MATERIALS AND METHODS

1.1 Animals

Adult male albino rats of Wistar strain weighing around 200-220g were maintained in standard laboratory conditions and were fed with standard pellet diet (M/s Hindustan Lever Limited, Mumbai, India) and water ad libitum. The animals were handled according to the principles of laboratory care framed by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Govt of India. The experimentation protocol was reviewed and approved by Institutional Ethical Committee (No 08/019/04).

Necessity of folate deficiency in rat

Rodents do not develop metabolic acidosis in MeOH poisoning unlike humans, owing to their high liver folate content and rapid formate metabolism. Folate deficient rodents only accumulate formate leading to metabolic acidosis (Lee et al, 1994, Eells et al, 2000). To induce folate deficiency, rats were fed a folic acid deficient diet that was prepared according to the procedure of Reeves et al. (1993) for a period of 45 days (composition is given in the appendix). Sulphamethoxazole (1% added to folic acid deficient diet) was added to suppress bacterial synthesis of folic acid in the intestine. Folate deficiency of the groups was further confirmed by estimating urinary excretion of formiminoglutamic acid (FIGLU) after 45th day (Tabor and Wyngarden, 1962).
**Experimental design**

Male animals were divided into seven groups each containing six animals.

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<th>Group</th>
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<td>VII</td>
<td>Folate deficient treated with lipoic acid and MeOH (FLM) group</td>
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Four (Group I, Group II, Group III and Group V) groups were fed with normal diet and water. To induce folate acid deficiency, three groups (Group IV, Group VI and Group VII) of albino rats (six weeks old) weighing between 60 and 90 gms were fed with folate acid deficient diet (Reeves *et al.*, 1993) for a period of 45 days. Sulphasemethoxazole was used to suppress bacterial synthesis of folate acid in the intestine. Then, the Group II, Group V and Group VII were treated with lipoic acid (Arivazhagan *et al.*, 2000) (100 mg/kg body weight) dissolved in alkaline saline (0.5M KOH in 0.9% of NaCl) intraperitoneally for two weeks. After that Group III, Group V, Group VI and Group VII were injected with 20% MeOH (one ml of MeOH absolute in 3 ml of normal saline). Control groups were treated similarly with 4 ml of normal saline.

**Collection of blood and tissue sample**

Stress free blood samples were collected with heparinized syringes and then the rats were sacrificed by cervical dislocation. A day after the MeOH
treatment, along with the control groups the blood samples and isolation of brain was performed between 8AM - 10AM to avoid circadian rhythm induced changes. The blood samples were used for quantification of free radicals.

After removal, the brain was weighed and kept in cold buffered solution for immediate processing. For histological study, rats were perfused with buffered formalin ((Figure 5), the kidney and liver tissues were removed and preserved in the buffered formalin.

**Parameters**

**Estimation of formiminoglutamic acid in urine:**

Formiminoglutamic acid was estimated according to the method of Tabor and Wyngarden (1958).

**Chemicals**

1. Sodium borate
2. Sodium hydroxide (NaOH)
3. Potassium ferricyanide
4. Sodium nitroprusside
5. Potassium hydroxide - KOH (2.5 N)
6. Hydrochloric acid - HCl (2.5 N)

Ferricyanide-nitroprusside reagent 4 gm each of NaOH, potassium ferricyanide and sodium nitroprusside in 120 ml of water.

Before sacrificing the animal, an overnight sample of rat’s urine was subjected to a FIGLU test to confirm the folic acid deficient state of the animal.
Procedure

Urine plus water (0.5 ml) is mixed with 2 ml of saturated sodium borate and 0.5 ml of the ferricyanide-nitroprusside reagent. The optical density at 485 nm was read after 30 min at room temperature against a corresponding blank. The latter is prepared by pre-incubating 0.3 ml of urine with 0.1 ml of 2.5 N KOH for 2 hr at 25°C to destroy the formamino glutamic acid present. The mixture is then neutralized with 0.1 ml of 2.5N HCl and then assayed by the above procedure. Only animals showing high Figlu excretion were used for the study.

pH measurement

pH of blood samples from various groups was measured using blood gas analyzer (Radiometer-Copenhagen ABL-5).

