Materials and Methods
Materials and Methods

Materials

An inbred strain of Swiss albino mice (Mus musculus), reared and maintained in the animal house of the Department of Zoology, (under the supervision of The Animal Welfare Committee) University of Kalyani served as the materials for the present study. Mice were provided food and water ad libitum. The food was generally made up of wheat, gram and powdered milk without any animal protein supplement, unless mentioned otherwise.

Experimental Design

In the present study formation of hepatic liver nodules and the subsequent hepatocarcinoma the chronic dietary feeding method used by several workers (Daoust and Cantero 1959, Daoust and Molnar 1964; Ogawa et al 1974; Kitagawa et al 1979) was adopted. The azodye p-dimethylaminoazobenzene (p-DAB) was chronically fed followed by chronic feeding of phenobarbital (PB) with water. For the cytogenetical (bone marrow chromosome aberration, micronucleated erythrocytes, mitotic indices, sperm with abnormal head shapes) and biochemical (lipid peroxidases, glutamate oxaloacetate transaminase, glutamate pyruvate transaminase, acid phosphatase, alkaline phosphatase, protein qualitative and quantitative) studies, which form the major part of the present investigation, extensive experiments have been conducted by including the data on six different fixation intervals, namely, 7, 15, 30, 60, 90 and 120 days maintaining suitable controls. However, for the works of preliminary nature as for example histology, scanning electron microscopy, transmission electron microscopy, p53 and Bax proteins either only a single fixation interval or two with lesser number of control and treated series have been done with a view to determining further direction in future studies. The limited number of experimentations performed in these pilot studies is mainly due to the lack of resources and infra-structural facilities in the department where the main bulk of the studies have been carried out.
Control and treatment series

For the cytogenetical and biochemical studies, healthy mice weighing between 18 and 25 grams (about two months old) were chosen at random for setting up the experimental series for chronic feeding of the azodye p-dimethylaminoazobenzene (p-DAB, initiator) and phenobarbital (PB) promoter. In general, for the shorter fixation intervals (7, 15 and 30 days), 5 mice each were used while for the longer fixation intervals (60, 90 and 120 days) 10 mice each were used for each series for each fixation interval.

Negative control (NORMAL)

A set of 30 healthy mice fed with normal diet were maintained and five each of them were sacrificed at each of the six fixation intervals for studying their cytogenetical and biochemical features selected as endpoints in the present study. This group of mice served as negative controls.

Phenobarbital series (PB)

For the shorter fixation intervals a group of 15 healthy mice were fed 0.05% phenobarbital solution dissolved in water right from the onset of the experiment, along with normal diet ad libitum. Of these five mice each were sacrificed at each of the three shorter fixation intervals. For the longer fixation intervals all together 30 mice were used 10 each for each fixation interval.

p-DAB (D) fed series

0.06% p-dimethylaminoazobenzene (p-DAB) [SIGMA, D-6760] was mixed with diet and fed to 45 mice along with distilled water. Of these 5 mice each were sacrificed at each of the shorter fixation and 10 mice were sacrificed at each of the longer fixation intervals.

p- DAB (D) +PB (PB) fed series

5 mice each for the shorter fixation intervals and 10 mice each for the longer fixation intervals were fed both 0.06% p-DAB and 0.05% PB chronically till sacrificed.

p- DAB (D) +PB (PB) +Chelidonium mother fed series

5 mice each for the shorter fixation intervals and 10 mice each for the longer fixation intervals were fed both 0.06% p-DAB and 0.05% PB plus 0.06% mother tincture of Chelidonium.

p- DAB (D) +PB (PB) +Chelidonium-200 (CHELI-200) fed series

5 mice each for the shorter fixation intervals and 10 mice each for the longer fixation intervals were fed both 0.06% p-DAB and 0.05% PB along with 0.06 ml of the stock solution of the
Materials and Methods

drug-CHELI-200 twice a day (6 A.M, 6 P.M.) all along till they were sacrificed at all fixation intervals starting from 7 days through 120 days

**p-DAB (D) +PB (PB) +Chelidonium-30 (CHELI-30) fed series**
This set of mice were given p-DAB and PB in the same way as that of the previous group but instead of CHELI-200 they were fed CHELI-30 thrice a day (6 A.M, 12 Noon, 6 P.M) from the first day onward of p-DAB feeding for seven days, and then twice a day till they were sacrificed (as per general practice rule of homeopathic medicines in chronic toxicity cases).

**p-DAB (D) +PB (PB) +Chelidonium-200 (CHELI-200) +Carcinosin 200 (CAR-200) fed series**
5 mice each for the shorter fixation intervals and 10 mice each for the longer fixation intervals were fed both 0.06% p-DAB and 0.05% PB along with 0.06 ml of the stock solution of CHELI-200 and CAR-200 twice a day in the same way as described in the previous sets.

**p-DAB (D) +PB +Carcinosin 200 (CAR-200) fed series**
5 mice each for the shorter fixation intervals and 10 mice each for the longer fixation intervals were fed both 0.06% p-DAB and 0.05% PB along with 0.06 ml of the stock solution of the drug Carcinosin-200 twice a day all along till they were sacrificed at the said intervals.

**p-DAB (D) +PB + Alcohol (ALc) fed series**
This group of mice conformed the materials for the positive control (Ethyl alcohol being the "vehicle" of all potencies of Chelidonium (namely, Chelidonium mother, Chelidonium-30, Chelidonium-200) and Carcinosin-200. This group consisted of 5 mice each for the shorter fixation intervals and 10 mice each for the longer fixation intervals and the feeding of p-DAB and PB was followed exactly as described in the previous groups but instead of CHELI-30 or CHELI-200, 0.06 ml of diluted alcohol (0.06 ml of 90% ethyl alcohol diluted with 20 ml of double distilled water) was fed.

**p-DAB (D) +PB (PB) + Vitamin-C (VC) fed series**
5 mice each for the shorter fixation intervals and 10 mice each for the longer fixation intervals were fed both with 0.06% p-DAB and 0.05% PB and additionally fed 0.06 ml 1% Vitamin C (L-ascorbic acid, Product code 0149100, SISCO Research Laboratories).

**p-DAB (D) +PB (PB) + Vitamin-C (VC) + Chelidonium-200 (CHELI-200) fed series**
This group of 5 mice each for the shorter fixation intervals and 10 mice each for the longer fixation intervals were fed p-DAB, PB and VC similar to the previous set except that this group was additionally fed with Chelidonium-200 in doses as mentioned above.
Materials and Methods

*p- DAB (D) + PB (PB) + Vitamin-C (VC) + Carcinosin-200 (CAR-200) fed series*

This group of 5 mice each for shorter fixation intervals and 10 mice each for the longer fixation intervals were fed p-DAB, PB, vitamin C exactly in similar way to the previous set but here Chelidonium-200 was replaced with Carcinosin-200.

