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Human subjects

Patients suffering from liver dysfunction, with and without hepatic encephalopathy and normal healthy volunteers were included in this study, only after taking their informed consents. Annexure-1 provides the sheet containing the format of informed consent. Patients without HE were those with chronic liver disease with portal hypertension, ascites, may be with GI bleed. Adult patients attending the out- and in-patient departments of Gastroenterology, Medicine and Neuromedicine at Calcutta National Medical College and Hospital, Kolkata, India were recruited. The diagnosis of HE has generally been performed by clinical and pathological examination. Table 1 provides information on the number of volunteers thus recruited, their average age in years, and gender. Clinical data, including the patients’ case history, signs and symptoms, clinical biochemistry, etc. along with justification by the specialized clinicians determined the gradation of hepatic encephalopathy (HE) (see Table 2). The present study was conducted with the approval of the Human Ethics Committee of the participating institutes and it is in accordance to the ethical guidelines of 1975 Declaration of Helsinki.

Table 1. Normal, with and without HE patients with age and sex

<table>
<thead>
<tr>
<th></th>
<th>Age (Years-Average)</th>
<th>Number</th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>41 ± 1.44</td>
<td>84</td>
<td>57</td>
<td>27</td>
</tr>
<tr>
<td>WHE</td>
<td>46 ± 1.48</td>
<td>75</td>
<td>53</td>
<td>22</td>
</tr>
<tr>
<td>HE</td>
<td>48 ± 1.43</td>
<td>81</td>
<td>56</td>
<td>25</td>
</tr>
</tbody>
</table>

Table 1. Total number, age and gender of patients included in the study. WHE = without hepatic encephalopathy, HE = hepatic encephalopathy.
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Table 2. Gradations of HE in the study population

<table>
<thead>
<tr>
<th>Grades of HE</th>
<th>Number of patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>6 (male-5; female-1)</td>
</tr>
<tr>
<td>I</td>
<td>15 (male-8; female-7)</td>
</tr>
<tr>
<td>II</td>
<td>15 (male-13; female-2)</td>
</tr>
<tr>
<td>III</td>
<td>10 (male-8; female-2)</td>
</tr>
<tr>
<td>IV</td>
<td>4 (male-3; female-1)</td>
</tr>
</tbody>
</table>

Table 2. The table depicts the gradation of HE in the patients who are included in the study. Gender of patients and their numbers are provided against the HE grade in which they belonged.

Experimental Animals

Adult, male Sprague Dawley rats (230-300 g body weight) and 18-22 days-old litters were used in the present study. Two month old male Sprague Dawley rats were used in some experiments. They were issued from IICB animal care facility, which is registered with the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), under the Ministry of Social Justice and Empowerment, Govt. of India (Regn. # 147/CPCSEA, 1999, renewed in 2005 and 2008). Animals were housed under standard conditions of temperature (22 ± 2 °C), humidity (60 ± 5%), and illumination (12 h light–dark cycle). The animals were provided with free access to food and water ad libitum. Animals were always sacrificed in the mornings to avoid diurnal variations in the levels of neurotransmitters, their metabolites and changes in other biochemical parameters. All the animal experimentation procedures met the CPCSEA National Guidelines on the ‘Care and Use of Animals in Scientific Research’ (INSA, 2000) and were approved by the Animal Ethics Committee of Indian Institute of Chemical Biology, Kolkata [IICB-AEC Code Nos. KPM and KPM-I]. Precautions were taken to minimize animal sufferings, if any and to keep the number of animals used for experimentations to a minimum.

Sprague-Dawley rats were divided into three groups (see Table 3). TAA was prepared in normal saline freshly before each injection. Rats were treated with saline (Control: Group I) or two different doses of TAA injected intraperitoneally (i.p.) for 3 consecutive days. The animals were administered TAA at 100 (Group: II) and 200 mg/kg
(Group: III) doses, once daily for three days (see Fig. 1). By 24 h the treated rats showed general weakness, lethargy and slowness in movement. The effect was much prominent in Group III, which received 200 mg/kg dose of the toxic molecule.

Table 3. Rat experimental groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Saline</td>
<td>5-10</td>
</tr>
<tr>
<td>II</td>
<td>TAA (100 mg/kg)</td>
<td>6-10</td>
</tr>
<tr>
<td>III</td>
<td>TAA (200 mg/kg)</td>
<td>6-10</td>
</tr>
</tbody>
</table>

Table 3. Rats were treated with TAA (100 and 200 mg/kg., i.p.) once every day for three consecutive days. The animals were studied for normal behaviors prior to TAA administration, and one day after the last dose of the drug.

Fig. 1. TAA treatment regimen and experimental paradigm

Materials

Chemicals, Drugs and Reagents

The following chemicals/drugs were purchased from Sigma Chemical Company (St. Louis, MO., USA): purified acetylcholinesterase (AChE) from bovine erythrocyte, acetyl thiocholine iodide, acetyl coenzyme-A (Acetyl-CoA), neostigmine sulfate, 4,4'-dithiopyridine (4-TP), 5,5'-dithiobis-2-nitrobenzoic acid (DTNB), acrylamide, ammonium persulphate (APS), bovine serum albumin (BSA), bromophenol blue, 3,3'-diaminobenzidine tetrahydrochloride (DAB), ethylenediaminetetraacetic acid (EDTA), glycine, β-mercaptoethanol (β-ME), triethylamine HCl (TEA), N,N'-methylene-bis-acrylamide, N,N,N,N'-tetramethylethylene diamine (TEMED), tris (hydroxymethyl)
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aminomethane (Tris), polyoxyethylene-sorbitan monolaurate (Tween 20), Triton-X 100, TRI® reagent, 3-hydroxytyramine hydrochloride (dopamine, DA), 3,4-dihydroxyphenyl acetic acid (DOPAC), homovanillic acid (HVA), 5-hydroxyindole-3-acetic acid (5-HIAA), 5-hydroxytryptamine creatinine sulphate (5-HT), norepinephrine (NE), 1-heptane sulfonylic acid (HSA), 4-hydroxyquinoline (4-HQ), clorgyline hydrochloride, kynuramine, D,L-homocysteine (Hcy), reduced glutathione (GSH), potassium ferricyanide [K₃Fe(CN)₆], copper sulfate (CuSO₄) and ammonium chloride (NH₄Cl). Thioacetamide (TAA) and trichloroacetic acid (TCA) were purchased from SISCO Research Laboratories (SRL) PVT, Ltd, Mumbai, India.

Aspartate, pyruvate standard, alanine, α-ketoglutarate (α-KG), DNPH, Diazo-A & B, bilirubin standard, methanol and brain thromboplastin solution with CaCl₂ were purchased from Span Diagnostic Pvt. Ltd (Surat, India). Potassium chloride, sodium chloride and sodium dodecyl sulfate (SDS) were obtained from MP Biomedicals Inc. OH, USA. Chloral hydrate and absolute alcohol were bought from Fluka, Switzerland. Agarose was obtained from Gibco BRL, USA. Immuno-Blot PVDF membranes were brought from Millipore, USA. DPX mountant, hydrogen peroxide, imidazole, paraformaldehyde (PFA), perchloric acid and xylene were obtained from Merck (I) Ltd., Mumbai, India. HPLC-grade methanol was bought from Spectrochem Pvt. Ltd. (Mumbai, India). Glacial acetic acid and glycerol were procured from Qualigens Fine Chemicals division of Glaxo SmithKline Pharmaceuticals Ltd., Mumbai, India. 2, 4′-Dinitrophenylhydrazine (2,4-DNPH) was from British Drug Houses Ltd., England. Anesthetic ether was procured from Kabra Drugs Ltd., Indore, India. Other reagents and chemicals were of analytical grade and were produced from SRL (Mumbai, India), Glaxo (Mumbai, India) and BDH (Mumbai, India).

Antibodies: Mouse monoclonal IgG for AChE (MAB-304) and Goat polyclonal IgG for ChAT (AB-144P) were procured from Chemicon, USA. Anti-actin antibodies (SC-1615) was purchased from Santa Cruz Biotechnology, Inc., CA, USA.
Other consumables

* Cryotome blades (Shandon HP35; 35/75 mm) and embedding matrix for freeze sectioning were purchased from Thermo Electron Corporation, PA, USA.

