MATERIAL AND METHODS

3.1 MATERIALS

Experiments were performed on male Wistar rats (*Ratus nonvergicus*), obtained from the inbred colony of the animal house of Department of Nutrition and Biochemistry, Central institute of Fisheries Technology (CIFT), Kochi. Two categories of male rats, 3 months old (young) and 1 year old (adult) were used for the experiment. The rats were kept in non-pathological conditions in polypropylene cages of size 43.5x29.0x16cm and maintained on standard pellet diet supplied by Sai feeds, Bangalore and water *ad libitum*. The cages were maintained in a well-ventilated room at room temperature of 28 ± 2°C.

3.1.1 Experimental design

The experiments were conducted in three phases as elaborated in Table: 2. In the first phase, rats were studied under normal control conditions. The normal control condition referred to healthy rats reared under optimum environmental conditions and with food and water maintained *ad libitum*. Both 3 months and 12 months old rats were studied. In the second phase, the effect of isobaric hypoxia (10%) on the rat brain for two durations (1 & 3 hours) was studied. Effect of hypoxia was studied on both young and adult rats. Rats subjected to 10%
isobaric hypoxia in a specially designed and fabricated chamber for 1 hour and 3 hours followed by reoxygenation with 21% O₂ for 10 minutes and 30 minutes, respectively, formed the hypoxia-experimental groups. The rats of both the age groups exposed to 21% oxygen condition in the same chamber for the same durations acted as controls for these experiments.

The third phase dealt with the preparation of SOD entrapped liposomes, its intravenous injection (through jugular vein) to reach the rat brain and to study the effect of this enzyme on the pathological changes expected with hypoxia-reoxygenation. In this set of experiments, only young (3 months old) male rats were studied and hypoxia was given only for the duration of 3 hours. Young rats of the same age injected (through jugular vein) with buffer containing liposomes (Blank liposomes) and subjected to 3 hours of hypoxia followed by reoxygenation for 30 minutes formed the control group.

Table: 2

Experimental Design

Animal pool

<table>
<thead>
<tr>
<th>Normal - control</th>
<th>Hypoxia-control</th>
<th>Hypoxia-experimental</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age</strong></td>
<td>3m 12m</td>
<td></td>
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<tr>
<td><strong>Hypoxia-1</strong></td>
<td></td>
<td></td>
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<tr>
<td><strong>Duration</strong></td>
<td>1 hr 3 hrs</td>
<td>1 hr 3 hrs</td>
</tr>
<tr>
<td><strong>Age</strong></td>
<td>3m 12m 3m 12m</td>
<td>3m 12m 3m 12m</td>
</tr>
<tr>
<td><strong>Hypoxia-2</strong></td>
<td>Blank liposome injected (control)</td>
<td>SOD entrapped liposome injected (experimental)</td>
</tr>
<tr>
<td><strong>Duration</strong></td>
<td>3 hrs</td>
<td>3 hrs</td>
</tr>
<tr>
<td><strong>Age</strong></td>
<td>3m</td>
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</tbody>
</table>
3.1.2 Experimental hypoxia

A gadget for creating hypoxia was fabricated by modifying an inhalation chamber with an outlet and two inlets, one for the entry of O₂ and the other for N₂. A gauge cum regulator, which monitors the volume and pressure of gases entering the chamber, controls the flow of gases through the inlet (Fig: 1). During the experiments rats were placed inside the chamber and the amount of O₂ in the chamber was maintained to 10% by controlling the flow of N₂ and O₂.

![Image of the gadget for creating hypoxia](image)

*Fig: 1*
The Gadget for creating hypoxia

3.2 METHODS

3.2.1 Biochemical assays

After subjecting to hypoxia, rats were anesthetized with ketamin (0.5 ml). This was done as an attempt to maintain the uniformity in the experiments, as for the procedure of intravenous injection of SOD entrapped liposomes anesthetization was unavoidable. The whole brain was removed by opening the cranium. The brain was washed in 0.90% cold saline and immediately weighed in a single pan electric balance. The brain was then regionalized into cerebrum,
cerebellum, medulla, hippocampus and hypothalamus. Each region was weighed separately and 10% homogenate was prepared using phosphate buffer of pH 7.4. Homogenates were then centrifuged in a REMI refrigerator centrifuge.