*p- DAB (D) + PB (PB) + Alcohol (Alc) + Vitamin-C (VC) fed series*

5 mice each for the shorter fixation intervals and 10 mice each for the longer fixation intervals were fed 0.06% p-DAB, 0.05% PB along with 0.06 ml of diluted alcohol and 0.06 ml of 1% vitamin C.

Selection of tissues

Cytogenetical

For cytogenetical preparations, bone marrow cells were processed for observation of aberrations in somatic metaphase chromosomes and induction of micronuclei and mitotic index. For sperm head anomaly study testis from males were processed.

Biochemical, Histological and Electron Microscopy

Three organs namely liver, kidney and spleen were selected for biochemical studies. For histological and electron microscopy (both SEM and TEM), only liver, being the major target of p-DAB and PB in production of hepatocarcinoma, was considered for the preliminary study.

Experimental Protocols

The following protocols were used for the present study.

Cytogenetical

- Bone marrow chromosome aberration (BMCA) study
- Study of micronucleated erythrocytes (MNE)
- Sperm head anomaly study (SHA)
- Mitotic Index (MI)

Biochemical

- Lipid peroxidase (LPO)
- Glutamate oxaloacetate transaminase (GOT)
Materials and Methods

- Glutamate pyruvate transaminase (GPT)
- Acid phosphatase (ACPP)
- Alkaline phosphatase (ALKP)
- Quantitative estimation of total protein.
- Gel electrophoretic studies of total protein.

Methods

Cytogenetical studies

Study of Chromosomal Aberrations

Colchicine pre-treatment

Mice at all fixation intervals were intraperitoneally injected with 0.03% colchicine @ 1 ml per 100 gram body weight, 1 hour and 30 minutes before sacrifice.

Processing for somatic chromosomes

Treatment with hypotonic solution

- Marrow of the femur was flushed in 1% sodium citrate (hypotonic) solution, and brought into suspension by repeated flushing in and out of a pipette with rubber tilt and incubated for 7-10 minutes at 37°C
- The materials were centrifuged for 6-8 minutes at 6000g and the supernatant was discarded.
- The materials collected at the bottom of the centrifuge tube were fixed in freshly prepared Carnoy’s fixative (1 part acetic acid : 3 parts methanol) and resuspended by flushing.
- By centrifugation and decantation the fixative was changed twice at an interval of 20 minutes

Preparation of slides

- Materials were dropped with the aid of a pipette from a distance of 1-1½ feet above on clean grease-free slides pre-chilled in 50% alcohol (slides kept in 50% alcohol in a freezing chamber of a domesticated refrigerator).
- The slides were air-dried by keeping in a slanting position and were flame dried by touching on to a flame and allowing the alcohol and fixative to burn out and air dry.
Staining procedure

Ordinary Giemsa staining

The slides were stained with Giemsa for scanning of suitable metaphase spreads in the following way:

Preparation of Giemsa stock solution

Reagents used

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Giemsa powder (E-Merck, Germany)</td>
<td>1 gm</td>
</tr>
<tr>
<td>Glycenn (E-Merck, Germany)</td>
<td>66 ml</td>
</tr>
<tr>
<td>Methanol</td>
<td>66 ml</td>
</tr>
</tbody>
</table>

Procedure

- 1 gm Giemsa powder was mixed thoroughly with 66 ml of glycenn by mortar and pestle.
- Then the mixture was incubated at 60°C for 2 hr
- After cooling, 66 ml of methanol was added to it and filtered.
- The solution was kept in refrigerator for maturation.
- After 15 days this was used as stock staining solution.

Preparation of Giemsa working solution

Phosphate buffer stock solution

Solution A: 3.12 gm Sodium di-hydrogen phosphate 2-hydrate (NaH$_2$PO$_4$, 2H$_2$O) dissolved in 100 ml of distilled water.

Solution B: 3.56 gm di-Sodium hydrogen phosphate (Na$_2$HPO$_4$) dissolved in 100 ml of distilled water

Phosphate buffer working solution (pH 6.8)

51 ml of solution A was mixed with 49 ml of solution B to make working buffer solution at pH 6.8. This was made fresh at the time of use.

Final staining solution

1 ml of Giemsa stock solution was diluted with 20 ml of buffer solution to make the working stain solution.

Procedure of staining

- Slides were kept on glass rods in a horizontal position and flooded with the diluted Giemsa stain.
- After about 45 minutes, the slides were rinsed in tap water and air dried.
Materials and Methods

Then they were scanned for suitable metaphase spreads.

Scoring of data

Different types of chromosomal abnormalities were grouped into "major types" (e.g. break, fragment, pulverisation, centnc fusion, translocation, terminal association, ring, polyploidy and aneuploidy) and "other types" (e.g. precocious centromeric separation, C-mitotic effect, stretching, stickiness, pycnosis, constriction etc.) in order to make qualitative analysis of data more meaningful. The "major types" include the more deleterious and difficult-to-restitute aberrations. Percentage of frequencies of different types of aberrations in each series was scored.

Statistical analysis of chromosomal abnormalities

The differences in the frequencies of different types of aberrations between different experimental series were critically analyzed by Student's t-test. The level of significance was tested according to Fisher and Yates statistical tables (see Fisher & Yates 1963; Samueles 1989; Bailey 1995; Bland 1995, Strickberger 1996).

Study of Micronucleated Erythrocytes

Preparation of slides

- Bone marrow cells of control and treated series were smeared uniformly on clean grease free slides.
- Semidried slides were dipped in 90% ethyl alcohol briefly and allowed to air-dry.
- Air-dried slides were stained for 5 minutes in May-Grunwald-Giemsa stain as per the routine procedure by mixing 1 part of stock solution and 1 part of distilled water. The slides were rinsed in distilled water and finally stained in dilute Giemsa (1 part stock Giemsa and 10 part distilled water (Schmid 1976).

Composition of May-Grunwald stain

- May-Grunwald stain
- Methanol

Scoring of data

5000 erythrocytes from each individual were randomly observed (1000 cells from each slide and at least 3 slides from each individual). Percentages of MNE and abnormal nuclei among 3000 erythrocytes were scored.
Study of Mitotic Indices

Preparation of slides
The preparation of slides for mitotic index study of bone marrow divisional activity was done by the same method as described above for preparation of slides for micronuclei assay.