* Disposable plastic wares such as micropipette tips and microcentrifuge tubes were procured from Tarsons Products Pvt. Ltd., Kolkata, India.

* Double distilled water (DDW) was prepared using a quartz double distillation system. To obtain deionized water for use in HPLC and in cultures, a high quality water purification system (TKA-MICRO, Niederelbert, Germamy) was used.

* Micro slides and cover slips were obtained from Blue Star Polar Industrial Corporation, Mumbai, India

* Teflon tubes, plastic disposable syringes, etc. were purchased locally.

Preparation of drugs and solutions

1. Phenol-nitroprusside solution: 50 mg of phenol and 350 mg of sodium nitroprusside, dissolved in 3.75 ml DW.

2. Sodium hypochloride solution: 8.4 gm of sodium hydroxide and 8.92 gm of dihydrogen orthophosphate and 10 of 4% sodium hypochloride, dissolved in DW and made the final volume 1 L.

3. Acrylamide (30%) / bis-acrylamide (0.8%): Bis-acrylamide (0.8 g) was dissolved in 70 ml of DDW. To this solution, 30 g of acrylamide was dissolved. The final volume was made up to 100 ml with DDW. The solution was stored at 4 °C in dark up to 1 month.

4. Gradient separating gel (4 and 10%) for SDS-polyacrylamide gel electrophoresis (PAGE): Cleaned, dried glass plates were assembled with 1 mm spacers in a BioRad gel caster. A small gradient maker connected to a peristaltic pump was placed on a magnetic stirrer with two small magnetic fleas inside each chamber. The chamber nearer to the outlet was assigned for heavier solution (10%) and the other one for the lighter solution (4%). The ingredients for the two gel concentrations (see Table 4) were mixed very slowly (without introducing bubbles) in the assigned chambers.
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(outlet and inter chamber tap closed) with continuous stirring. After adding APS and TEMED, the outlet was opened first to allow the higher concentration gel to flow into the tubing and then the inter-chamber tap was opened for formation of proper gradient. Once the flow was over, the gel was overlaid with double distilled water and kept for polymerization at room temperature.

Table 4. The separating with stacking gels was prepared as follows:

<table>
<thead>
<tr>
<th>Item names</th>
<th>Separating gel (10%)</th>
<th>Stacking gel (4%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DDW (ml)</td>
<td>2.00</td>
<td>1.22</td>
</tr>
<tr>
<td>30% Acrylamide (ml)</td>
<td>1.67</td>
<td>0.26</td>
</tr>
<tr>
<td>Tris-HCl (1.5M) (ml)</td>
<td>1.25</td>
<td>0.50</td>
</tr>
<tr>
<td>10% SDS (μl)</td>
<td>50</td>
<td>20</td>
</tr>
<tr>
<td>10% APS (μl)</td>
<td>25</td>
<td>20</td>
</tr>
<tr>
<td>TEMED (μl)</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Total volume (ml)</td>
<td>4.997</td>
<td>2.024</td>
</tr>
</tbody>
</table>

5. Mobile phase for high performance liquid chromatography electrochemical detection (HPLC-ECD) of Hcy, GSH and biogenic amines were prepared 24 h before use, in deionised water. The composition of the mobile phase for (i) Hcy and GSH was monosodium phosphate (0.1 M), EDTA (0.5 mM), methanol (20%) and the pH was adjusted to 4.5 by 1 M phosphoric acid, (ii) Biogenic amines was heptosulphonic acid (8.65 mM), EDTA (0.27 mM), acetonitrile (13%, v/v), trimethylamine (0.4- 0.45%, v/v), phosphoric acid (0.30-0.35%, v/v). Before use, it was filtered and degassed employing a solvent filtration unit (Millipore, MA, and USA) attached to a vacuum pump (Millipore).

6. PBS (0.01 M, pH 7.4) was prepared by adding 17.52 g NaCl and 400 mg KCl to 100 ml of 0.2 M phosphate buffer stock, pH 7.4 and finally made up to 2000 ml with DDW.

7. PFA 4% was prepared by adding 4 g of PFA in 100 ml of 0.01 M PBS, pH 7.4 and heated to 50 °C until complete dissolution. The solution was filtered and stored at 4 °C for 1-2 days.
8. Potassium phosphate buffer (0.1 M, pH 7.8): Solution A: 0.2 M monobasic potassium phosphate in DDW; Solution B: 0.2 M dibasic potassium phosphate in DDW; 28 ml of solution B was added to 72 ml of solution A and then diluted up to 200 ml.

9. Running buffer was prepared by mixing 3.0 g Tris, 14.4 g glycine, and 1.0 g SDS in 1000 ml DDW to a final concentration of Tris (24.8 mM), glycine (192 mM) and SDS (0.1%).

10. Sample loading buffer (4x) for SDS-PAGE: SDS (0.8 g), 87% glycerol (4.7 ml), 1 M Tris-HCl, pH 6.8 (2.5 ml), and bromophenol blue (100 mg) were mixed and the final volume was made up to 10.0 ml with DDW.

11. SDS (10%) was prepared by dissolving 1 g SDS in 10 ml of double distilled water and stored at room temperature.

12. Sodium phosphate buffer (0.2 M, pH 7.8): Solution-A: 0.2 M monobasic sodium phosphate in DDW; Solution-B: 0.2 M dibasic sodium phosphate in DDW; 19 ml of solution A was added to 81 ml of solution B to prepare 100 ml of the buffer.

13. Stacking gel for SDS-PAGE: Acrylamide (30%) / bis-acrylamide (0.8%) solution (666.7 μl), 4X Tris-HCl 0.5 M, pH 6.8 (1250 μl), DDW (2972.8 μl) and 10% APS (55 μl) were mixed thoroughly before TEMED (5.5 μl) was added. The solution was swirled gently to mix and poured immediately on top of the separating gel. The Teflon comb was inserted to make wells for loading. The setup was left undisturbed until polymerization of the gel was complete.

14. TBS-Tween (TBS-T): Tween 20 (0.1%, v/v) was added to TBS (pH 7.6).

15. Transfer buffer was prepared by mixing 3.0 g Tris, 14.4 g glycine and 200 ml of methanol in 1000 ml DDW with a final concentration of Tris (24.8 mM) and glycine (192 mM).

16. Tris buffered saline (TBS) (pH 7.6) was prepared by mixing 2.42 g Tris base, 8 g NaCl in 1000 ml of DDW to a final concentration of Tris base (20 mM), NaCl (137 mM) and the pH of the solution was adjusted to 7.6 by adding 1.0 M HCl.

17. Tris-HCl (4x), pH 6.8 (0.5 M Tris-HCl): Tris (12.1 g) was dissolved in 80 ml DDW and the pH was adjusted to 6.8 with 1.0 M HCl. The final volume of the solution was
made up to 200 ml with DDW. The solution was filtered, and stored at 4 °C up to 1 month.

18. Tris-HCl (4x), pH 8.8 (1.5 M Tris-HCl): Tris (36.4 g) was dissolved in 120 ml DDW and the pH was adjusted to 8.8 with 1.0 M HCl. The final volume of the solution was made up to 200 ml with DDW. The solution was filtered, and stored at 4 °C up to 1 month.

19. Clorgyline HCl was freshly prepared in normal saline.

20. EDTA, acetylthiocholine iodide, choline chloride and neostigmine sulfate solutions were freshly prepared in distilled water (DW).

21. Purified acetylcholinesterase enzyme (bovine erythrocyte, type XIII) was freshly prepared in sodium phosphate (0.1 M) buffer (pH 8.0).

22. DTNB (0.01 M) solution was freshly prepared in 0.1 M sodium phosphate buffer (pH 7.0) and to every 10 ml was added 15 mg of sodium bicarbonate. This solution was always kept in dark at 4 °C.

23. Acetyl CoA was dissolved in 0.01 N HCl to make 10 mM stock, which was refrigerated till further use. Further dilution to working stock of $6.2 \times 10^6$ M was made in DDW.