Procedure

Fresh blood samples were collected from jugular vein of rats, small quantity of blood (approximately 50μl) was immediately injected into the blood gas analyzer. Then the result was noted from the analyzer.

Quantification of superoxide radicals (O₂⁻)

By the method of Nishikimi et al (1972)

Superoxide is a very labile radical and superoxide radicals were estimated in erythrocytes with superoxide dismutase inhibition (diethyl dithiocarbomate) and without the superoxide dismutase inhibition. The NBT reduction difference gave the superoxide radicals present at the time of assay.
Chemicals

1. Diethyl dithio carbonate (DDC) – 1 mM
2. C₂H₅OH
3. 10 mM hypotonic sodium phosphate buffer containing 67 mM NaCl and 0.1 mM EDTA (pH 7.6)
4. CHCl₃
5. Ascorbic acid – 150μM
6. 1,4 Dioxane
7. 0.1 M sodium hydroxide solution containing 24 mM of sodium bicarbonate
8. Standard A stock solution containing 100 mM of NBT was prepared in water and diluted to make working standard solution of 10 mM of NBT containing 340 mM sucrose

Procedure

Fresh blood samples containing heparin were centrifuged immediately to separate plasma from the cells. The cell count was adjusted to 10¹² cells/ml with normal saline. From this 2 ml was taken in two different tubes with and without 1 mM of DDC (inhibitor of SOD) and 0.5% of haemolysate was prepared using 10 mM hypotonic phosphate buffer. Haemoglobin was precipitated by adding 0.25 ml of ethanol and 0.15 ml of chloroform. The mixture was shaken well in 4°C and centrifuged at 3000 g. To 4.8 ml of the supernatant, 0.2 ml of 4 mM NBT was added followed by 150 μM of ascorbic acid and incubated at 37°C for 10 min. Then 0.2 ml of 0.1M NaOH containing 24 mM sodium bicarbonate was added and precipitate obtained after centrifugation was dissolved in 5 ml of 1,4 dioxane. The absorbance was read at 520 nm against the blank of 1,4 dioxane. The difference
between the two gives the NBT reduction specifically by the superoxide radicals present. Superoxide levels in erythrocytes are expressed in terms of millimoles of NBT reduced \(10^{12}\) cells min

**Quantification of hydroxyl radicals (OH\(^{-}\))**

By the method of Gutteridge (1981)

Hydroxyl radicals were estimated by their reaction (hydrogen abstraction) with 2-deoxy ribose, resulting in the formation of thiobarbituric acid (TBA). The assays were done with the same haemolysate prepared for superoxide.

**Reagents**

1. 10 mM hypotonic sodiumphosphate buffer containing 67mM sodium chloride and 0.1 mM EDTA (pH 7.6)
2. Deoxyribose 20 mM
3. 1% TBA contained 50 mM NaOH
4. 2.8% TCA contained 0.1 M sodium arsenite
5. Standard solution A stock standard solution of malondialdehyde (MDA) (400 nM/ml). 0.2 ml of MDA was made up to 2 ml with water.

Working standard From the stock 100 μl was diluted to 10 ml. Then 100 μl was taken from this and diluted to 10 ml to get the 50 mM of MDA.