Study of Sperm Head Anomaly

Preparation of slides
- The epididymis of each side of the male mice was dissected out and taken separately into 5 ml of 0.87% normal saline. It was made free of fats, vas deferens and other tissues. The inner content of each side of the epididymis was taken out in normal saline and the material was thoroughly shaken to make the sperm free to suspend in saline solution. The sperm suspension was filtered through silken cloth to remove the debris and the filtrate was collected in a graduated tube, more saline was added to make the volume 10 ml.
- The sperm suspension thus collected, was put in the center of a clean slide over which 0.02 ml methanol was added
- The material was allowed to dry.
- A drop of distilled water diluted Giemsa stock solution (6:1) was put on the material.
- The material was covered with a cover glass and sealed temporarily for observation as per the routine procedure (Wyrobek 1984).

Scoring and analysis of the data
Percentage frequencies of occurrence of sperm with abnormal head shape in each series were scored and the differences in the frequencies between different experimental series were critically analyzed by Student's t-test. The observer was initially "blinded" as to the exact "treatment" or "control" series he was studying and the coded slides were later deciphered. A uniformity in scoring data of the "control" and the "treated" series was all along maintained.

Biochemical studies

Preparation of tissue samples

Collection of tissues
Three different organs/tissues, namely liver (L), spleen (S) and kidney (K) were dissected out from the adult specimens and were kept separately in petridish (-40°C) till homogenization. The wet weight of the tissues were measured and known amounts of tissue were taken after suitable trials and errors for perfect band resolution and biochemical assay.
Homogenisation and centrifugation
Different tissues, weighing between 25-100 mg were homogenized separately in 2.5-5 ml of
normal saline (0.9% sodium chloride solution), depending upon the amount of tissue. The
homogenized tissues were then spun in refrigerated centrifuge (REMI C 24 Model, India) at
3000g for 15 minutes at 0°C. After centrifugation the supernatant of the tissue extracts were
mostly used directly as samples or aliquotes or else in some cases were stored at -40°C till
they were used within the next 3 days (generally within a day for enzymatic studies).

These samples served as the stock solution for both analysis of the various enzymes,
quantitative and qualitative analysis of proteins.

Quantitative Estimation of Total Proteins
For the quantitative estimation of total protein the method of Lowry et al (1951) was followed

Preparation of sample for known (standard) protein
Stock Standard solution: 50 mg of Bovine Serum Albumin (BSA) (Sigma, USA) was
dissolved in double distilled water to make the volume up to 50 ml in a standard flask.

Working Standard solution: 10 ml of the stock solution was diluted with double distilled
water to make up a total volume of 50 ml in standard flask (Lowry et al 1951; see Plummer
1988).

Preparation of sample for unknown protein
As described before.

Preparation of reagents

0.1 N Sodium hydroxide (NaOH) solution

\[
\text{NaOH} \quad 1 \text{gm}
\]
\[
\text{Double distilled water} \quad 250 \text{ml}
\]

Alkaline Copper Solution
Reagent A: 2% Sodium carbonate (Na$_2$CO$_3$) in 0.1 N NaOH solution.

\[
\text{Na}_2\text{CO}_3 \quad 2 \text{gm}
\]
\[
0.1 \text{ N NaOH} \quad 100 \text{ml}
\]

Reagent B: 0.5% Copper sulphate (CuSO$_4$·5H$_2$O) in 1% Sodium potassium tartarate.
Materials and Methods

After suitable mixing, 20 mg of Sodium potassium tartarate was added to this solution to make the reagent B.

The 50 ml of reagent A was mixed with 1 ml of reagent B prior to use to make the alkaline copper solution finally.

**Folin-Ciocalteu reagent**

Commercial Folin-Ciocalteu reagent was diluted with double distilled water (1:1 v/v) on the day of use. This is a solution of sodium tungstate and sodium molybdate in phosphoric and hydrochloric acid.

**Procedure for estimation of protein**

**Estimation of known (Standard) protein**

Different concentrations of working solution of BSA were pipetted out into series of test tubes taking at least 3 replica for each concentrations. Then different reagents were mixed step by step as described in the following table.

<table>
<thead>
<tr>
<th>Conc. of BSA (µg/ml)</th>
<th>Sample BSA (ml)</th>
<th>0.1 N NaOH (ml)</th>
<th>Alkaline Copper solution (ml)</th>
<th>Folin-Ciocalteu solution (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Blank</td>
<td>1.0</td>
<td>5</td>
<td>0.5</td>
</tr>
<tr>
<td>10</td>
<td>0.1</td>
<td>0.9</td>
<td>5</td>
<td>0.5</td>
</tr>
<tr>
<td>20</td>
<td>0.2</td>
<td>0.8</td>
<td>5</td>
<td>0.5</td>
</tr>
<tr>
<td>30</td>
<td>0.3</td>
<td>0.7</td>
<td>5</td>
<td>0.5</td>
</tr>
<tr>
<td>40</td>
<td>0.4</td>
<td>0.6</td>
<td>5</td>
<td>0.5</td>
</tr>
<tr>
<td>50</td>
<td>0.5</td>
<td>0.5</td>
<td>5</td>
<td>0.5</td>
</tr>
<tr>
<td>60</td>
<td>0.6</td>
<td>0.4</td>
<td>5</td>
<td>0.5</td>
</tr>
<tr>
<td>70</td>
<td>0.7</td>
<td>0.3</td>
<td>5</td>
<td>0.5</td>
</tr>
<tr>
<td>80</td>
<td>0.8</td>
<td>0.2</td>
<td>5</td>
<td>0.5</td>
</tr>
<tr>
<td>90</td>
<td>0.9</td>
<td>0.1</td>
<td>5</td>
<td>0.5</td>
</tr>
<tr>
<td>100</td>
<td>1.0</td>
<td>0</td>
<td>5</td>
<td>0.5</td>
</tr>
</tbody>
</table>

After giving the different concentrations of sample BSA solution in each test tube, 0.1 N NaOH was added so that the total volume of each sample would be 1 ml. After mixing alkaline copper solution and Folin-Ciocalteu solution, the test tubes were shaken well till a bluish colour would appear in the solution. Then the tubes were allowed to incubate at room
Materials and Methods

Temperature in dark for at least 30 minutes. The sample containing no BSA was taken as the "blank" solution.

Estimation of unknown protein

0.1 to 0.5 ml of tissue samples ( aliquot) were pipetted out in test tubes keeping at least 3 replica. Then the other steps were followed as described in case of known protein sample.

Reading: The extinction (Optical Density – OD) was read at 750 nm against suitable blank in a spectrophotometer (Double-Beam UV-180 Spectrophotometer, Shimadzu, Japan).

Scoring of data

Preparation of standard curve

At least 3 replicated readings were taken for each concentration of BSA and the mean OD values were plotted against different concentrations of the standard samples prepared (10 µg/ml – 100 µg/ml). A linear curve was obtained which represented the standard curve.