24. The stock solution of 4,4'-dithiopyridine (10 mM) was prepared in 10% ethanol. Further dilution to make the working solution of $10^{-9}$ M was done in DDW.

25. Kyrunamine and 4-HQ were freshly prepared in potassium phosphate buffer (10 mM, pH 7.2). These solutions were used within 6 h from the time of preparation.

26. ETDA (0.01%) in perchloric acid (HClO₄, 0.1 M) was used as the deproteinizing solution for the brain tissue. This solution was stored for months at 4 °C.

27. Sodium citrate (0.1 M) was freshly prepared in double distilled water.

28. Copper sulfate (30 mM) was freshly prepared in DW.

29. Potassium ferricyanide [K₃Fe(CN)₆] (5 mM) was freshly prepared in DW.

30. Phenol-nitroprusside solution: 50 mg of phenol and 350 mg of sodium nitroprusside, dissolved in 3.75 ml DW.

31. Sodium hypochloride solution: 8.4 gm of sodium hydroxide and 8.92 gm of dihydrogen orthophosphate and 10 of 4% sodium hypochloride, dissolved in DW and made the final volume 1 L.
32. Potassium EDTA (0.25 M) was prepared in DW.
33. Ammonium chloride standard (0.1 to 1.0 mM) was freshly prepared in DDW.
34. Sodium citrate (3.2%) solution: Taken 3.2 gm of sodium citrate and dissolved in 100 ml of DW.
35. Solution A for protein estimation was prepared by dissolving 2 gm of NaOH, 10 gm of Na$_2$CO$_3$, and 100 mg of potassium sodium tartrate in 500 ml of double distilled water.
36. Solution B for protein estimation was prepared by dissolving 500 mg of CuSO$_4$5H$_2$O in 100 ml of DDW and stored in amber colored bottle.

**Instruments used**

1. Confocal microscope (Leica DM IRB, Germany).
2. Cryostat (Shandon Cryotome E, Thermo Electron Corporation, USA).
3. Electrophoretic apparatus and power supply (BioRad, CA, USA)
4. Fine balance (Model: 71/C, Adam Equipment Co. Ltd., UK)
5. Gel Documentation System (BIORAD Gel Doc EQ System, CA, USA).
6. Homogenizer (Remi Motors, Mumbai, India)
7. HPLC-ECD system (BAS EPSILON LC System version 2.30 from Bioanalytical systems, West Lafayette, USA) consisting of a pump (PM-80), a Rheodyne injector (7725i), an amperometric LCEC detector (CC-5e with epsilon).
8. Inverted microscope with fluorescence accessories (Axiovert 200 with HBO-100, Carl Zeiss, Germany)
9. Micropipettes (Microlit, India; Merck Ltd., Germany; Thermo Electron Corp., USA)
11. Peristaltic pump (LKB BROMMA 2120 VARIOPERPEX, Sweden).
12. Plus Maze Apparatus (custom made)
13. Power supply for electrophoresis (BIO-RAD PowerPac Universal, CA, USA)
14. Rat-Walk gangway (custom made)
15. Refrigarated High Speed Centrifuge (Sorvall RC 5B PLUS, CT, USA)
16. Rotarod (Techno Electronics, Lucknow, India)
17. Solvent filtration system (Millipore, MA, USA)
18. Sonicator (Misonix Ultrasonicator, USA)
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22. Spectrophotometer (GBC Cintra 10e, Australia).
23. Stereo Zoom Microscope (Stemi SV 6, Carl Zeiss, Germany)
24. Tabletop centrifuge (Hermle Z 230 M, Germany)
25. Table-top refrigerated centrifuge (Hermle Z 233 MK-2; Germany)
26. Ultracentrifuge (Sorvall Ultra 80, CT, USA).
27. Vertical electrophoresis apparatus (BIO-RAD, CA, USA).
28. Vortex (Tarsons, Kolkata, India)

Softwares used for data/image analysis:

1. Axiocam (Carl Zeiss, Germany)
3. FL Winlab (Perkin Elmer, USA)
4. Microsoft Word and Excel, Adobe photoshop
5. Quantity One (BIO-RAD, CA, USA)
6. SPECTRAL 1.70 (GBC Scientific, USA)

Methods

Collection and preparation of samples:

Blood from human: From healthy and diseased, fasted (male/female) human subjects, blood were drawn from the vein by using a 10 ml sterilized syringe.

Blood from rats: From control and TAA treated rats blood were collected from the retro orbital plexus by using fine glass capillary tubes.

Blood samples were transferred into plain vials and anti-coagulated vials containing potassium EDTA (0.25 M)/sodium citrate (3.2%). For P-time test, both (human and rat) blood were collected into a tube containing 3.2% sodium citrate (100 μl of sodium citrate for 900 μl of blood). For the analysis of AChE, ChAT and MAO, both (human and rat) blood were collected into tubes containing 0.25 M EDTA (100 μl of 0.25 M EDTA for 5.0 ml of blood).
Preparation of serum: Blood samples were allowed to stand for half an hour at room temperature, and then centrifuged at 4000 rpm at 4 °C for 10 min. Aliquoted the sera into Eppendorf tubes and kept at 4 to -70 °C for biochemical investigations.

Preparation of plasma: Anti-coagulated blood was centrifuged at 4000 rpm at 4 °C for 10 min. Aliquoted the plasma and kept at 4 to -70 °C for biochemical investigations. In case of Hey and GSH assays, it was again centrifuged at 12,500 rpm for 15 min at 4 °C.

Rat brain

Dissection of discrete rat brain regions

Animals were sacrificed by decapitation using a guillotine, always in the mornings prior to 11.00 AM in order to avoid any diurnal variations of the endogenous amines, enzymes and other antioxidant molecules. The schematic diagrams of motor cortex (MC), nucleus caudate putamen (NCP), hippocampus (HP) and nucleus raphe dorsalis (NRD) regions (Fig. 2) were dissected out for the estimation of biochemical analysis. NCP and NRD were micro-punched (Palkovits and Brownstein, 1983), and cortex and hippocampus were dissected out from one mm frozen sections, cut free hand with a sharp razor blade.

Fig. 2. Schematic diagrams showing different regions of rat brain: motor cortex (MC), nucleus caudatus putamen (NCP), hippocampus (HP) and nucleus raphe dorsalis (NRD) were excised from the shaded regions (Figures are modified from from Paxinos and Watson, 1998).
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Rat liver

Rat livers were collected from the control and the treated animals into 50 ml normal saline and washed properly and immersed in 3.7% buffered formaldehyde prepared in 0.1 M phosphate buffer, pH 7.0, for 24 h.

Behavioral analyses:

Gait analysis

Gait abnormalities in the control and TAA-treated rats were investigated by placing them in an inclining gangway as described by Klapdor et al. (1997). In short, rats with their fore- and hind-limbs painted with two different non-toxic water colors were placed on the small gangway (100 cm long, 12 cm wide with 12 cm high side walls and a slope of 30°). The test was conducted twice for each animal per session. Proper care was taken to wash off the paints with water and to wipe the feet of the animals with a dry towel before placing them back in their home cage. They were pre-trained before the injection to walk up the gangway into a dark compartment. The time taken to walk up the gangway and the foot prints, taken on white papers lined in the gangway, were recorded and analyzed as reported (Klapdor et al., 1997; Chandra et al., 2006; Pandey et al., 2007).

Akinesia

Akinesia was measured by noting the latency in seconds (s) of the animals to move all four limbs and the test was terminated if the latency exceeded 180 s. Rats were kept at one edge of a wooden platform. Using a stopwatch, the time taken (s) by the animals to initiate movement was recorded (Weihmuller et al., 1989; Mitra et al., 1992; Muralikrishnan and Mohanakumar, 1998).