**Procedure**

To 2 ml of 0.5% of haemolysate, 2 ml of deoxy ribose was added and incubated at 37°C for an hour. To this, 0.5 ml of TBA solution and 0.5 ml of TCA solution were added and heated in a boiling water bath at for 15 min. Then the
mixture was cooled and absorbance was read at 530 nm. Blanks were included without 2-deoxy ribose to assess the baseline TBARS in the haemolysate for each blood sample analyzed. The set of standards in the range of 5-30 nanomoles were used. Amount of hydroxyl radicals present in the erythrocyte are expressed in terms of nanomoles of MDA $10^{17}$ cells hour

**Brain dissection**

Since the study involved the estimation of free radical scavenging enzymes and no anesthesia was used as it affects the level of these substances in brain (Vogt, 1954). After sacrifice the brain was rapidly removed. To expose the brain, the tip of curved scissors was inserted into the foramen magnum and a single lateral cut was made into the skull extending forward on the left and right side. With a bone cutter, the dorsal portion of cranium was peeled off and using a blunt forceps, brain was dropped onto the ice-cold glass plate leaving the olfactory bulbs behind. The whole process of isolating the brain takes less than 2 min. After isolating the brain, it was blotted and chilled. Further dissection was made on ice-cold glass plate.

First rhombencephalon (Part A) was separated by a transverse section made in the brain, to make it into two parts. Cutting the cerebellar peduncles leaving medulla oblongata isolated the cerebellum. Second transverse section was made at the level of optic chiasma, which delimits the anterior part of the hypothalamus and passes through the anterior commissure. This section separates the cerebrum into two parts B and C. Part B was divided into 5 fractions. First, the hypothalamus was dissected by taking anterior commissure as a horizontal reference and line between the posterior hypothalamus and the mammillary bodies as caudal limit. The corpus striatum was dissected with the external walls of the lateral ventricles as internal limit and the corpus callosum as external limit. The
frontal parts of the corpus striatum those were in portion ‘C’, were dissected separately and combined with the posterior parts of the portion ‘B’. The midbrain is gently separated from the remaining part of the brain. The hippocampus was then dissected. The remaining part of ‘B’ is combined with the remainder part of ‘C’ to form cortex.

The medulla oblongata corresponds to the medulla and the Pons. The midbrain corresponds to the midbrain thalamus and sub thalamus. The corpus striatum contains the putamen, caudate nucleus and globus pallidum. The cortex corresponds to the telencephalon without corpus striatum. The different brain regions were weighed and used for the experiment.

10% homogenates of individual regions were prepared using a Teflon-glass homogenizer with TRIS-HCl buffer (0.1M pH 7.4). The homogenate of each region was centrifuged separately in refrigerated centrifuge at 300 g for 15 min. The supernatants were used for the following study.

**Estimation of protein**

Protein was estimated by the method of Lowry *et al.* (1951).

**Reagents**

1. Alkaline copper reagent (Lowry’s Reagent)

   **Solution A**: 2% sodium carbonate in 0.1N NaOH and

   **Solution B**: 0.5% copper sulphate in 1% sodium potassium tartarate

   50 ml of solution A was mixed with 1 ml of solution B just before use.
2 Folin's phenol reagent (commercial reagent, 12 dilutions with water)
3 Bovine serum albumin (BSA)

Procedure

To 0.1 ml of brain tissue homogenate from individual regions, 0.9 ml of water and 4.8 ml of alkaline copper reagent were added and kept at room temperature for 10 min. Then 0.5 ml of Folin's phenol reagent was added and the color developed was read after 20 min at 640 nm. The level of protein was expressed as mg gram of tissue.

MeOH toxicity in optic nerve and retina

Since acute MeOH toxicity claim medical assistance only with blurring of vision and in severe intoxication loss of eye sight, the free radical changes were monitored in retina and optic nerve up to optic chiasma along with the dissected brain regions.