Calculation of amount of protein for unknown sample

The concentration (µg/ml) of unknown protein was measured against the standard curve. The amount of protein was calculated as follows:

\[
\text{Amount of protein} = \frac{\text{Amount of NaCl} \times \text{Conc of protein} \times 10}{\text{Sample amount} \times \text{Weight of tissues}} \text{ mg/gm}
\]

Sample amount= 0.1-0.5 ml of homogenised tissue solution

Analysis of the data

The difference in amount of protein in a particular tissue of different series was statistically analyzed by Student's t-test.

Estimation of Lipid peroxidase

The colorimetric assay of Thiobarbituric acid (TBA) was performed by the method of Buege and Aust (1978) with some minor modifications.
Materials and Methods

Preparation of tissue samples

Different tissue samples viz. liver, kidney, spleen were dissected out from both treated and control mice sets and weighed 100mg, which was then homogenized in 4 ml of cold 1N NaOH. kept at 4°C and the above procedure was followed to obtain tissue aliquots.

Preparation of the reagents

Stock TCA-TBA-HCl: 15% w/v Trichloro Acetic acid (TCA, Merck, 822342) and 0.375% w/v TBA (SIGMA, T-5500) were dissolved in 0.25N hydrochloric acid. This solution was mildly heated to assist in dissolution of the TBA

- 1 ml sample homogenate containing 0.1-0.2 mg of protein was mixed thoroughly with 2 ml of TCA-TBA-HCl reagent in a centrifuge tube
- This resultant solution was heated for 15 minutes in a boiling water bath.
- After cooling the flocculent precipitate was removed by centrifugation at 1000g for 10 minutes
- The absorbance of the sample was determined at 535 nm against a "blank" that contains the reagent and double distilled water instead of the biological sample.
- The MDA (Malonaldehyde) concentration of the sample was calculated by using the extinction coefficient of 1.56×10^5 m^2 cm^-1

Estimation of Glutamate Oxaloacetate Transaminase (GOT)

The enzyme catalyses the transamination of α-ketoglutarate to L glutamate; in the process L-aspartate is converted to oxaloacetate and then spontaneously to pyruvate. Pyruvate may be estimated by the formation of the brown complex with dinitrophenylhydrazine (DNPH). The results are obtained by comparing with the standard solution of the pyruvate and expressed as μ mol pyruvate formed/min/mg protein. For the analysis of GOT the method of Bergmeyer and Brent (1974) was followed with some minor modification.

Preparation of the Reagents

0.1(M) potassium phosphate buffer pH 7.4

14.2 gm of anhydrous disodium hydrogen phosphate (E. MERCK, Cat No. 17549) was dissolved in double distilled water and the volume made up to 100 ml. 3.4 gm of potassium dihydrogen phosphate (S.D Fine Chem, Cat No. 39608) was dissolved separately and made up to 250 ml. The two solutions were mixed and the pH was adjusted to 7.4.

Buffered substrate

2.66 gm of L aspartic acid (Sisco Research Laboratories, India, Product code 014881) and 0.272 gm of α-ketoglutarate (Sisco Research Laboratories, India, Product Code114914) were
dissolved in 5 ml of 10% sodium hydroxide (EMERCK, Cat No. 17573)+30-40 ml of phosphate buffer and the pH was adjusted to 7.4 by adding more of sodium hydroxide, if necessary. To make the solution clear sometimes slight heating was necessary. The volume was made up to 100 ml with phosphate buffer and stored at 0°C in refrigerator.

**Pyruvate standard solution**

110 mg of sodium pyruvate (Sisco Research Laboratories, India, Product Code 194796) was dissolved in 10 ml of concentrated HCl. The volume was made up to 100 ml with double distilled water. It was stored in a brown bottle flask. The stock standard was diluted 1 in 5 with phosphate buffer. It was prepared fresh which contains 200 μmol/100ml.

**10% sodium hydroxide solution**

10 grams (EMERCK) was dissolved in 100 ml of double distilled water.

**0.4(N) sodium hydroxide solution**

**Dinitrophenylhydrazine (DNPH) solution**

19.8 mg of dinitrophenylhydrazine (SRL, Product Code 044946) was dissolved in 10 ml of concentrated hydrochloric acid and made up to 100 ml with distilled water. It was stored in a brown bottle.

**Procedure**

<table>
<thead>
<tr>
<th>Additions in ml</th>
<th>Control (C)</th>
<th>Treated (T)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffered substrate pre-heated at 37°C for 5 minutes</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Tissue homogenate</td>
<td>-</td>
<td>0.1</td>
</tr>
<tr>
<td>Incubated for 60 minutes and then</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DNPH was added</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Tissue homogenate was added to control</td>
<td>0.1</td>
<td>-</td>
</tr>
<tr>
<td>0.4(N) NaOH was added after 20 minutes</td>
<td>5</td>
<td>5</td>
</tr>
</tbody>
</table>

It was mixed by cyclomixture at 37°C and after 10 minutes the absorbance of T versus C was recorded at 520 nm.
Materials and Methods

Two more test tubes "S" and "B" was taken and the protocol was set up as below.

<table>
<thead>
<tr>
<th>Additions in ml</th>
<th>S</th>
<th>B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Working pyruvate standard</td>
<td>0.1</td>
<td>-</td>
</tr>
<tr>
<td>Buffered substrate</td>
<td>0.4</td>
<td>0.5</td>
</tr>
<tr>
<td>Water</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>DNPW</td>
<td>0.5</td>
<td>0.5</td>
</tr>
</tbody>
</table>

All the solutions were mixed thoroughly and after 20 minutes 0.4(N) NaOH was added.

The solution was mixed well and after 10 minutes readings of "S" versus "B" was taken at 520 nm.

Calculation

Activity of glutamate oxaloacetate transaminase in the tissue homogenate was calculated and expressed as mM/min/mg protein.

Estimation of Glutamate Pyruvate Transaminase (GPT)

GPT catalyses the formation of pyruvate from alanine.

Reagents

Buffered substrate

1.8g of alanine (SRL, Product Code 014878) and 0.292g of α-ketoglutarate (SRL, Product Code 114914) was dissolved in 20-30 ml of phosphate buffer and 2.5(N) NaOH was added drop wise to bring the pH to 7.4. The volume was made 100 ml with phosphate buffer which can be preserved at -15°C for 5 days only.

Calculation

The calculation was same as that of GOT and the liberation was expressed as mM / minute / mg protein.

Quantitative study of Acid and alkaline phosphatase

For the estimation of acid and alkaline phosphatase activities the method of Walter and Schutt (1974a, b) was followed with some minor modifications.
Materials and Methods

Homogenization and Centrifugation

Known amount of tissue samples were homogenized in 0.9% normal saline solution and were centrifuged at 3000g for 10 minutes at 4°C in a refrigerated centrifuged machine (REMI, C-24). The supernatants were divided into two parts, one part as the sample solution for the estimation of acid and alkaline phosphatases and other for quantitative estimation of proteins.