Elevated Plus-Maze test

Animals were tested for any disturbance in their motor activity employing a Plus-Maze placed at an elevated position. The apparatus was made of a wooden plus-shaped Maze elevated at 100 cm from the floor having two open arms, 50 x 10 cm, crossed at right angles by two arms of the same dimensions enclosed by 50 cm high walls. A 1 cm
high edge made of plywood surrounded the open arms to prevent the animals from falling. Rat was placed at the center of the arms and allowed to explore the Maze freely (File et al., 1992). The number of entries in the open and closed arms was recorded during a 5 min period. Total number of entries into either type of arm indicated overall motor activity of the animals.

**Rotarod test**

The test was conducted following Moreira et al. (2006). Adaptation to the rotarod commenced on day before the injection. Rats were acclimatized to walk on a Rotarod apparatus at different speeds from 5 to 25 rpm for 5 min and those which could not continue on the rotating rod for 300 s at the maximum speed were not included in the study. The actual testing began on 4th day, one day after the last injection. The treadmill constantly rotated at a speed of 10 rpm and the time that rats could stay on the rotarod during a 300 s period was recorded. The rotarod scores of rats were recorded as average of two separate trials at 5 min interval.

**Biochemical assays**

**Estimation of serum aspartate aminotransaminase (AST; EC: 2.6.1.1) and alanine aminotransaminase (ALT; EC: 2.6.1.2) activities by colorimetric method (Reitman and Frankel, 1957)**

**Principle:** The oxaloacetate and pyruvate formed by the action of AST and ALT from appropriate substrates (alanine and aspartate-α-ketoglutaric acid) are coupled with 2,4-dinitrophenyl hydrazine to form a golden brown color of 2,4-dinitrophenyl hydrazone in alkaline medium (Fig. 3). The color thus produced is measured employing a spectrophotometer at 540 nm wavelength, and compared with pyruvic acid (1- 10 μm) as standard.

**Reaction:** The reaction for aminotransferase (ALT and AST) assay is provided in figure 3 below.

**Sample:** Serum samples are required for the estimation of amiotransferase.

**Reagents:** (i) Reagent 1 for ALT: Buffered alanine and α-ketoglutarate substrate, pH 7.4
(ii) Reagent 1 for AST: Buffered aspartate and α-ketoglutarate substrate, pH 7.4
(iii) Reagent 2: DNPH color reagent
(iv) Reagent 3: Sodium hydroxide, 4 N
(v) Reagent 4: Working pyruvate standard, 2 mM

**Preparation of working solutions:** Diluted 1 ml of reagent 3 to 10 ml with purified water. Reagent 1, 2, and 4 are ready to use.

**Procedure:** ALT and AST activities in human and animal serum samples were measured by employing a diagnostic reagent kit from Span diagnostics, which follows the method of Reitman and Frankel (1957). Briefly, 50 µl of serum was mixed with 250 µl of reagent 1 (buffered alanine, pH 7.4) and were kept at 37 °C for 5 min. To this, 250 µl of Reagent 2 (DNPH color reagent) was added to each tube, mixed well and allowed to stand at room temperature (RT) for 20 min. The reaction was stopped by adding 2.5 ml of 0.4 N NaOH. The solution was kept at RT for 10 min and measured the absorbance at 505 nm against distilled water as blank. The activities of these enzymes were expressed in µmol/min/L. The units of these enzymes were expressed as µmol of the brown dinitrophenyl hydrazone formed per min per liter of serum.

![Reaction of aminotransferases](image_url)

**Fig. 3.** Catalyses the reaction of aminotransferases (ALT, alanine aminotransferase; AST, aspartate aminotransferase; PLP = pyrioxidal phosphate).
Estimation of serum total bilirubin (TB) and direct bilirubin (DB) levels

**Principle:** Bilirubin reacts with diazotized sulphonic acid in acidic medium to produce a red purple coloured compound called azobilirubin. The colour thus produced is measured in a spectrophotometer at 520 nm and compared with that of bilirubin standard. Direct (conjugated) bilirubin produced immediate colour with diazo reagent, but the unconjugated bilirubin (indirect) produced colour only in the presence of caffeine (Fig. 4).

**Reagents:** The reaction for aminotransferase (ALT and AST) assay is as follows:

![Mechanism of reaction of Bilirubin](image)

**Sample:** Serum sample is used for this test.

**Reagents:** (i) Reagent-1 (sulfanilic acid and conc. HCl), (ii) Ragent-2 (sodium nitrite), (iii) Reagent-3 (caffeine), and (iv) Bilirubin standard.

**Procedure:** The serum TB and DB in human and animal samples were measured by employing a reagent kit from Reckon Diagnostics, which follows the method of Jenderas, N. and Grof (1938). Briefly, 500 µl of serum was mixed with 100 µl of prepared working reagent (mixture of Reagent 1 and Reagent 2). To this mixture 1 ml of Reagent 3
(containing caffeine) was added for the estimation of TB and 1 ml of normal saline (0.9%) was added for estimation of DB. After thorough mixing, the tubes were incubated at RT for 5 min for TB and 30 s for DB. The absorbance was measured at 546 nm against blank (reaction mixture without reagent 2). The concentration of bilirubin was expressed in mg/dl of serum.

**Estimation of plasma Prothrombin-time (P-time):**

**Principle:** Prothrombin-time was estimated employing a kit from Tulip diagnostics. Tissue Thromboplastin in the presence of calcium activates the extrinsic pathway of human blood coagulation mechanism. When LIQUIPLASTIN reagent is added to normal anticoagulated plasma, the clotting mechanism is initiated, forming a solid gel clot within a specific period of time. The time required for clot formation would be prolonged if there is a deficiency of factors/factor activity in the extrinsic pathway of the coagulation mechanism (Fig. 5).

**Reaction:** The mechanical reaction for prothrombin-time assay is as follows:

Fig. 5. Coagulation cascade of prothrombin. V = Labile factor, VII = Stable factor, IX = Christmas factor, X = Stuart - Power factor, XII = Hageman factor.

**Sample:** Plasma sample is required for this examination

**Reagents:** (i) “LIQUIPLASTIN” reagent
Procedure: Briefly in a tube 100 μl of the plasma was taken and incubated in water bath at 3 °C for 5 min. To this tube, 200 μl of “LIQUIPLASTIN” reagent (pre-warmed at incubated at 37 °C for at least 3 min) was added and simultaneously started a stopwatch to note the time taken for clot formation. The time required for the appearance of the clot was recorded. The clot formation was expressed in second (s).

Determination of ammonia

Principle: Concentration of ammonia was estimated following the colorimetric method of Ramakumari and Murthy (1990). Serum ammonia reacts with phenol-nitroprusside in the presence of sodium hypochlorite reagent to form a colored complex that is directly proportional to the concentration of ammonia. It gives absorbance maxima at 630 nm. The reaction is as follow:

Phenol + sodium nitroprusside $\rightarrow$ Phenol-nitroprusside solution

Sodium hypochlorite

Phenol-nitroprusside solution + sample $\rightarrow$ Blue color complex

Sample preparation: Serum was deproteinized by addition of an equal volume of 10% perchloric acid (v/v) and waited for 15 min at RT for the complete precipitation of protein. The tubes were centrifuged at 5000 rpm for 10 min at 4 °C. Collected the supernatant and neutralized with saturated sodium carbonate till pH was 7.0. Tubes were kept in ice for 15 min and then they were then centrifuged at 10000 rpm for 15 min at 4 °C to remove precipitated potassium chlorate. The supernatant was used for the estimation of ammonia.

Reagents: (i) Phenol, (ii) sodium-nitroprusside, (iii) sodium hypochlorite (5%), (iv) sodium hydroxide, (v) disodium hydrogen phosphate, (vi) sodium carbonate, (vii) ammonium chloride

Preparation of reagents: (i) Phenol-nitroprusside solution: 50 g of phenol, 250 mg of sodium-nitroprusside in 3.75 liters of water, (ii) Sodium hypochloride solution: 8.4 g sodium hydroxide, 92 g disodium hydrogen phosphate and 10 ml of 5% sodium hypochlorite made 1 liter. (iii) Stock ammonium chloride (100 mM): 5.35 mg of
ammonium chloride dissolved in 1 ml of D/W, (iv) Working standard (0.1 to 10 μmol): It was prepared from stock ammonium chloride standard.

