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&
METHODS
Albino rats of body weight ranging 150 ± 10 g were used for the present study. The animals were maintained under constant environmental conditions and were fed on standard pellet diet (Agro Corporation Pvt. Ltd., Bangalore, India) and water was given ad libitum. The animals were maintained in separate cages in accordance with the guidelines of the National Institute of Nutrition (Indian Council of Medical Research, Hyderabad, India). They were fasted for 24h prior to the experiment.

**Chemicals:** Carbon tetrachloride was used in the present investigation. The molecular structure and other details are given below.

![Carbon tetrachloride structure](image)

<table>
<thead>
<tr>
<th>Property</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Systematic name</td>
<td>Carbon tetrachloride</td>
</tr>
<tr>
<td>Molecular formula</td>
<td>CCl₄</td>
</tr>
<tr>
<td>Molar weight</td>
<td>153.82 g/mol</td>
</tr>
<tr>
<td>Appearance</td>
<td>colorless liquid</td>
</tr>
<tr>
<td>Density and phase</td>
<td>1.5842 g/ml, liquid</td>
</tr>
<tr>
<td>Solubility in water</td>
<td>0.08 g/100 ml (20 °C)</td>
</tr>
<tr>
<td>Melting point</td>
<td>-22.9 °C (250 K)</td>
</tr>
<tr>
<td>Boiling point</td>
<td>76.8 °C (350 K)</td>
</tr>
</tbody>
</table>

Carbon tetrachloride was purchased from BDH Chemicals Co., India. H (L-Arginine was obtained from Dupont New England Nuclear (Boston,
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USA). Dowex columns were obtained from Fisher scientific. All other chemicals used were of technical grade.

Preparation of stock solution:

In vitro study: Stock solutions of CCl₄ (1 mM) were prepared in 1 ml of acetone and the stock solution was diluted in such a way to obtain a desired final concentration 10 – 100 μM.

In vivo study: CCl₄ (1 mM) was diluted with acetone at a concentration of 100μl / 1ml. First group of experimental animals were administered with 50μl and second group of animals received 100 μl of CCl₄ while controls received equal amount of acetone.

Treatment of animals:

Before treatment of animals, food was withdrawn the evening prior to the study but the animals were permitted free access to water. Experimental animals were intraperitoneally administered with different concentrations (50μl and 100 μl) of CCl₄ Controls received equal volume of acetone.

Isolation of cells:

The MAC and LYM were isolated from rat blood according to the procedure of Bigold and Ferrante (1987) as done by Rao et al. (1997). Approximately 5ml of heparinised blood was diluted with an equal volume of RPMI-1640. The diluted samples were layered on to 5 ml Ficol-Hypaque (density of 1.114 ± 0.002) and centrifuged at 800 g for 10 min. The mononuclear cell gradient in the middle was removed and carefully suspended in 10 ml RPMI-1640, incubated at 37°C in 5% CO₂ incubator. The adherent
and non-adherent portions containing MAC and LYM were separated, suspended in saline and centrifuged twice at 400 g. The cells from the adherent portion contained MAC and non-adherent portion LYM which immediately were used for NOS assay after suspending them in NOS Buffer.

The isolation of ET cells from the arteries was accomplished according to procedure as described by Saltis and Bobic (1992) and Freshney (1994). Briefly the arteries were cleared off fat and connective tissue and infused for 30 min with normal saline (5ml / min) and were thus cleared from blood and other debris. The vessels were cut longitudinally to open the lumen, further cut into 5 cm sections and incubated with trypsin, EDTA (with 0.5 g Porcaine trypsin and 0.2 g EDTA, Sigma, USA) and were incubated in 5% CO2 incubator at 37°C/1h. The separated cells containing ET were isolated by centrifugation with saline and were finally suspended in NOS homogenizing buffer. The cell number of all above preparations was maintained as 2 X 10^6/ml of NOS buffer. The viability of cellular preparations in these studies was 90% as determined by trypsin blue exclusion technique.

Hepatocytes were isolated by collagenase perfusion (Moldeus et al. 1978) and the cells were dispersed into Kreb's – Henselit buffer, pH 7.4, and supplemented with 12.6 mM Hepes. Viability of hepatocytes was around 90 - 95% as determined by trypsin blue exclusion. After CCl4 treatment, the viability of hepatocytes decrease to about 80% by 6 h. Hepatocytes were incubated at 37 °C for 15 min in Kreb's – Henselit buffer, pH 7.4, supplemented with 12.6 mM Hepes at a concentration of 5 X 10^6 cells/ml.