Assay of superoxide dismutase (SOD) (EC.1.15.1.1)

By the method of Marklund and Marklund (1974)

Reagents

1 0.1M Tris-HCl buffer pH 8.2
2 0.05M Tris
3 Freshly prepared 2.5 mg of pyrogallol (photosensitive chemical) in 10 ml of 0.05M Tris
4 Ethanol Chloroform mixture in the ratio of 3:2 (prepared fresh)
**Procedure**

To 0.5 ml of homogenate was mixed with 0.5 ml of ethanol chloroform mixture the content was shaken well for 15 min and centrifuged for 10 min. To 0.5 ml of supernatant taken 2 ml of Tris-HCl buffer (pH 8.2) was added. Finally 0.5 ml of freshly prepared pyrogallol solution was added and read at 470 nm in 0, 1, 2, 3 min intervals. The activity was expressed as Units/min/mg protein.

**Catalase (EC 1.11.1.6)**

By the Method of Sinha (1972)

**Reagents**

1. 0.01 M Phosphate buffer pH 7
2. 0.2 M H₂O₂
3. Potassium dichromate acetic acid reagent (2.5 gm of K₂Cr₂O₇ in 50 ml of H₂O + 150 ml of glacial acetic acid)

**Procedure**

Four sets of 0.1 ml of homogenate was mixed with 1 ml of phosphate buffer and 0.5 ml of H₂O₂ and this reaction was arrested by addition of 2 ml potassium dichromate acetic acid reagent at various intervals of 0, 15, 30, and 60 sec. Then all the samples were kept in a boiling water bath for 10 min and were cooled gradually. Developed green color was read at 610 nm. The activity was expressed as amount of H₂O₂ utilized/min/mg protein.
Glutathione peroxidase (GPx) (EC.1.11.1.9)

By the method of Rotruck et al. (1973)

Reagents

1. 0.32 M phosphate buffer, pH 7
2. 10 mM Sodium azide
3. 3 mM Reduced glutathione
4. 2.5 mM H$_2$O$_2$
5. 10% v/v TCA
6. 0.3 M Disodium hydrogen phosphate
7. DTNB solution (40 mg of DTNB in 100 ml of 1% sodium citrate)

Procedure

To three sets of test tubes, 0.4 ml of phosphate buffer, 0.1 ml of sodium azide, 0.2 ml of standard GSH solution and 1 ml of H$_2$O were sequentially mixed. To this 0.1 ml of H$_2$O$_2$ was added and the reaction in individual set was arrested with 1 ml of 10% TCA at 0, 15, 3 min intervals and then centrifuged for 10 min. To the 0.2 ml of supernatant, 1.8 ml of H$_2$O followed by 1 ml of 5% TCA was added and allowed to stand at room temperature for 20 min. Then 4 ml of phosphate solution was added, followed by 0.5 ml of DTNB. The optical density was taken at 412 nm within 5 min. The activity is expressed as units/min/mg protein

Reduced (GSH)

The level of reduced glutathione was measured by the method of Moron et al. (1979).
Reagents

1. 10\% TCA
2. 0.2 M Phosphate buffer, pH 8
3. 0.6 mM 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) in 0.2 M sodium phosphate solution
4. 0.3 M sodium phosphate solution

Procedure

To 0.2 ml of homogenate, 1.8 ml of H₂O, 1 ml of 5\% TCA were sequentially added and kept at room temperature for 20 min. Then, 4 ml of 0.3 M sodium phosphate solution followed by 0.5 ml of DTNB was added and mixed well. Optical density was taken at 412 nm within 5 min from the addition of DTNB. The level of GSH is expressed as \( \mu \)g of GSH/mg of protein.

Oxidized glutathione (GSSG) estimation

By the method of Hissin and Hilf (1976)

Principle

In oxidized glutathione estimation, N-Ethylmaleimide is used for removal of endogenous GSH, and the estimation is based on the method that uses o-phthalaldehyde as the fluorescent reagent, which binds with the free thiol groups of glutathione producing a highly fluorescent derivative.

Reagents

1. Sodium hydroxide : 0.1 N
2. N ethyl maleimide (NEM) : 0.04 M
3. o-Phthalaldehyde (OPT) : 1 mg/ml
centrifuged for 10 min. Supernatant was taken and was read at 532nm in spectrophotometer. The level of LPO is expressed as nanomoles of MDA/mg protein.