**Procedure:** To 1 ml of the supernatant 1.5 ml of phenol-nitroprusside reagent and 2 ml of sodium hypochlorite reagent were added. After 20 min at RT, the colour intensity was measured at 630 nm against distilled water blank.

**Measurement of protein by Lowry et al. (1951) method**

**Principle:** Under alkaline conditions Cu²⁺ forms a complex with the peptide bonds and reduce to Cu⁺. The Cu⁺ reacts with the side chain group of tyrosine, tryptophan, and cysteine residues then react with the Folin-phenol reagent. The reagent reacts by first producing an unstable product, which slowly reduces to a stable molybdenum/tungsten blue complex.

**Procedure:** Protein was estimated according to a modified method of Lowry et al. (1951). For protein estimation 5-10 μl of protein was taken in micro-centrifuge tubes and diluted up to 250 μl using double distilled water and added 1 ml of freshly prepared mixture of solution A and B (50:1). The solution was mixed thoroughly and incubated for 10 min at room temperature in dark. Hundred μl of 1 N Folin phenol reagent was added and again mixed thoroughly. The reaction was incubated for 30 min at room temperature in dark and absorbance was recorded at 750 nm in a double beam spectrophotometer.

**Determination of acetylcholinesterase (AChE; EC 1.3.1.7):**

**Principle:** The principle of this method is the measurement of the rate of production of thiocholine as acetylcholine is hydrolyzed. This is accomplished by the continuous reaction of the thiol with 5,5'-dithiobis-2-nitrobenzoate ion to produce the yellow anion of 5-thio-2-nitrobenzoic acid. The rate of color production is measured continuously at 412 nm by spectrophotometer. The reaction with the thiol has been shown to be sufficiently rapid so as not to be rate limiting in the measurement of the enzyme and in the concentrations used does not inhibit the enzymatic hydrolysis. The yellow anion formed is measured by taking the molar extinction coefficient as 1.36 x 10⁴.
Preparation of sample: A suspension of the whole blood cells in ice cold 0.1 M sodium phosphate buffer (pH 8.0) was prepared (in vitro), using a glass-teflon homogenizer. The dilution was 1:600 (e.g. 5 μl blood into 3 ml buffer).

Reagents: (i) Sodium phosphate buffer (pH 8.0), (ii) Sodium phosphate buffer (pH 7.0), (iii) 0.075 M Acetylthiocholine iodide, (iv) 0.01 M DTNB.

Procedure: The reaction mixture contained 0.1 M sodium phosphate buffer (pH 8.0), 5, 5'-dithio-bis-2-nitrobenzoic acid; (10 mM), acetyl thiocholine iodide (75 mM) and whole blood suspension (200 μl). The rate of increase in absorbance was recorded in a Cintra 10e (GBC) Spectrophotometer at 412 nm and the unit of the enzyme activity was expressed as μmole of anion produced per minute per milligram protein, taking the extinction coefficient of the yellow anion formed as ε_{412} = 1.36 \times 10^4. Protein was estimated following the procedure of Lowry et al. (1951), taking bovine serum albumin as standard.

Reaction: The reaction for AChE assay is as follows:

\[
\begin{align*}
\text{H}_2\text{O} + (\text{CH}_3)_2\text{N}^+\text{-CH}_2\text{-CH}_2\text{-S-C-CH}_3 & \overset{\text{AChE}}{\longrightarrow} (\text{CH}_3)_2\text{N}^+\text{-CH}_2\text{-CH}_2\text{-S'} + \text{CH}_3\text{C-O}^- + 2\text{H}_2\text{O} \quad \text{(Acetylthiocholine)} \text{ (Thiocholine) (Acetate)} \\
(\text{CH}_3)_2\text{N}^+\text{-CH}_2\text{-CH}_2\text{-S'} + \text{R-S-S-R} & \longrightarrow (\text{CH}_3)_2\text{N}^+\text{-CH}_2\text{-CH}_2\text{-S-S-R} + \text{R-S'} \quad \text{(Thiocholine)} \text{ (5'-DTNB) (5-DTNB)} \\
\end{align*}
\]

R = O\text{N}^-

Assay of AChE in brain tissue:

The tissues were weighted and homogenized in ice cold 0.1 M sodium phosphate buffer (pH 8.0) to obtain a 2% homogenate, using a glass-teflon homogenizer. The homogenate
was centrifuged at 7500 x g for 30 s in a Hermle micro-centrifuge and the supernatant was used for AChE assay immediately.

**Procedure:** Eighty μl of the supernatant was added to a cuvette containing 2.8 ml of 0.1 M sodium phosphate buffer (pH 8.0), 100 μl of DTNB (0.01 M) reagent and was mixed thoroughly. To this solution 20 μl of acetyl thiocholine iodide (0.075 M) was added. The blank for such a run consisted of buffer, substrate and DTNB solution. The change in absorbance at 412 nm in a spectrophotometer over a period of 2 min was noted and change of absorbance per minute was calculated. The unit of enzyme activity is defined as μmol of substrate hydrolyzed/min/mg protein.

**Determination of choline acetyltransferase (ChAT; EC: 2.3.1.6):**

**Principle:** ChAT activity was assayed following by Chao and Wolfgram (1972) method, where the reduced CoA produced by the acetylation of choline was reacted with 4,4'-dithiopyridine to produce 4-thiopyridine (4-TP), which has an absorption maximum at 324 nm and a molar extinction coefficient of 1.98 x 10^4. This involves a reaction as follows:

\[
\text{Cl} \quad \text{ChAT} \\
\text{CH}_2\text{CO-S-CoA} + \text{HO-(CH}_2)_3\text{N-(CH}_2)_3 \quad \rightarrow \quad \text{CH}_2\text{CO-O-(CH}_2)_3\text{N-(CH}_2)_3 + \text{CoA-SH}
\]

\[
\text{CoA-SH} + \text{4-4'-Dithiopyridine} \quad \rightarrow \quad \text{4-Thiopyridine} + \text{4-Thiopyridine}
\]

**Preparation of sample from blood:** A suspension of the whole blood cells in ice cold 0.1 M sodium phosphate buffer (pH 7.4) was prepared, using a glass-Teflon homogenizer. The dilution was 1:240 (e.g. 5 μl blood into 1200 μl buffer).
Reagents: (i) 0.5 M disodium phosphate buffer (pH 7.2), (ii) 20 mM sodium phosphate buffer (pH 7.4), (iii) Acetyl-CoA, (iv) choline chloride, (v) neostigmine sulphate, (vi) 4,4'-dithiopyridine, (vii) 3 M NaCl, (viii) 1.1 mM EDTA, and (ix) 0.01 N HCl.

Procedure: The reaction mixture contained 200 μl of the suspension and 25 μl each of the following reagents: 0.5 M sodium phosphate buffer (pH 7.2), acetyl-CoA (96.2 x 10^{-3} M), choline chloride (1 M), neostigmine sulfate (7.6 x 10^{-4} M), NaCl (3 M) and EDTA (1.1 x 10^{-3} M). The volume was adjusted with distilled water so that after the reaction of the enzyme, the final volume was 0.5 ml. The reaction mixture without the enzyme was pre-incubated in centrifuge tubes in a water bath at 37 °C for 20 min. The reaction was stopped by boiling the tubes in a water bath for 2 min, and 1 ml of oxygen-free distilled water was added to the tubes. One ml of this mixture was added to a tube containing 50 μl of 4,4'-dithiopyridine (10^{-3} M). The absorbance at 324 nm was measured employing a spectrophotometer after 15 min. Protein was estimated following the procedure of Lowry et al. (1951).

Preparation of brain tissue: The tissues were weighted and homogenized in ice-cold 0.1 M sodium phosphate buffer (pH 7.4) to obtain a 5% homogenate, using a glass-Teflon homogenizer. The resulting supernatant from centrifugation at 7500 x g for 30 s in a Hermle micro-centrifuge was used for the assay immediately.