Cardiac cells were obtained using a procedure modified from that of Banardeo et al. (1996). Briefly, atrial specimens were collected and finely
minced with iridectomy scissors in Kreb's buffer containing 30 mM 2,3 butanedione monoxime (BDM) and 0.5 mM EGTA. The tissue was subjected to enzymatic digestion in Kreb's buffer supplemented with 0.5% BSA (Invitrogen, carlsbad CA, USA). A first digestion was performed with 6 µl/ml collagenase type V (Invitrogen). Samples were gently triturated using a fine - polished Pasteur pipette in a washing buffer. Isolated cells were filtered to remove undissociated pieces of tissue and gradually resuspended in DMEM (Dulbecco's Modified Eagle's Medium, Biowittakar, Walkersville, MD, USA). Culture medium supplemented with 1% antibodies (100 µl/ml penicillin -G-Na; 50 µl streptomycin sulfate). 1mM insulin, and 10% fetal calf serum. Cells were seeded into 35 mm pertidishes pretreated with laminin (Invitrogen) and maintained at 37° in an H2O-saturated 95% air-5% CO2 environment.

NO Synthase Assay:

The NOS activity of various cell preparations from the rats was measured by monitoring the formation of [3H] citrulline according to the procedure of Bredt and Snyder (1990) Knowles et al. (1990) and as modified by Rajeswara Rao et al. (1995 and 1996). Briefly, the cells were homogenized in 1 ml of NOS homogenizing buffer containing 10 mM HEPES, 0.32 M sucrose, 0.5 mM EGTA, 1 mM dithiothreitol, 10 µg/ml soybean trypsin inhibitor, 2 µg/ml aprotinin and 1 mg/ml PMSF (phenyl methyl sulfonyl fluoride), pH 7.4. The homogenates were centrifuged at 100000 g for 30 minutes and the supernatant containing the soluble fraction was used for NOS assays. The reaction mixture of 500 ul contained 40 mM potassium phosphate buffer (pH 7.2), 6 uM L-arginine, 0.4 u Ci/ml [3H]-arginine and 2 mM NADPH for Ca^{2+}/CaM-independent (iNOS) activity. ("In addition to the above, the reaction mixture for Ca^{2+}/CaM-dependent (eNOS) also contained 1 uM each of calcium chloride
and CaM, 1uM each of FAD and FMN-and 1 mM bioprotein. Incubations were carried out for 30 min at 37°C and the reaction was stopped by adding 20 ul of stop buffer containing 20 mM HEPES and 10 mM EGTA (pH 7.5) The samples were passed over Dowex-50 w (Na' form) and the columns were washed with 1.5 ml distilled water. NOS activity was quantified by measuring the radioactivity of flow-through fraction containing [3H]-citrulline. NOS activity was expressed as mol citrulline/mg protein/min.

The protein content of experimental samples was measured according to the procedure of Lowry et al. (1951).

**Determination of NO\textsubscript{2}^- and NO\textsubscript{3}^- :**

The serum was collected by centrifuging the blood at 2000 rpm for 10 min. NO\textsubscript{2}^- and NO\textsubscript{3}^- determinants were done according to the procedure of Guarner et al. (1993). Briefly the control and experimental animal serum samples were deproteinized before analysis with 35% sulfosalicylic acid. Supernatants of serum were analysed for NO\textsubscript{2}^- concentration, but determination of NO\textsubscript{3}^- necessitated reduction of NO\textsubscript{3}^- to NO\textsubscript{2}^-, which was achieved by reducing the samples in the presence of nitrate reductase and NADPH. The concentration of NO\textsubscript{2}^- was determined with the Griess reaction. The Griess reagent consisted of one part 0.1% naphthylandiamine dihydrochloride and one part of 1% sulfanilamide (sulfanilic acid) in 5% sulfanilamide (sulfanilic acid) in 5% phosphonic acid, which were mixed and kept chilled. The colour was developed by incubation for 1 hr at 60°C, and the absorbance was measured spectrophotometrically at 546 nm. Total nitrite and nitrate concentrations were calculated based on the curve obtained with sodium nitrite standards. NO\textsubscript{3}^- was determined by subtracting NO\textsubscript{2}^- values from
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$\text{NO}_2^+ + \text{NO}_3^-$ values (measured as $\text{NO}_2^-$). The data was expressed as ng of $\text{NO}_2^-$ ml of serum.