**Assay of protein carbonyl**

By method of Levine *et al.* (1990)

**Reagents**

1. 10 mM 2,4-dinitrophenyl hydrazine (DNPH) in 2N HCl
2. Ethanol/Ethyl acetate mixture (1:1)
3. 10% TCA
4. 6M Guanine hydrochloride

**Procedure**

One ml of brain homogenate from various regions were centrifuged individually at 1,00,000g for 20 min to separate cytosol. To 0.5 ml of cytosolic fraction, 0.5 ml of TCA was added. After precipitation, the sample was treated with 0.5 ml of DNPH, maintained at room temperature for 1 hour, vortexed for every 10-15 min, and centrifuged at 1,00,000g (Ultracentrifuge, Hitachi, Japan) for 10 min. Then the pellet was washed thrice with 1 ml of ethanol/ethyl acetate. The pellet was dissolved in 1 ml of guanine hydrochloride and read at 366nm.

**Protein thiol**

By the method of Sedlack and Lindsay (1968)

**Reagents**

1. Tris-HCl buffer pH 8.2 (0.2M contains 0.02M EDTA)
2. 5% TCA
3. Disodium salt of EDTA 0.02M
4. DTNB [5, 5′ dithiobis (2-nitrobenzoic acid)] 0.01M in absolute MeOH
5. Standard solution: 10 mg GSH/100 ml H₂O

Procedure

To 1 ml of the homogenate was added 15 ml of Tris-HCl buffer (pH 8.2) which contained 0.02M EDTA. This was followed by the addition of 0.1 ml of DTNB solution and 7.5 ml MeOH. The contents were mixed well in a vortex mixture and then centrifuged at 3000g for 10 min. The color developed was read at 412 nm. The level of thiol was expressed as μg/mg protein.

Western Blot analysis (hsp70)

Protein concentration was determined for the individual regions, according to the method of Lowry et al. (1951) using bovine serum albumin as a standard. The protein concentration of each region was adjusted to have 50 μg of protein/ml and 20 μl was electrophoresed in a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (1970). The stacking gel was 5% and the resolving gel was 10%. After electrophoresis, the gel was placed over an appropriately cut nitro cellulose membrane. The gel and the nitro cellulose membrane were packed by three cut-pieces of Whatman’s filter paper (No 3). This set-up was covered on both sides with the absorbers (provided with the system) and clipped. The whole set-up was immersed in a tank containing blotting buffer. A current of 25 mA was passed for 4 hrs. The membrane was then removed from the system and immersed in MeOH for a minute. Empty sites in the membrane were blocked by treating the membrane with blocking solution for 1 hour at 37°C. After washing, the membrane was incubated with the mouse monoclonal hsp70 antibody (1:1000) for 1 hour at 37°C. This was followed by
washing with 1% TWEEN 20 -PBS The membrane was then incubated with peroxidase conjugated rabbit anti-mouse IgG antibody (1 10000) for 1 hour at 37°C After washing, the bound Horse Radish Peroxidase (HRP) activity was monitored by treating the membrane with diaminobenzidine (DAB) reagent After development of color, the membrane was washed in water (Blondeau et al. 2000) Densitometric analysis was done with ChemiImager software (Alpha Innotech, CA, USA)