Procedure: The final volume of the assay mixture was made to 0.5 ml with D/W. The reaction mixture without the enzyme was pre-incubated in a water bath at 37 °C for 20 min. Stopped the reaction by boiling in a water bath for 2 min and added to this 1 ml of oxygen-free distilled water and 50 μl of 4,4'-dithiopyridine (10^{-3} M). The absorbance at 324 nm was measured employing a spectrophotometer after 15 min, and the enzyme activity is calculated from the extinction value of 4-thiopyridine as 1.98 x 10^4. MAO activity is expressed as nmol of 4-hydroxyquinoline formed/h/mg protein. Albumin replaced the enzyme sample in the control reaction.

Determination of monoamine oxidase (MAO; EC: 1.4.3.4) activity

Principle: MAO is a deaminating flavin-containing enzyme. Total MAO and MAO-B activities were estimated in isolated blood platelet and brain mitochondrial (P2) fraction.
Fluorimetric assay procedure of Morinan and Garratt (1985) was used for the assay of MAO activity using kynuramine as the enzyme substrate, the product of which could be measured employing fluorimetry. The amount of 4-hydroxyquinoline, which fluoresces at alkaline pH, formed due to oxidation of kynuramine by MAO represents the total enzyme activity. For determining MAO-B activity, the MAO-A inhibitor clorgyline was used in the assay mixture. The excitation wavelength of 315 nm and an emission wavelength of 380 nm were used.

**Reaction:** The reaction for monoamine oxidase (MAO) assay is as follows:

\[
\text{MAO} \quad C-CH_jCH_jNH_j \quad * \quad 2HBr \\
\text{Alkaline pH} \\
(Kynuramine) \quad \text{\rightarrow} \quad \text{4-hydroxyquinoline}
\]

**Preparation of standard curve:** A standard curve for 4-hydroxyquinoline is prepared at a range of 1-10 nmol/ml.

**Stock standard:** Dissolved 1.45 mg of 4-hydroxyquinoline in 10 ml of 10 mM potassium phosphate buffer.

**Isolation of platelets rich fraction:**

Blood platelets from both human and animal samples were isolated by the procedure of Christopher et al. (2006). In this process, the collected blood was centrifuged at 200 x g for 10 min to obtain platelet-rich-plasma (PRP). The PRP was centrifuged at 3000 x g for 25 min at 4 °C. The supernatant fraction was discarded and the pellets were washed twice using PBS. After the wash, the pellets were resuspended in potassium phosphate buffer (10 mM, pH 7.2), and sonicated until the tissue is disrupted evenly. Samples were stored at -80 °C until the assays were performed.

**Reagents:** (i) 10 mM potassium phosphate buffer, pH 7.2, (ii) Kynuramine (1 mg/ml, 3.07 mM dissolved in buffer), (iii) Clorgyline (10 mM) was prepared in the phosphate buffer, (iv) 0.4 M HClO₄, (iv) 1.0 N NaOH.
**Procedure:** One hundred μl of isolated platelet fraction was incubated with 770 μl of phosphate buffer for 5 min at 37 °C. To this tube 100 μl of clorgyline was added and incubated for 15 min at 37 °C. After incubation, 30 μl of kynuramine was added and again kept for 15 min at 37 °C. The reaction was terminated at 15 min, by the addition of 300 μl of ice-cold 0.4 N perchloric acid. The mixture was kept for 20 min on ice, and centrifuged at 10,000 x g for 5 min. Two ml of 1 N NaOH was added to one ml supernatant of the reaction mixture. Fluorescence intensity was measured at excitation/emission wavelengths of 315/380 nm. The specific enzyme activity is expressed as nmol of 4-hydroxyquinoline formed/h/mg protein. A standard curve was obtained for commercially available 4-hydroxyquinoline.

**Brain tissue:** Mitochondrial P₂ fraction from rat brain was prepared by the method of Whittaker (1971). A 10% homogenate of the whole brain was prepared using a glass-Teflon homogenizer in ice-cold sucrose (0.32 M, in 10 mM potassium phosphate buffer, pH 7.2). The homogenate was centrifuged at 1000 x g for 10 min at 4 °C using Sorvall centrifuge. The supernatant fraction was again centrifuged at 10,000 x g for 30 min. The pellets were collected and resuspended in ice-cold 50 mM Tris in 10 mM potassium phosphate buffer (pH 7.4) as 1:1 (v/v). The suspension was centrifuged at 10,000 x g for 30 min. The resulting pellets were resuspended the same buffer (same volume), and kept over night at -20 °C. The suspension was sonicated and used for assay the next day.

**Procedure:** Mitochondrial P₂ fraction (100 μl) was incubated with 770 μl of phosphate buffer for 5 min at 37 °C. To this tube 100 μl of clorgyline was added and incubated for 30 min at room temperature. After incubation, 30 μl of kynuramine was added and again kept for 15 min. The reaction was terminated by addition of 300 μl of ice-cold 0.4 N perchloric acid. The mixture was centrifuged (10,000 x g for 5 min) and 2 ml of 1 N NaOH was added to one ml supernatant of the reaction mixture. Fluorescence intensity was measured at excitation/emission wavelengths of 315/380 nm. The specific enzyme activity is expressed as nmol of 4-hydroxyquinoline formed/h/mg protein. A standard curve was obtained for commercially available 4-hydroxyquinoline.
Hcy and GSH estimation:

**Principle:** HPLC uses a combination of high pressure with small particle diameter, which allows high resolution of the components. The C-18 reverse-phase column was used that provided hydrophobicity to the stationary phase and thus when the polar solvent is used as the mobile phase, interested samples can be separated according to their relative hydrophobicity, i.e., non-polar solvents elute later. The detection was performed using an electrochemical detector. The sample components separated in the column is oxidized at the glassy-carbon working electrode that is kept at a constant potential of +0.85 V and the integrator records the current generated. Standards of all the biogenic amines were run prior to and following the sample injections on every day of analysis.

**Preparation of sample:** Fasting blood samples from patients and healthy volunteers were collected into EDTA-tubes and plasma separated out after centrifugation at 400 x g for 15 min at 4 °C. For determination of total Hcy and total GSH, 50 µl of a solution (containing 1.43 M sodium borohydride, 1.5 mM EDTA, 66 mM NaOH and 10 µl n-amyl-alcohols) was added to 200 µl of plasma, and incubated at 40 °C in a water bath for 30 min (Melnyk et al., 1999). Precipitation of plasma proteins were performed by addition of 250 µl of ice-cold 0.4 M perchloric acid and after centrifugation at 18,000 x g for 15 min at 4 °C. The supernatant was filtered through 0.2 µm membrane filter (Merck, Mumbai, India) and 20 µl of the sample was injected into the HPLC system coupled with an electrochemical detector.

**Procedure:** The mobile phase consisted of 50 mM sodium phosphate monobasic, 1.0 mM OSA, 2% acetonitrile (v/v); and pH adjusted to 2.7 with 85% ortho-phosphoric acid. The flow rate was 0.7 ml/min and the electrochemical detection was performed at 850 mV (Melnyk et al., 1999). The HPLC system consisted of an isocratic pump (Bioanalytical Systems, West Lafayette, USA), an amperometric detector (Epsilon, Bioanalytical Systems) and C-18, ion pair, analytical column (4.6 x 250 mm; Ultrasphere IP; Beckman, USA), with a particle size of 5 µm and pore size of 80Å. The values were calculated against standards containing 20 pmol/20 µl of Hcy and GSH. A representative HPLC chromatogram of the standard Hcy and GSH levels in the plasma of normal subjects, without HE, and HE patients are provided in Fig 6A-D.
Fig. 6. HPLC profile of commercial standards of Hcy & GSH. The thiols were separated on a C$_{18}$ reverse phase ion-pair column (250 x 4 mm) and detected by electrochemistry. (A) Standard Hcy and GSH had retention time of 12.2 min, and 13.5 min, respectively. (B) Perchloric extract of a sample of blood plasma in normal subjects. (C and D) Perchloric extract of samples of blood plasma from hepatic encephalopathy. (C) Without hepatic encephalopathy. (D) Hepatic encephalopathy.