Estimation Procedure

Estimation of standard solution

The enzyme phosphatase hydrolyses p-nitrophenol phosphate. The released p-nitrophenol is yellow in colour in alkaline medium. The optimum pH for acid and alkaline phosphatases are 5.3 and 10.5 respectively. The activity was expressed in mM of phenol liberated per 100mg of protein at 25°C after 30 minutes of incubation. Therefore, the standard solution was same for both acid and alkaline phosphatase enzymes.

Preparation of standard solution

p-nitrophenol (E-MERCK Japan, Cat. No. 6798) - 6.955 mg
Distilled water - 5 ml

The concentration of the solution became 10mM

Preparation of reagents for estimation of standard solution

0.085(N) NaOH solution

NaOH-0.85 gm
Distilled water -250ml

Estimation procedure for standard solution

Different concentrations of the standard solution of p-nitrophenol were pipetted out into series of test tubes taking at least three replica for each concentrations. Then different reagents were mixed step by step as described in the following table.

<table>
<thead>
<tr>
<th>Concentration of p-nitrophenol (mM)</th>
<th>Sample of p-nitrophenol solution (ml)</th>
<th>0.085N NaOH (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Blank</td>
<td>10</td>
</tr>
<tr>
<td>0.001</td>
<td>0.2</td>
<td>908</td>
</tr>
<tr>
<td>0.002</td>
<td>0.4</td>
<td>906</td>
</tr>
<tr>
<td>0.003</td>
<td>0.6</td>
<td>9.4</td>
</tr>
<tr>
<td>0.004</td>
<td>0.8</td>
<td>9.2</td>
</tr>
<tr>
<td>0.005</td>
<td>1.0</td>
<td>9.0</td>
</tr>
<tr>
<td>0.006</td>
<td>1.2</td>
<td>8.8</td>
</tr>
</tbody>
</table>
Materials and Methods

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Extinction</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.007</td>
<td>1.4</td>
<td>8.6</td>
</tr>
<tr>
<td>0.008</td>
<td>1.6</td>
<td>8.4</td>
</tr>
<tr>
<td>0.009</td>
<td>1.8</td>
<td>8.2</td>
</tr>
<tr>
<td>0.010</td>
<td>2.0</td>
<td>8.0</td>
</tr>
</tbody>
</table>

After giving the different concentrations of standard solution in each test tube 0.085N NaOH was added so that the total volume of each sample would be 10 ml. Then the test tubes were incubated in a water bath at 37°C for 30 minutes.

Reading: After incubation the solutions were cooled and the extinction was read at 405nm in a spectrophotometer.

Estimation of acid phosphatase activity in unknown samples

Preparation of reagents for unknown acid phosphatase

- Acid buffer (pH 4.8)
  - Citric acid – 0.41 gm
  - Sodium citrate – 1.125 gm
  - p-nitrophenyl phosphate – 0.203 gm

These were added to double distilled water to make the final volume 100 ml. The pH of the buffer was checked and kept at 4°C, under this condition the buffer can be preserved and used for the next seven days. Care should be taken that the buffer was brought to 37°C before use.

- 0.1N NaOH solution
- NaOH – 400mg
- Double distilled water – 100ml

Procedure

- 0.2 ml of the supernatant was taken in each test tube 0.2 ml NaCl was taken for the blank solution.
- 1 ml of acid buffer was added to each test tube to make the total volume 1.2 ml always.
- After mixing thoroughly with a cyclomixer the solution was incubated at 37°C in a water bath for 30 minutes.
- After incubation 2 ml of 0.1N NaOH was added to each test tube and mixed well.
- Then the extinction was read at 405nm against a blank.
**Estimation of alkaline phosphatase activity in unknown samples**

**Preparation of reagents for unknown alkaline phosphatase**

**Alkaline buffer (pH 10.5)**

- Glycine-375 mg
- MgCl₂-10 mg
- p-nitrophenyl phosphate-165 mg

These were dissolved in 42 ml of 0.1N NaOH and the whole solution was diluted with double distilled water to make the final volume up to 100 ml. The pH of the buffer was checked and was kept at 4°C. In this condition the buffer could be used for the next 7 days. Before using the buffer it was brought to 37°C

**0.05N NaOH solution**

- NaOH-200mg
- Distilled water-100 ml

**Procedure**

- 0.05 ml of the supernatant was taken in each test tube and 0.05 ml of NaCl was taken for the blank solution
- 2 ml of alkaline buffer was added to each of the test tube to make the total volume 2.05 ml always.
- The solutions were mixed well by a cyclomixer and then were incubated at 37°C for 30 minutes in a water bath
- 10 ml of 0.05N NaOH was added to each of the test tubes after incubation and mixed well.
- The extinction was read at 405 nm against a blank.

**Determination of activities of acid and alkaline phosphatases**

**Preparation of the standard curve**

At least 3 replica readings were taken for each concentration of p-nitrophenol phosphate and the mean O.D readings were plotted against different concentrations of the standard samples so prepared (0.001mM to 0.01mM) A linear curve was obtained, which is the standard curve.
**Determination of acid phosphatase activity**

The concentration of phenol liberated after 30 minutes in unknown solution was determined against the standard curve. The activity of acid phosphatase (the phenol liberated after 30 minutes of incubation) in a tissue per 100 mg of protein was calculated as follows:

\[
\text{Phenol liberated after 30 minutes of incubation (acid phosphatase activity)} = \frac{\text{Concentration} \times \text{Amount of NaCl} \times 100000}{0.2 \times \text{tissue weight} \times \text{amount of protein}} \text{ mM /100mg of protein}
\]

Amount of NaCl = Amount of NaCl used for homogenisation of the tissue

Amount of protein in mg/g of tissue

**Determination of Alkaline phosphatase activity**

The concentration of phenol liberated after 30 minutes of incubation in unknown solution was determined against the standard curve. Then the activity of alkaline phosphatase i.e. the phenol liberated after 30 minutes in a tissue per 100 mg of protein was calculated as follows:

\[
\text{Phenol liberated after 30 minutes of incubation (alkaline phosphatase activity)} = \frac{\text{Concentration} \times \text{Amount of NaCl} \times 100000}{0.05 \times \text{tissue weight} \times \text{amount of protein}} \text{ mM /100mg of protein}
\]

Amount of NaCl = Amount of NaCl used for homogenisation of the tissue

Amount of protein in mg/g of tissue

**Scoring and analysis of the data**

The activities of acid and alkaline phosphatases for each tissue from each mice of both control and treated series were scored and the mean and standard errors were determined. Statistical analysis of the data in different series was done (*vide supra*).
Gel-electrophoretic Study of Total Protein

For SDS-PAGE study of total protein the method of Laemmli (1970) was followed with some minor modifications. Here only 10% gel was used as it gave us better results.