**Estimation of Free Calcium Content**

<table>
<thead>
<tr>
<th></th>
<th>(B)</th>
<th>(S)</th>
<th>(T)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Working Reagents</td>
<td>2.5 ml</td>
<td>2.5 ml</td>
<td>2.5 ml</td>
</tr>
<tr>
<td>Calcium Standard</td>
<td></td>
<td>0.02 ml</td>
<td></td>
</tr>
<tr>
<td>Soluble Fraction</td>
<td></td>
<td></td>
<td>100 μl</td>
</tr>
</tbody>
</table>

Calcium levels in the cell free soluble fractions was measured using Qualigens Diagnostics Calcium Kit ($0 - \text{Cresolphthalein Complexoneone Method}$) manufactured by Sigma Diagnostics (India) Pvt. Ltd. Baroda. The 1,00,000 g cell soluble fractions were obtained by homogenizing and centrifuging the samples at 1,000g initially and 1,00,000 g later in phosphate buffer. The buffer solution and colour reagents were mixed in equal volumes. Three clean test tubes were labeled as blank (B), standard (S) and test (T). The following were added to 100μl of soluble fractions as shown above.

After adding the above, the control and experimental samples were mixed well and allowed to stand at lab temperature for 5 minutes. The absorbance was measured in a spectrophotometer at 578 nm. The values were expressed as n mol $\text{Ca}^{2+}/$ mg of cell soluble fraction.
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Assay of cGMP

The cGMP activity was measured following the procedure a given by Colowick and Kaplan (1974). Briefly, the cellular preparations were homogenized in glass homogenizers in 2 - 3 volumes of ice-cold 5% trichloroacetic acid. The precipitate was removed by centrifugation (1,000 g/10min) and the supernatant was centrifuged again at 1,00,000 g and the resultant supernatant was passed on to a 0.5 x 2.0 Cm column of AG1 - X 8 (200-400 mesh, formate form, Himedia, Chennai, India), the resin having been previously washed with distilled water. The columns then were washed with 6 ml of 0.5 N formic acid and the elute, which contained cAMP, however was discarded. The cGMP then was eluted from the column with 4 ml of 4 N formic acid and this elute individually was lyophilized. The dried material alone was taken up in 200μl of sterile water for cGMP assays. To the incubation tubes containing sodium acetate buffer (0.1M, pH 6; magnesium acetate 2 μM; 20 pM cGMP) in 1000μl of reaction medium; 40 μg of histone; 30μl of sterile water; cGMP-dependent protein kinase (40 units, Sigma), 2 nM 32p - ATP (Bhaba Research Institute, Trombay, Mumbai, India, 2 X 105 CPM - 10 μl).

The tubes were kept in ice during the addition of the enzyme. The reaction was commenced by the addition of 32p - ATP. The entire sample was incubated for 7 minutes at 37°C, with shaking and the reaction was terminated by addition of 200 μl of 10% TCA. 10 μg of bovine serum albumin was added as carrier protein and further all contents of the tubes were shaken well by adding 300 μl of precipitating solution (ice-cold TCA- tungstate-H2SO4). The over all mixtures of the samples were centrifuged at 2500 rpm/15 min. The resulting supernatant solution was removed by
aspiration. The obtained precipitate from control and experimental samples individually were dissolved in 100 µl of 1 N NaOH + 1000 µl of precipitating solution and were centrifuged at 2000 rpm and the resulting supernatants were recovered individually. The precipitate was continually dissolved in alkali and the resulted protein was collected and dissolved in 125 µl of 1N NaOH and the radioactivity was counted in 5ml of scintillation fluid. The amount of cGMP present in the samples was determined from a standard graph obtained by assaying a known quantity of cGMP. The cGMP values were expressed as pmole cGMP/mg protein/min.

**Determination of acetylcholine (ACh) content:**

ACh content was estimated by the method of Hestrin as described by Augustinsson (1957). After isolating different cells, they were transferred into clean test tubes and the tubes were kept in boiling water bath for 5 min to inactivate the acetyl cholinesterase (AChE) enzyme activity and to release bound ACh as described by Vasantha et al (1975). The tubes were cooled and the contents were homogenized in 2 ml of distilled water, 2 ml of alkaline hydroxylamine hydrochloride and 1 ml of hydrochloric acid (1:1 HCl : H₂O) were added to the homogenates. The contents were centrifuged at 2000g /10 min and 1 ml of ferric chloride was added to the supernatant. The optical density of the sample was measured at 540 nm in a spectrophotometer using the reagent blank. ACh content was expressed as µmoles of ACh/gm wet weight of tissue.
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Statistical Analysis

For each parameter, the Mean and SD (for both control and experimental groups) were calculated and Statistical significance of the data was analyzed through two way ANOVA (Analysis of Variance) and S-N-K (Student-Newman-Keul's) test using SPSS (Statistical Package for Social Sciences).