A representative HPLC-ECD chromatograms of the standard Hcy and reduced glutathione levels in the plasma of control and TAA treated-rats are provided in Fig. 7A-D.
Fig. 7. HPLC-ECD chromatograms of commercial standard and rat plasma Hcy and GSH: Employing C\textsubscript{18} reverse phase column (250 x 4. mm) GSH and Hcy were separated. (A) Standard Hcy and GSH had a retention time of 11.5, and 13.0 min, respectively. (B) Perchloric extract of a sample of blood plasma in control rats. (C and D) Perchloric extract of samples of blood plasma from TAA treated rats. (C) Lower dose, (D) higher dose of TAA.

HPLC analysis of dopamine, serotonin and their metabolites

**Principle:** The detection was performed using an electrochemical detector. The sample components separated in the column is oxidized at the glassy-carbon working electrode that is kept at a constant potential of + 0.74 V and an integrator recorded the current generated. Standards of all the biogenic amines were run prior to and following the sample injections on every day of analysis.

**Preparation of sample:** Animals were sacrificed on the 4\textsuperscript{th} day following i.p. injection of TAA. Whole brain from rats were dissected out within sixty seconds and rinsed in chilled normal saline; blotted dry on ash-free filter paper and the NCP, NRD were micro-punched (Palkovits and Brownstein, 1983) and cortex and hippocampus were dissected out separately and processed for the analyses of DA employing an HPLC-electrochemical procedure (Muralikrishnan and Mohanakumar, 1998).
Reagents: (i) 0.1 M HClO₄, (ii) EDTA, (iii) Mobile phase [8.65 mM heptane sulphonic acid, 0.27 mM EDTA, 13% acetonitrile, 0.4-0.45 % triethylamine and 0.32 - 0.35 % phosphoric acid (v/v)].

Procedure: The tissue was sonicated (50 Hz for 30 s) in ice cold 0.1 M HClO₄ (1 mg/10 µl of HClO₄) containing 0.01% EDTA. Ten µl supernatant collected after a spin of 10000 x g for 5 min (Hermle, Germany) was injected into the HPLC system for the analysis. The mobile phase consisted of 25 mM citric acid, 125 mM sodium phosphate, 100 mg/L EDTA and 30 mg/L octane sulfonic acid, pH adjusted to 2.5 with 85% ortho-phosphoric acids. Flow rate was maintained at 0.7 ml/min and the electrochemical detection was performed at +740 mV. The HPLC system consisted of an isocratic pump (Bioanalytical Systems, West Lafayette, USA), an amperometric detector (Epsilon, Bioanalytical Systems) and C18, ion pair, analytical column (4.6 x 250 mm; Ultrasphere IP; Beckman, USA), with particle size of 5 µm and pore size of 80 Å.

Electrophoresis

Determination of AChE activity by poly-acryl amide gel electrophoresis (SDS-PAGE)

Principle: Electrophoresis allows the separation of charged molecules in an applied electric field. The relative mobility of individual molecules depends on several factors like net charge, charge/mass ratio, molecular shape and temperature. Proteins are denatured in the presence of SDS and β-ME. SDS coats proteins with a uniform layer of negative charges, which causes them to migrate towards anode when placed in an electric field, regardless of the net charge of the uncomplexed proteins. This charge-density is independent of the mass of the polypeptide, thus the mobility of SDS-protein complexes in polyacrylamide gels depends on their mass. Once proteins are separated by SDS-PAGE they can be transferred to nitrocellulose/PVDF membranes by electro-transfer and stained with specific antibodies.
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**Preparation of samples:** We have taken 100 µl of whole blood in Eppendorf tube and added 100 µl of phosphate buffer (pH 8.0). The samples were mixed and sonicated for 10 s. The samples were mixed with sampling buffer [containing 50% (w/v) sucrose and 0.01% (w/v) bromophenol blue] in the ratio 1:200 (10 µl of samples and 190 µl of buffer).

**Preparation of gel:** 7.5% slab gels with 0.5% Triton X-100 in glycine/Tris buffer 50 mM at pH 8.5.

**Procedure:** To study the activity of AChE in the whole blood suspension, we used PAGE with Triton X-100 under non-denaturing conditions (Filomena et al., 2005) and specific staining for AChE activity (Kamovsky and Roots, 1964). The gel was pre-run at 75 V for 1 h at RT using a Mini Protean® 3 Cell (vertical gel electrophoresis apparatus; Bio-Rad). All the samples to be analyzed were loaded onto the gel in a volume of 5 µl per well (containing 25 µg of protein). The human erythrocytes AChE standard was loaded onto the gel as positive control. The gel was run at 100 V for 3 h in running buffer [containing 50 mM of glycine/Tris, pH 8.1 with 0.5% (v/v) Triton X-100]. The gel was incubated with acetyl thiocholine 2 mM, sodium citrate 5 mM, copper sulfate 3 mM and potassium ferricyanide 5 mM in 67 mM phosphate buffer at pH 6.1. The gel was gently shaken in an orbital shaker at RT for 2 h, and the bands appeared were photographed.

**Identification of AChE and ChAT protein by immunoblot**

**Preparation of samples:** One hundred µl of whole blood was added to 100 µl of radio-immunoprecipitation assay (RIPA) buffer (pH 8.0), mixed, incubated on ice for 30 min, sonicated 10 s and centrifuged at 12,000 x g for 10 min. The supernatant was mixed with gel loading dye, boiled for 5 min and stored in -20°C till further analysis.

**Preparation of gel:** The separating with stacking gels was prepared as shown in Table 1.

**Procedure:** The gel was run in 0.25 M Tris with 1.9 M glycine, 0.01 M EDTA and 0.017 M SDS at 80 V for the stacking gel and 100 V for the running gel, for approximately a total of 75 min. Following the run, proteins were transferred to a PVDF membrane at a
constant voltage of 50 mV for 1 h using a Mini Tran-Blot® Electrophoretic Transfer Cell (Bio-Rad) wet transfer system. The membrane was removed, washed twice in TBST (5 min each) and blocked for 1 h in 10% non-fat milk (skimmed milk powder) prepared in TBST. After that it was again washed twice in TBST solution. Blots were subsequently incubated with the AChE or ChAT primary antibody (Mouse monoclonal IgG for AChE and goat polyclonal IgG for ChAT) at a dilution of 1:1000 in 10% non-fat milk under gentle shaking at 4 °C overnight. Next day, the blots were washed with TBST and incubated with the conjugated horse-raddish-peroxidase-linked (HRP) secondary antibody (anti-mouse and anti-goat IgG) at a dilution of 1:1000 for 2 h at room temperature in 5% non-fat milk. The blots were washed and developed with diaminobenzidine (DAB) solution containing H₂O₂. The blots were stripped and re-probed with mouse monoclonal anti β-actin (1:2000) in TBST containing 5% skimmed milk overnight at 4 °C and processed in the same way as before. The blots were air dried; scanned and densitometry analyses were carried out by employing Quantity One software (BioRad, USA).

Behavioral analyses in rats:
Male Sprague Dawley rats administered with two different doses of TAA (100 and 200 mg/kg) for three consecutive days were assessed for any changes in their normal behaviours. Animals receiving the higher dose showed distinct behavioral abnormalities from third day which became maximum by 4th day, 24 h following the last injection. Animals were almost unable to move; hind limbs were mostly affected with complete loss of toe tonus and toe spread. Those animals which could move at this stage usually dragged the whole body with the help of their fore limbs.

In general, the rats were placed individually in a transparent Perspex boxes (45 cm diameter, 40 cm height). Following a 2-min acclimatization the following tests were
conducted. Each animal was allowed to take rest for 5 min in between two experiments. At the end of each session, the concerned instruments were cleaned thoroughly with a swab of 70% alcohol to remove any dirt and door.

Gait analysis
Gait abnormalities of the control and TAA-induced rats by placing them in an inclining gangway as described by Klapdor et al. (1997). In short, rats with their fore- and hind-limbs painted with two different non-toxic water colors were placed on the small gangway. The test was conducted twice for each animal per session. They were pre-trained before the injection to walk up the gangway into a dark compartment, placed at the end of the gangway, where a food pellet was kept. The time taken to walk up the gangway and the foot prints, taken on white papers lined in the gangway, were recorded and analyzed as reported (Klapdor et al., 1997; Chandra et al., 2006; Pandey et al., 2007). The tests were conducted in a silent, properly illuminated and temperature controlled (21 ± 2 °C) animal experimentation room.