Isolation of RNA

RNA was isolated from six regions of rat brain using TRIZOL reagent

Reagents

1  TRIZOL reagent
2  Chloroform
3  Isopropanol
4  Absolute alcohol (Ethanol)
5  MilliQ water

Procedure

100 mg of tissue was homogenized in 1 ml of TRIZOL reagent, the homogenate was kept in ice for 10-15min and it was followed by the addition of 0.2 ml of chloroform. The contents were vortexed vigorously for 15 sec, kept in ice for 5 min and centrifuged at 12,000g for 15 min at 4°C. Then the aqueous layer was carefully transferred completely to a new centrifuge tube without disturbing the other two layers and equal volume of isopropanol was added. The contents were mixed well, kept in ice for 10 min, centrifuged at 12,000g for 10 min at 4°C and the supernatants were discarded without disturbing the RNA pellet. Then one
ml of 75% absolute alcohol was added and vortexed well for 1 min. centrifuged at 500g for 5 min at 4°C. The supernatant was discarded and the RNA pellet was air-dried by keeping the tube open inside the hood for about 10 min. To the RNA pellet 50μl of autoclaved MilliQ water was added, vortexed well and kept in -20°C - 60°C water for 10 min. Again the content was mixed well, 5 μl of RNA solution was made to 1 ml with autoclaved MilliQ water and read at 260/280 nm using a λ-spectrophotometer. Then the isolated RNA was aliquoted and stored at -80°C. The purity range of RNA = A260/A280 - 1.8 - 2 (for pure RNA) and should either be below 1.2 nor above 2. The purity was found to be 1.65 - 1.97 for the RNA isolated from various regions.

Reverse transcription-polymerase chain reaction (RT-PCR) for c-Jun N-terminal kinase

Total RNA from rat brain regions were isolated according to the RNA isolation kit instructions (Prefect RNA IM Eukaryotic Mini Kit, Eppendorf AG, Hamburg, Germany). The level of JNK3 mRNA in the brain regions were quantified by RT-PCR with an endogenous internal standard, β-actin as described by Nakahara et al. (2002) RT was performed on 1 μg total RNA for 90 min at 42°C in a 5 μl reaction mixture containing 25 mmol/l Tris-HCl (pH 8.3), 50 mmol/l KCl, 5 mmol/l MgCl2, 2 mmol/l dithiothreitol, 1 mmol/l each deoxynucleotide, 10 U AMV reverse transcriptase, 10 U ribonuclease inhibitor, and 0.8 μg oligo (dT) primer. The RT was terminated by heating the sample at 95°C for 2 min. The multiplexed PCR was performed in a 20 μl reaction mixture containing 10 mmol/l Tris-HCl (pH 8.3), 50 mmol/l KCl, 1.5 mmol/l MgCl2, 2% (v/v) dimethyl sulfoxide, 0.2 mmol/l each deoxynucleotide, 0.1 μmol/l each of 5’ and 3’β-actin-specific primers, 1 μmol/l each of 5’ and 3’ hsp70 specific primers, 25 ng of reverse transcribed total RNA, and 0.5 U Taq DNA polymerase. The PCR amplification was performed, denaturation at 95°C for 2 min,
Annealing at 55°C for 45 sec and extension at 72°C for 75 sec for 29 cycles. After 8 (JNK) cycles, 0.1 μmol/l each of β-actin primer pair was added to the reaction mixture, and PCR cycles were further continued. The primer sequences used for amplification of the coding regions of JNK3 and β-actin were as follows:

**JNK3**

- Sense: 5'-AACAATCGCTACACCTCCAAAGAC-3'
- Anti-sense: 5'-GGCAATAGATGACACATCCACG-3'

Target sequence 350 bp.

**β-actin**

- Sense: 5'-TCATGCCATCCTGCCTGTGGACTT-3'
- Anti-sense: 5'-CGGACTCATCGTACTCCTGCTTG-3'

Target sequence 598 bp.

To compare the amount of steady state mRNA, 5 μl of each PCR product was resolved in 2% agarose gel (stained with ethidium bromide). After electrophoresis, the gels were viewed under UV light, and digital images were captured on BioRad Gel Doc 2000 System. The levels of JNK3 mRNA were calculated as the ratios of optical density of the PCR products to that of the β-actin PCR product.

**Histopathology**

Histological procedure was carried out according to the method of Culling (1974).