Preparation of sample for known (standard) protein

The standard molecular weight (MW) protein marker for SDS gel electrophoresis with molecular weight ranging between 29 and 200 kilo Dalton (kD) was procured from GENEI, Bangalore, India (Cat. No. PMW-B). This is a solution containing the mixture of the proteins listed in the following table, the total protein concentration of which is 2.5 mg/ml.

<table>
<thead>
<tr>
<th>Name of Proteins</th>
<th>Approx. Molecular weight (kD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myosin, Rabbit muscle</td>
<td>205.0</td>
</tr>
<tr>
<td>Phosphorylase b</td>
<td>97.4</td>
</tr>
<tr>
<td>Bovine Serum albumin</td>
<td>66.0</td>
</tr>
<tr>
<td>Ovalbumin</td>
<td>43.0</td>
</tr>
<tr>
<td>Carbonic Anhydrase</td>
<td>29.0</td>
</tr>
<tr>
<td>Soyabean Trypsin Inhibitor</td>
<td>20.1</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>14.3</td>
</tr>
<tr>
<td>Aprotinin</td>
<td>6.5</td>
</tr>
<tr>
<td>Insulin</td>
<td>3 (2.5-3.5)</td>
</tr>
</tbody>
</table>

Preparation of working standard solution

- A sample buffer was prepared with 0.0625 M Tris, pH 6.8, 2% SDS, 5% Mercaptoethanol, 15% glycerol and 0.001% bromophenol blue (BPB).
- Then 10μl of the protein molecular weight marker was mixed with 50μl of the sample buffer and incubated in boiling water bath for a minute.
- The entire solution was then ready for electrophoresis.

Preparation of sample for unknown protein

As described before.

Preparation of different electrophoretic solutions

All the solutions were stored in dark and cool place before use.
Materials and Methods

Solution A (30% Acrylamide solution)

| Acrylamide (Sigma, USA, Cat. No. A-8887) | 28.2 g/ml |
| bis-acrylamide (N,N'-Methylene-bis-Acrylamide) (Sigma, USA, Cat. No. M-7256) | 0.8 g/ml |

The mixture was brought up to a total volume of 100 ml with double distilled water.

Solution B (Separating gel buffer/0.1M Tris-HCl buffer, pH 8.8)

| Tris (Tris(hydroxymethyl)aminomethane, Sigma, USA, Cat. No. T-1378) | 18.2 g/ml |
| SDS (sodium dodecyl sulphate/Lauryl sulphate, Sigma, USA, Cat. No. L-4390) | 0.4 g/ml |
| HCl (Hydrochloric acid) | 2.0 ml |

The mixture was brought up to a total volume of 100 ml with double distilled water.

Solution C (Stacking gel buffer/0.5 M Tris-HCl buffer, pH 6.8)

| Tris | 6.1 g/ml |
| SDS | 0.4 g/ml |
| HCl | 4.2 ml |

The mixture was brought up to a total volume of 100 ml with double distilled water.

Solution D (10% Ammonium persulphate solution)

100 mg of Ammonium persulphate (Sigma, USA, Cat. No. A-3678) was dissolved in 1 ml of double distilled water. This solution was always prepared freshly before use.

TEMED (N,N,N',N'- Tetramethyl-ethylenediamine, Sigma, USA, Cat. No. T-8133)

This was always used fresh from the refrigerator.

Treatment Buffer

| SDS | 0.1 g/ml |
| 2-Mercaptoethanol (Sigma, USA, Cat. No. M-7454) | 0.1 ml |
| 50 mM Solution C (0.5 M Tris-HCl buffer, pH 6.8) | 1.0 ml |
| Glycerol | 2.0 ml |
The mixture was brought up to a total volume of 10 ml with double distilled water.

**Tracking Dye**

1-2 mg of Bromophenol Blue (BPB, 3', 3",5',5"-Tetramethylthiuramdisulfide, Sigma, USA, Cat No B-8026) was added to the treatment buffer solution and stored at 0°C.

**Running Buffer (pH 8.2-8.4)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris</td>
<td>15.0 ml</td>
</tr>
<tr>
<td>Glycine</td>
<td>7.2 ml</td>
</tr>
<tr>
<td>SDS</td>
<td>0.5 ml</td>
</tr>
</tbody>
</table>

The mixture was brought up to a total volume of 500 ml with double distilled water.

**Electrophoresis**

**Assemblage of glass plates for gel casting**

- 2 glass plates (102 mm x 120 mm x 3 mm) and gaskets (procured from Atto, Japan, AE-6400 Dual Mini Slab Kit) were assembled with the clips, to make plate sandwiches which produced the gel with a size of 80 mm x 90 mm x 1 mm.
- A level was marked, 5-6 mm bottom of the comb on the notched glass plates and the comb was removed. This mark served as the interface between the stacking and separating gel.

**Preparation of separating gel**

The 10% separating gel was prepared as follows:

- 4.5 ml of solution A and 4.5 ml of solution B was mixed with 9.0 ml of double distilled water.
- 0.01 ml of TEMED and 0.08 ml of solution D was added to it.
- After mixing well the solution was poured immediately between the sandwiches upto the marked level.
- A overlay of water was put at a level of 2-3 mm over the gel solution to see a clear interface.
- The sandwiches were kept for about 1 hr for polymerization.
- After 1 hr, the water overlay was discarded and the sandwiches were filled with stacking gel solution.
Materials and Methods

Preparation of stacking gel
The 4.5% of stacking gel was prepared as follows

- 0.9 ml of solution A and 1.5 ml of solution D was added to 3.6 ml of double distilled water
- 0.01 ml of TEMED and 0.02 ml of solution D was added to it.
- After mixing well the solution was poured immediately between the sandwiches.
- The combs were inserted carefully leaving no air bubbles between the sandwiches and were kept for 30 min for polymerization
- After 30 min of polymerization the combs were removed slowly without damaging the sample wells

Charging of the samples

- Sample buffer including the tracking dye was mixed with each tissue aliquotes in 1:2 ratio (approximately).
- The mixture was heated at 100° C in a boiling water bath for 1-2 min.
- The samples were cooled to 25° C before loading in the gel.
- The samples wells were rinsed with small amount of running buffer before use.
- 25-50 μl volume of protein (50-200μg amount of protein) was charged onto slab gel, one sample per slot/well
- Pre-cooled (0°C) running buffer (pH 8.2-8.4) was filled in both the chambers of the electrophoretic unit.
- The electrophoretic unit was then connected with an electrophoretic power module, a regular flow of electric current was allowed to pass through it.
- 3 mA of current per slot at a potential difference of 200 volts was passed through the unit.
- The unit was kept inside the refrigerator throughout the period of run to prevent anomalous electrophoretic migration or damage to the SDS-PAGE apparatus due to heat generation during electrophoresis (Makowski and Ramsby 1998).
- Electrophoresis was performed until the dye front reached 0.5 cm from the bottom of the gel and then the current flow was discontinued

Staining of the gel

Preparation of the staining solution

| Coomassie Brilliant Blue R (Sigma, USA, Cat. No. B-0149) | 2.5 gm |
| Methanol | 500 ml |
| Acetic acid | 100 ml |
The mixture was brought up to a total volume of 1 liter with double distilled water.