Akinesia
The akinesia was measured by noting the latency in seconds (s) of the animals to move all four limbs and the test was terminated if the latency exceeded 180 s. Each animal was initially acclimatized for 5 min on a wooden elevated (1m) platform (40 cm x 40 cm) for measuring akinesia in rats. Using a stopwatch, the time (s) taken by the animal to move all the four limbs was recorded (Mitra et al., 1992). This exercise was repeated three times for each animal (Weihmuller et al., 1989; Mitra et al., 1992; Muralikrishnan and Mohanakumar, 1998).

Exploratory activities
Animals were tested for any disturbance in their motor activity employing a Plus-Maze placed at an elevated position. The number of entries in the open and closed arms was recorded for 5 min. Total number of entries into either type of arm indicated overall motor activity of the animals.
Treadmill test

Rotarod test was performed to measure the ability of the animal to maintain balance on a continuously rotating rod by coordinating movements of its four feet and making necessary postural adjustments (Rozas et al., 1997). Adaptation to the rotarod was commenced one day before the first injection. The recording began from the day 1 (before injection) and continued till 5th day. The treadmill constantly rotated at a speed of 15 rpm and the time rats could stay on the rotarod (latency) was recorded. The rotarod scores of rats were recorded as average of two separate trials at 5 min interval.

Histological analysis

Liver tissue

Rat liver sections were stained with hematoxylin-eosin, to analyze nuclear morphology of hepatocytes.

Brain tissue

Brain sections were stained for AChE activity by employing the method of Karnovsky and Roots (1964).

Principle: The basis of this method is that the thiocholine liberated by the enzyme reaction is believed to reduce ferricyanide to ferrocyanide. The latter reacts with copper ions to form an insoluble ferrocyanide. The copper in the medium competes with the citrate ions to prevent the formation of copper ferricyanide (Karnovsky and Roots, 1964).

Reactions: The reaction for acetylcholinesterase stain is as follows:

\[
\text{AChE} \\
\text{Acetyl thiocholine iodide} + \text{Ferricyanide} \rightarrow \text{Thiocholine ferrocyanide} \\
\text{Ferro cyanide} + \text{Cu}^{++} \rightarrow \text{Copper ferrocyanide (insoluble pinkish brown precipitation)}
\]
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Reagent: Acetyl thiocholine substrate solution mix was prepared by dissolving 5 mg of acetyl thiocholine iodide in 6.5 ml of 0.1 M phosphate buffer (pH 6.0). The following solutions were then added to the above solution in the order mentioned, stirring well after each addition. All the components are mixed very fast and added to the sections as quickly as possible:

- 0.1 M sodium citrate: 0.5 ml
- 30 mM copper sulphate*: 1.0 ml
- Double distilled water: 1.0 ml
- 5 mM potassium ferricyanide*: 1.0 ml

* Prepared freshly every time

Procedure: Selected free-floating sections (20 μm) were washed thrice with 0.1 M phosphate buffer (pH 6.0) to remove unwanted tissue debris, if any. The substrate solution mix (clear greenish in color) was poured very carefully in a petri plate containing the sections and incubated for 45 min at 37 °C. The sections were washed thrice in DDW, mounted on a lysine-coated slide and air dried. Later the sections were dehydrated in gradation of alcohol and finally mounted in DPX.

Haematoxyline and Eosin staining (Luna, 1968)

Principle: The oxidation product of haematoxyline is haematin, and is the active ingredient in the staining solution. Haematoxylin is not classified as a dye since the molecule possesses no chromophore. The in situ oxidation of haematoxyline is effected by the addition of a strong oxidant to the stain, in this case sodium iodate.


Preparation of haematoxyline solution: Prepared by dissolving 1 g of haematoxyline solution in 10 ml of ethyl alcohol. Potassium / ammonia alam (20 g) was dissolved in 200 ml of double distilled water and boiled. To this haematoxyline solution was added
and again boiled for 30 min. Mercuric (0.5 g) was added to this and cooled this solution rapidly. A few drops of glacial acetic acid were added to this solution to keep away metallic cluster.

**Preparation of Eosin solution:** Two gram of eosin powder was dissolved in 15 ml of alcohol solution.

**Procedure:**

**Fixation, Processing and Sectioning:**

The right lobe of rat liver was immediately fixed by immersion in 3.7% buffered formaldehyde in 0.1 M phosphate buffer, pH 7.2 for 24 h. Following fixation, the tissues were thoroughly washed with distilled water to remove all traces of blood or other tissues. The tissue was dehydrated by passing successively through upgrades of ethanol, cleared in xylene. Small pieces of the tissue were embedded in a cryomix, to form blocks and cut at -24 °C in the chamber of the cyrotome (Thermo, Shandon Cryotome E). Twenty μm sections were cut and transferred the sections on glass slides coated with poly-L-lysine.

**Staining:** Sections were dried and then kept at 4 °C. Before staining they were rehydrated through gradations of alcohol and then stained with haematoxyline, and dehydrated with gradations of alcohol. After 100% alcohol, the sections were immersed in to 1% eosin solution for 30 s. Excess stain was removed with ethanol and the slides were kept in xylene for 5 min, and mounted in DPX mountant. The sections were viewed under bright field using a Zeiss stereomicroscope and photographed using a SLR camera.
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Haematoxyline and eosin staining protocol:

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<tbody>
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<td>1</td>
<td>Absolute alcohol</td>
<td>5 min</td>
<td>9</td>
<td>70% alcohol</td>
<td>5 min</td>
</tr>
<tr>
<td>2</td>
<td>90% alcohol</td>
<td>5 min</td>
<td>10</td>
<td>90% alcohol</td>
<td>5 min</td>
</tr>
<tr>
<td>3</td>
<td>70% alcohol</td>
<td>5 min</td>
<td>11</td>
<td>100% alcohol</td>
<td>5 min</td>
</tr>
<tr>
<td>4</td>
<td>50% alcohol</td>
<td>5 min</td>
<td>12</td>
<td>Eosin</td>
<td>30 s</td>
</tr>
<tr>
<td>5</td>
<td>Water</td>
<td>5 min</td>
<td>13</td>
<td>Absolute alcohol</td>
<td>wash</td>
</tr>
<tr>
<td>6</td>
<td>Haematoxyline</td>
<td>5 s</td>
<td>14</td>
<td>Xylene</td>
<td>3 min</td>
</tr>
<tr>
<td>7</td>
<td>Water</td>
<td>5 min</td>
<td>15</td>
<td>DPX</td>
<td>Mount</td>
</tr>
<tr>
<td>8</td>
<td>50% alcohol</td>
<td>5 min</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Statistics
Student’s t-test was used for finding statistically significant data comparing two means, on parametric data. Microsoft Excel programs were used for the analyses. A probability of \( p \leq 0.05 \) was considered be significant. Error bars represent standard error of means (S.E.M.). For non-parametric data, such as behavioral scores or neurological scores one-way Analysis of Variance (ANOVA) was performed, followed by post-hoc analyses using the Pearson’s-test.
Annexure I.

The sheet containing the format of consent for the patients.

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AND CALCUTTA NATIONAL MEDICAL COLLEGE & HOSPITAL, 32, Golaband Road, Kolkata-700 014.

Permission Letter based on the Information supplied by the Candidate concerned/Declaration of the Patient:

I ___ I am whole-heartedly willing to join for this Research work. I have read the above proposal with full knowledge.

Signature/Thumb impression/Date:
Chapter III
Materials & Methods

References


INSA (Indian National Science Academy) Guidelines for care and use of animals in scientific research, INSA Publication, New Delhi, 2000;1-26.


Chapter III
Materials & Methods


Mralkrishnan D, Mohanakumar KP. Neuroprotection by bromocriptine against 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced neurotoxicity in mice. FASEB J 1998;12:905-12.


Chapter III
Materials & Methods