3.2.1.1 Estimation of protein

Protein content was measured by the method of Lowry et al (1951) using Bovine Serum Albumin (BSA) as a standard protein. The following reagents were used:

Reagent A : 2% Na$_2$CO$_3$ in 0.1N NaOH
Reagent B : 5% CuSO$_4$.5H$_2$O in Sodium potassium tarterate
Reagent C : Mixture of 50 ml of reagent A with 1ml of reagent B.
Reagent D : Folin-ciocalteu 's reagent diluted 1:1 with distilled water.
Reagent E : Standard BSA solution (0.4mg/ml) was prepared

3.2.1.1.1 Procedure for preparing standard curve

Five test tubes were taken and various volumes of BSA solution (Reagent E) added so to get 5 ranges of BSA concentrations in the five test tubes. The total volume in each test tube was made up to 0.5ml by adding the required volume of distilled water. 5 ml of the freshly prepared reagent C was added to each test tube. After 10-minute interval, 0.5ml of phenol reagent was added to stand for 30 minutes. The absorbance at 650 nm was recorded and the data were depicted graphically (Fig: 2).
3.2.1.2 Determination of superoxide dismutase activity

Superoxide dismutase activity was measured by the method of Stephen Marklund and Gudrun Marklund (1974).

The inhibition of Pyrogallol autooxidation brought about by superoxide dismutase was employed as a convenient method for the determination of the enzyme. The reaction mixture consists of 0.2mM Pyrogallol in air equilibrated 50mM Tris-Cacodylic acid buffer (pH 8.20) containing 1mM diethylenetriaminopentaacetic acid. 1ml of 1% homogenate centrifuged at 12000 rpm for 30 minutes was added to the above reaction mixture. The rate of autooxidation is taken from increase in absorbance at 420nm, which is 0.02/minute in the absence of superoxide dismutase. Diethylenetriaminopentaacetic acid was found to prevent interference from Fe²⁺(as well as from Cu²⁺ and Mn²⁺) and used as a chelator in the assay medium.
3.2.1.3 Biochemical assay for catalase

The method of Kar and Mishra (1976) was used with some modifications.

A mixture of 3 ml phosphate buffer (0.1M, pH 7.0) and 1ml H$_2$O$_2$ (0.1M) was incubated at 25°C for 10 minutes. One ml of enzyme extract and 1ml of distilled water were added to the test and blank respectively. The reaction was stopped after 5 minutes by adding 5 ml of sulphuric acid (4%V/V). The enzyme extract to the control was added after the addition of the sulphuric acid. The undecomposed H$_2$O$_2$ was titrated against potassium permanganate (0.1 N) solution until a pink colour appeared. CAT activity was expressed as micromoles of H$_2$O$_2$ decomposed per 100mg tissue per minute at 25°C. A unit of CAT activity is defined as one micromole of H$_2$O$_2$ decomposed per minute at 25°C.

3.2.1.3.1 Procedure for preparing standard curve

The standard curve was prepared by taking 0.02-1.0 ml H$_2$O$_2$ (0.1 M) in a series of test tubes and diluting to 1.0 ml so as to obtain H$_2$O$_2$ concentration in the range of 20 micromoles to 100 micromoles. To each of the test tubes 3 ml of phosphate buffer, 1 ml of distilled water and 4 ml of 4% sulphuric acid was added and was titrated against potassium permanganate (0.1 N) solution until a pink colour appeared. And the results were depicted graphically (Fig: 3).
3.2.1.4 Biochemical assay for glutathione peroxidase

Glutathione peroxidase activity was analyzed using method of Hafeman et al (1974).

The enzyme assay tubes were incubated at 37°C and contained: 1.0 ml of 2 mM GSH, 1.0 ml of 0.4M sodium phosphate buffer (pH 7.0) also containing 4 x 10⁻⁴ EDTA, 0.5 ml of 0.01M sodium azide (to inhibit catalase), 0.03 ml of 10% homogenate and water to bring the total volume to 4.0 ml. After a 5 minutes preincubation, 1.0 ml of 1.25 mM H₂O₂ (prewarmed at 37°C) was added. Thereafter at 3 minutes intervals 1.0 ml aliquots of incubation mixture were removed and added to 4.0 ml metaphosphoric acid precipitation solution. GSH in protein free filtrates was determined by mixing 2.0 ml of filtrate with 2.0 ml of 0.4M sodium hydrogen phosphate and 1.0 ml of DTNB (5,5'-dithiobis nitrobenzoic acid) reagent. A₄₁₂ was recorded within 2 minutes after mixing. A blank with H₂O was carried through the incubation simultaneously with the samples since non-enzymatic GSH oxidation by

Fig: 3
Standard curve - Catalase
H$_2$O$_2$ occurred during incubation. Both enzymatic and non-enzymatic reactions proceeded at rates directly proportional to GSH concentrations, since a plot of log [GSH] Vs reaction time was linear both with and without addition of enzyme source. An enzyme unit of activity was conveniently defined as decrease in the log [GSH] of 0.001 per minute after the decrease in log [GSH] per minute of the non-enzymatic reaction was subtracted.