**Procedure of staining**

After the run was over the gel was carefully removed from between the plates and was immersed in staining solution for at least 3 hr or overnight.

**De-staining and Fixation of the gel**

**Preparation of the de-staining solution**

- Methanol: 250 ml
- Acetic acid: 70 ml

The mixture was brought up to a total volume of 1 liter with double distilled water.

**Procedure of de-staining and fixation**

After decanting the staining solution, de-staining solution was added with periodical changes until desired background was achieved. Then the gel was preserved in 7% acetic acid solution (fixative) for further analysis.

**Scoring of data**

**Analysis of the banding pattern**

The slab gel was placed against a ground glass sheet suitably illuminated from behind to have a clear view of different bands and photographs were taken. The polarities (‘+’ and ‘-’) of the gel were marked on the photographs. The nature and intensity of the bands were measured as follows:

**Nature of bands:** For the convenience of our study the bands were arbitrarily classified as follows depending on their thickness

<table>
<thead>
<tr>
<th>Thickness of bands (mm)</th>
<th>Nature</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Linear (L)</td>
</tr>
<tr>
<td>&gt;0 ≤ 0.1</td>
<td>Thin (T)</td>
</tr>
<tr>
<td>&gt;0.1 ≤ 0.2</td>
<td>Medium (M)</td>
</tr>
<tr>
<td>&gt;0.2</td>
<td>Broad (B)</td>
</tr>
</tbody>
</table>

**Intensity of Bands:** The staining intensity of the bands were measured by visual observation and was described by “+” signs, one “+” indicating one unit and the extra “+” denoting more intensity.
Measurement of the Relative Mobility (Rm): relative mobility (Rm) is the ratio of the two distances. It is measured by dividing the distance that the protein zone has migrated from the interface between the stacking and running gels by the distance that the tracking dye has migrated from the same interface (Conn et al 1995) Rm values vary from 0 to 1.

\[
\text{Relative Mobility (Rm)} = \frac{\text{Migration distance of individual band}}{\text{Migration of the tracking dye (BPB)}}
\]

In this way the migration of a protein was compared from gel to gel, regardless of the physical length of the gel. The distances were measured from the interface between stacking and separating gels (Conn et al, 1995)

**Preparation of the standard curve**

Standard curve was obtained by plotting log of molecular weight versus the Rm values of the standard proteins as shown in the right hand side. This gave the straight line equation (Weber and Osborn 1969; Fnefelder 1982; Stryer, 1995 etc.).
**Analysis of molecular weights of unknown protein**

With the help of Rm values, the molecular weights of unknown proteins were determined from the standard curve (Weber and Osborn 1969; Friefelder 1982; Stryer 1995; Makowski and Ramsbi 1998). A number of gels were scored of single series and the most occurring values of the bands were taken into consideration. Sometimes, 1-2 bands in some gels of the same series, very faint in nature and of doubtful existence, appeared not to tally with the others, these slight differences, erratically appearing in some tissues, were generally ignored as due to technical artifacts, or due to little difference in the physiological conditions of the experimental fish, and were excluded during scoring and comparing data.

The molecular weights were grouped into different class-intervals (at an interval of 5kD) and the number of bands of a particular tissue in an experimental series were counted according to those class-intervals. The differences in occurrence of bands of a particular tissue in different series within a class-interval were critically analyzed.
**Histological studies**


For histology the liver samples isolated quickly from the sacrificed animals were fixed in Bouin’s fluid and then embedded in paraffin wax. The liver sections cut and stretched on clean grease free slides were brought down to distilled water then stained in Delafield haematoxylin for 10 minutes. Then the sections were immersed in tap water for 5 minutes until the sections appeared blue to the naked eye. The tissue slides were rapidly washed with distilled water and stained for 2 minutes with 1% alcoholic eosin. This was followed by washing and dehydration with 95% and absolute alcohol. It was cleared in xylol and mounted by DPX.

Preparation of reagents for Scanning and Transmission electron microscopy

**0.2M phosphate buffer**

Two solutions A and B were added to make 0.2M phosphate buffer (pH 7.2).

**Solution A:** 17.47 g of sodium di hydrogen orthophosphate was added to 560 ml of double distilled water.

**Solution B:** 51.3 g of sodium phosphate dibasic was mixed to 1440 ml of double distilled water. Then solution A was added to solution B to make 2000 ml of 0.2M phosphate buffer.

**Working solution:** 0.1M phosphate buffer was prepared by adding equal volumes of 0.2M phosphate buffer and distilled water.

To 100 ml of 2.5% Glutaraldehyde (LOBA Chemie, India. Product code 3965) 400 ml of distilled water was added. Then 500 ml 0.2M phosphate buffer was added.

**Modified Karnovsky fixative (1984)**

<table>
<thead>
<tr>
<th>0.2M phosphate buffer</th>
<th>500 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Paraformaldehyde (E-MERCK)</td>
<td>40 gm</td>
</tr>
</tbody>
</table>

It was heated to 60°C to dissolve it; till the solution becomes clear. It was cooled at room temperature, the volume was made up to 960 ml with double distilled water. It was cooled and 40 ml of 2.5% glutaraldehyde solution was added. Then it was stored at 4°C, here the concentration of paraformaldehyde remains 4% and concentration of glutaraldehyde remains 1%.
2% Osmium tetraoxide solution

1 g ampules of OsO₄ were obtained from Sisco Research Laboratones[Product Code 154022] and dissolved in 50 ml of double distilled water, it takes overnight to dissolve completely. Then it was stored at 4°C in brown bottle flask.

