**

Benzyl amine undergoes oxidative deamination in the presence of MAO and benzadehyde is formed.

\[
\text{R-CH}_2\text{CH}_2\text{NH}_2 + \text{O}_2 + \text{H}_2\text{O} \rightarrow \text{R-CH}_2\text{CHO} + \text{H}_2\text{O}_2 + \text{NH}_3
\]

**Procedure**

The reaction mixture in a final volume of 2.0 ml consisted of 0.4 ml of 0.5 M Phosphate buffer (pH 7.2), 0.1 ml of M
benzylamine hydrochloride and 0.2 ml of brain homogenate (10% w/v in Phosphate buffer). The reaction mixture was incubated at 37°C for 30 minutes. The reaction was stopped by the addition of 1.0 ml of 10% TCA (proteins were precipitated). The above reaction mixture was centrifuged at 2,500 rpm for 10 minutes in cold. The optical density of benzaldehyde formed was read in the supernatant at 25 nm against the blank containing 0.2 ml of 0.44 M sucrose instead of brain supernatant. Protein concentrations were also determined by the method of Lowry et al., 1951.

**Calculation**

Activity of MAO was estimated using the formula

\[ \frac{15.385 \times \text{OD/min}}{\text{Protein conc. (mg)}} \]

The activity of MAO was expressed as n moles benzaldehyde formed/min/mg protein.

**BEHAVIOURAL EXPERIMENTS**

**Y- Maze test**

A y-shaped three-arm wooden maze was selected. The rat was placed at the center of the maze; consecutive alteration and
errors traversed by the rat were noted. One alteration was counted when the rat successfully traverses all three arms of Y-maze. If a rat entered the same arm again it was considered as an error. The activity of rat was noticed for a period of eight minutes.

**Rotarod test**

The rota rod was rotated at 10 rpm and the animal was placed at the rotating rod. The time was noticed till the animal was able balance it self on the rod. The maximum allocated time for observation was slotted at 300 seconds i.e., 5 minutes. The activity was repeated six times after a gap of 10 minute for each rat taken.

**Photoactometer test**

Digital photoactometer from Techno was used. The animal was placed inside the photoactometer for a period of one hour, the readings on the display were noted after every 15 minutes thus taking 4 readings in all during the test period. Observation for one rat was taken at a time.

**ESTIMATION OF BILIRUBIN**

The method by Malloy and Evelyn was used for the quantitative determination of bilirubin in serum.
**Principle**

**Direct:** Conjugated bilirubin couples with diazotized, Sulfanilic Acid forming Azobilirubin. A red purple coloured product was found in acidic medium.

**Indirect:** Unconjugated bilirubin is diazotized only in the presence of its dissolving solvent the red purple coloured Azobilirubin produced in presence of methanol originated from both direct and indirect fractions and thus represents total bilirubin concentration. The difference of total and direct bilirubin gives indirect (unconjugated) bilirubin.

The intensity of red-purple colour so developed was measured colorimetrically at 540 nm and it was proportional to the concentration of the appropriate fraction of bilirubin.

**Reagents**

Kit supplied by Techno Pharm Chem was used.

**Calculations**

Bilirubin concentration in mg/dl:

\[
\text{Total (A)} = \frac{\text{O.D.T}.^1 - \text{O.D.T}.^2}{\text{O.D. of standard}} \times 10
\]

\[
\text{Direct (B)} = \frac{\text{O.D.D}.^1 - \text{O.D.D}.^2}{\text{O.D. of standard}} \times 10
\]
Indirect = (A) – (B).

ESTIMATION OF ALANINE AMINO TRANSFERASE (ALT)

The method by Reitman and Frankel, 1957 was used for estimation of ALT (SGPT).

Principle

Pyruvate produced by transamination of GPT reacts with 2,4-dinitrophenylhydrazine (DNPH) to give a brown coloured hydrazone which is measured at 510 nm.

Reagents

1. Phosphate buffer pH 7.4
2. GPT substrate – 200mM alanine and 2Mm α-Ketoglutarate
3. Stock pyruvate standard – (200 mM) 220 mg sodium pyruvate per 100 ml in phosphate buffer.
5. 2,4-dinitrophenylhydrazine 1Mm- dissolve 19.8 mg of dinitrophenylhydrazine in 10 ml HCl and make 100 ml with water keep in brown bottle.
6. 0.4 N NaOH.
Procedure

Warm 0.5 ml substrate in a water bath at 37 °C for 3 min, add 0.1 ml serum, mix gently and incubate for 30 minutes exactly. Remove tubes from bath immediately add 0.5 ml of DNPH solution and mix well. Keep at room temperature for 20 minutes, then add 0.4 N NaOH , leave further for 10 minutes finally read at 510 nm. Run control (C), standard (S), blank (B) subsequently.

Calculation

\[
\frac{\text{T} - \text{C}}{\text{S} - \text{D}} \times 0.4 \ \mu\text{mole for 0.1 ml serum}
\]

Estimation of Creatinine

Alkaline Picrate method is used for quantitative determination of creatinine in serum.

Principle

Creatinine reacts with alkaline picrate and produces red coloured complex which is measured at 520 nm.

Reagent

Kit manufactured by Techno Pharmchem was used.

Calculation

Serum creatinine = \(\frac{\text{OD Test} - \text{OD Blank}}{\text{OD Standard} - \text{OD Blank}}\) \times 3 mg/dl.
Statistical analysis

The level of significance between different groups was based on Student’s T-test. The level of significance was chosen as p< 0.05 and p< 0.01.