3.2.1.5 Estimation of reduced glutathione

Amount of reduced glutathione was estimated by the method of Moron et al (1979).

125 microlitres of 25% trichloroacetic acid was added to 0.5 ml of homogenate to precipitate protein. Tubes were cooled in ice for 5 minutes and mixture was further diluted with 0.6 ml of 5% trichloroacetic acid and centrifuged at 1000 rpm for 10 minutes. 0.3 ml of supernatant was taken for estimation and volume was made up to 1 ml with 0.7 ml 0.2M sodium phosphate buffer (pH 8). To the above mixture add 2 ml of freshly prepared DTNB [0.6 millimolar in 0.2M sodium phosphate buffer (pH 8)]. The intensity of yellow colour was measured at 412 nm after 10 minutes.

3.2.1.5.1 Preparation of standard curve

Five test tubes numbered 1-5 were taken and various volumes of GSH solution (100 micromoles) were added to get 5 ranges of GSH concentration ranging from 5 n moles - 25 n moles. Total volume in each test tube is made to 1 ml with
0.2 M sodium phosphate buffer (pH 8). To this 2 ml of freshly prepared DTNB (0.6 millimoles in 0.2 molar sodium phosphate buffer) was added and absorbance at 412 nm was measured after 10 minutes and data depicted graphically (Fig: 4).

![Graph showing absorbance at 412 nm](image)

**Fig : 4**
Standard curve-GSH

### 3.2.1.6 Biochemical assay for acid phosphatase

Acid phosphatase activity was analyzed using method in Sigma technical bulletin No: 104 (1960)

**Reagents**

1. **Total acid buffered solution (pH 4.8):** Dissolve 18.907 gm of citric acid monohydrate in water; add 180 ml of 1N sodium hydroxide and 100 ml of 0.1N HCl. Dilute to 1 litre with water and adjust the pH to 4.8.

2. **Stock substrate solution:** Dissolve 0.10 gm of P-nitrophenyl phosphate in 25 ml of water. Store in a dark bottle in the refrigerator.
3. **0.1N NaOH**

A mixture of 0.5 ml of total acid buffered solution and 0.5 ml of stock substrate solution was incubated at 38°C. After attaining temperature equilibrium, add 0.1 ml of 5% homogenate. Mix and incubate for exactly 30 minutes. A reagent blank was also prepared. Adding 4 ml of 0.1 N NaOH stops the enzyme activity. Read the tubes against reagent blank at 410 nm. The results were expressed as micromoles of PNP released /minute /mg protein/ml at room temperature.

**3.2.1.7 Estimation of malondialdehyde**

The method of Placer et al. (1966) was used.

10% homogenate was centrifuged at 5000g for 10 minutes in a REMI cooling centrifuge. 0.5 ml of supernatant was mixed with 1 ml of 0.2 M Tris-maleate buffer at pH 5.9. The mixture was then incubated at 37°C for 30 minutes. 1.5 ml of thiobarbituric acid reagent (TBA) made by mixing 2 volumes of 8% TBA solution in perchloric acid (7%) with 1 volume of 7% perchloric acid, were added to the incubated mixture, which was heated for 10 minutes in the boiling water bath. The solution was allowed to cool and after cooling, 3 ml of Pyridine-n-butanol (3:1V/V) solution were added. Subsequently, 1 ml of 1 N NaOH was added to the above mixture. After thorough shaking resulting colour was measured at 548 nm against the reagent blank. The levels of lipid peroxides were expressed as n moles of MDA.
3.2.2 Liposome preparation and entrapment of SOD

Multilamellar neutral liposomes were prepared by the method of Gregoriadis & Ryman (1972b) with some modifications.

Phosphatidyl choline and cholesterol in the ratio 7:2:2 was dissolved in 5 ml of chloroform methanol (2:1) in a round bottom flask and dried under vacuum on a Buchi rotatory evaporator to form a thin film of lipid. The lipid mixture was then dispersed in 2 ml of PBS (Phosphate Buffer 50 mM, pH 7.4, saline 0.15 M) containing 10 mg of protein (SOD). The emulsion thus obtained by continuous stirring was left for 1 hour at room temperature. In order to reduce the hydrated lipids to vesicles of the smallest size possible, the lipid suspension was exposed to high-energy ultrasonic irradiation using MSE 150 W probe Ultrasonic Disintegrator. The hydrated lipid was sonicated for a short duration (30 seconds + 30 seconds). The probe was immersed in fluid, approximately 4 mm below the surface. Because a lot of heat was generated in the process, the round bottom flask was immersed in a cooling bath. Small multilamellar liposomes formed were sedimented at 105,000 XG for 45 minutes in an ultra centrifuge. The unentrapped proteins were removed by washing liposome pellets twice with PBS. Liposomes were then tested for protein. Simultaneously, blank liposomes were prepared by the same procedure except 2 ml of PBS (Phosphate Buffer 50 mM, pH 7.4, saline 0.15 M) was added instead of SOD.
3.2.3 Confirmation of liposome formation by electron microscopy

After the preparation of liposomes, they were further analyzed through electron microscopy to confirm the formation of liposomes and to detect the size of the vesicles. A sample of the preparation was set apart for negative staining with 2.15% ammonium molybdate at pH 7.2 for confirmation study.

3.2.4 Estimation of protein entrapment in liposomes

The protein entrapped in liposomes was measured by the method of Lowry et al. (1951). Protein estimation was carried out after disrupting the pellet using 0.9% sodium dodecyl sulphate. Liposomes entrapped with PBS (Phosphate Buffer 50 mM, pH 7.4, saline 0.15 M) were used as homogenate blank.

3.2.5 SOD entrapped liposome administration

SOD entrapped in liposomes was administered intrajugularly to reach the rat brain. The rats were first anesthetized with 0.5 ml ketamin-injected intraperitoneally. After anesthetization an incision was made on the neck region (on dermis and epidermis) and the underlying muscles were then set aside using artery forceps to expose the jugular vein. 0.5 ml of liposome suspension prepared earlier was injected into the jugular vein slowly using a syringe (needle gauge- 29 (12.7 mm)). After administration, the incision on epidermis and dermis were sutured using absorbable surgical suture (sterilized surgical suture (cat gut) and needle (half circle round body, 30 mm). After intravenous administration of liposomes, rats were
subjected to hypoxia (10%) for duration of 3 hours followed by 30 minutes of reoxygenation (21%).

3.2.6 Electron microscopic studies

Electron microscopic study was used to study the ultra structural changes in the brain due to hypoxia. To compare the ultra structural changes associated with hypoxia, hypoxia control set of rats was examined electron microscopically along with the experimental set of rats. Electron microscopy was also employed to confirm the presence of liposome in brain and to study the ultra structural changes noticed in rat brain after it’s intravenous administration. Both the blank liposomes injected group and SOD entrapped liposome injected group was compared. Electron microscopic studies were done at Electron microscopic department, Cancer Research Institute, Adayar Chennai and Central Marine fisheries Research Institute, Kochi.
Whole brain was removed as described above for biochemical analysis. Brain was regionalized into cerebrum, cerebellum, medulla, hippocampus and hypothalamus. Tissues were immediately fixed in 5% cacodylate buffered gluteraldehyde (0.1M) for 3 hours. After three washes in cacodylate buffer, each of 15 minutes, the tissues were trimmed as required. Following which tissues were again washed with fresh buffer twice. Post fixation was done in 1% osmium tetroxide. After post fixation in 1% osmium tetroxide for 2 hours, the tissues were again washed in fresh buffer, 3 washes each of 15 minutes. Tissues were stained with Uranyl acetate (0.5%) for twelve hours and dehydrated in different grades of acetone (30%-100%) at 4°C. Clearing was carried out in different concentrations of acetone and spur embedding media SPURR-D. Following grades were used for clearing:

A. 25ml SPURR D + 75 ml 100% acetone
B. 50ml SPURR D +50 ml 100% acetone
C. 75ml SPURR D +25 ml 100% acetone

The tissues were cleared in the above grades for 1 hour each. After infiltration for 24 hours at 70°C the tissue is embedded in SPURR-D.

3.2.7 Statistical analysis

The statistical package SPSS PC+ (statistical package for social science, version 4.0.1) was used for statistical analysis. Mean and standard deviation were estimated from the sample for each study group. Mean values were compared by Student’s independent t-test or one - way analysis of variance appropriately. Multiple Range Test by Tukey HSD (Honestly significant difference) procedure was
employed to identify the significant groups if P-value in one-way ANOVA is significant. All variables were tested for normality assumption within each group by using Kolmogrove Smirnove non-parametric procedure before undertaking the test of significance. In the present study, P<0.05 was considered as the level of significance.