Working solution: 1% OsO₄ (was prepared by adding equal volume of 0.2M phosphate buffer and 2% OsO₄)

Sample preparation and viewing under Scanning Electron Microscopy

- Liver samples (2-3 mm) were quickly isolated after the animals were sacrificed and kept in 2.5% Karnovsky's fixative for 4-5 hours.
- Then the samples were washed in 0.1M phosphate thrice.
- Post fixation was carried out in 1% OsO₄ for 2 hours.
- Again the samples were washed with 0.1M phosphate buffer.
- Then the dehydration steps were followed:
  - 30% acetone 15 minutes
  - 50% acetone 15 minutes
  - 70% Acetone 15 minutes
  - 90% Acetone 15 minutes
  - 100% Acetone 15 x 2 minutes
- Samples were further dried by a critical point drier (CPD-Biorad, Microscience division, Watford, England). Critical point drying is considered one of the best techniques to dry liver samples.
- Then the liver samples were mounted on the aluminium stubs with the help of two side adhesive tapes.
- Gold coating of the liver samples was accomplished by the help of sputter coater [Agar Sputter coater, Model-198, Stansted, United Kingdom], about 35nm thickness of pure gold was obtained.
- After gold coating the samples were observed under Scanning Electron Microscope [LEO, 435VP, United Kingdom]

Sample preparation and viewing in Transmission Electron Microscopy

- Liver sections, 1-2mm thick, isolated immediately after sacrifice of mice were directly immersed in Karnovsky fixative (4% paraformaldehyde and 1% glutaraldehyde made in 0.1M phosphate buffer pH 7.4) at 4°C for 4-5 hours.
After fixation the specimens were rinsed thoroughly with phosphate buffer to wash off excess fixative because traces of primary fixative may react with secondary fixative to produce a precipitate of reduced osmium in the specimen.

Secondary fixation was followed after primary fixation using 1% (v/v) OsO₄ (E MERCK) for 1 hour at 4°C in dark.

This was followed by washing and dehydration with acetone.

Few tissues were rigid enough permitting being cut into thin sections without an additional support, this was accomplished by infiltrating resins into tissues and further hardening it, so that it would take the form of a block embedded with the tissue.

Embedding was done in the embedding medium (Epon) using beam capsule, after filling the capsule with the embedding medium, tissues were placed and it was ensured that they settled at the tip and if necessary the capsule was filled with Epon.

Polymerization followed embedding by which the liquid resin was hardened, it was accomplished by keeping the beam capsule along with the specimen at a temperature of 40 - 50°C overnight, then the temperature was increased to 60°C.

Beam capsules were cut open easily with a razor blade.

These blocks so obtained were trimmed to produce a pyramid at the tip where the tissue was located.

Before proceeding with the ultrathin sectioning, thick sections (0.5 to 2.0μm) were cut for viewing the tissue under an optical microscope.

After viewing the sections under optical microscope, the area to be examined under TEM was marked and the blocks were trimmed further.

60 to 90 nm thick sections were cut in a ultramicrotome [Reichert Jung- Ultracut E, UK] with the help of glass knives. To allow the sections to be cut without sticking to the knife edge, a trough (boat) was placed behind the edge which was filled with water. Further the sections were stretched by exposing them to xylene / chloroform vapours.

The sections were lifted from below on platinum grids, generally sections were lifted using the matted surfaces of the grid.

The grid with sections was coated with 3% formvar in ethylene di chloride.

Staining of ultra thin sections was carried out using uranyl acetate (freshly prepared-saturated solution of uranyl acetate (Analar) to 10 ml of filtered 50% ethanol in a 15 ml centrifuge tube which was shaken vigorously for 2 minutes and spun hard to allow excess of uranyl acetate to settle) and lead citrate (one half pellet of NaOH to 12 ml of distilled water was added in a 15 ml centrifuge tube, shaken well to dissolve NaOH, then lead citrate was added and centrifuged, this solution too was freshly prepared).

Then the ultra structural parts of both control and treated liver samples were observed under a transmission electron microscope (TEM CM-10, Philips Microscope).
Materials and Methods

Reagents used

RIPA buffer (Radio Immunoprecipitation Assay)

- 1% (w/w) Nonidet P-40 (NP-40) [169748, protease inhibitor cocktail tablets, Boehringer Mannheim].
- 1% w/v sodium deoxycholate.
- 0.1% (w/v) SDS
- 0.15M NaCl
- 0.01M sodium phosphate, pH 7.2.
- 2mM sodium vanadate added fresh from 0.2 stock solution (can be stored at room temperature).
- 100U/ml aprotinin (Trasylool, Pentex/ Miles)
- DNA ase (SIGMA, USA)
- Genei Protein estimation kit by Bicinchoninic acid (BCA) method (KT-31, Bangalore Genei Pvt limited)

Method

The level of p53 protein in liver of treated and control mice were evaluated by Western blotting analysis previously described by Perego et al (1997).

- The liver were homogenized in phosphate buffer and 200 µl of RIPA buffer was added to the samples in ice
- To this DNAase (1µl) was added and mixed well
- This was followed by protein assay by bicinchoninic acid (BCA) method using Bangalore Genei protein kit assay KT-31.
- To prepare BCA working reagent (BWR), 50 parts of reagent A was mixed with 1 part of reagent B. 2 ml of this reagent was added to each sample, it was mixed well and
- The optical density was measured at 562 nm after incubation for 30 minutes at 37°C
- Then the protein calculation was followed
- 40 µg of protein was resolved by SDS PAGE on a 10% polyacrylamide resolving gel and a 5% stacking gel at a constant voltage of 50 V for about 2 hours.
- After the dye has reached the mark, after 2 hours the electrodes were disconnected and the proteins were transferred on to a 0.45µm nitrocellulose membrane at 50V constant voltage for 7 hour.
Membranes were blocked at room temperature in TBST containing 5% (w/v) non-fat milk. The membranes were suspended in primary antibody p53Ab-8 (DO-7+BP53-12), [NEOMARKERS, FREMONT, CA] along with the assay buffer for 30 minutes and then washed with washing buffer three times for at least 10 minutes each.

The blots were then immersed in secondary antibody solution Rabbit anti mouse IgG-ALP conjugate, (Genei, Cat ALP-M) at 1:2000 dilution, it was gently shaken for about 1 hour.

A primary antibody for β-actin 1:30,000 dilution was also used as a control for sample loading.

Antibody binding to nitrocellulose was detected using substrate solution BCIP/NBT (Bangalore Genei, India) and bands were visible within 20 minutes.

40 µg of protein was resolved by SDS PAGE on a 10% polyacrylamide resolving gel and a 5% stacking gel at a constant voltage of 50 V for about 3 hours.

After electrophoresis, proteins were transferred to nitrocellulose membranes (Hybond-C, UK) using miniblot equipment supplied by Genei, as previously described.

After transfer the membranes were blocked at room temperature in TBST containing 5% (w/v) non-fat milk.

The membranes were suspended in primary antibody Bax Ab 5 (2C8), [Neomarkers, Fremont, CA] along with assay buffer for 45 minutes and then washed with washing buffer four times at least 10 minutes each.

Then the blots were suspended in secondary antibody IgG-Alp (Genei, Cat ALP-M) at 1:2000 dilution and shaken for 1 hour.

Antibody binding to nitrocellulose was detected using substrate solution BCIP/NBT (Bangalore Genei, India) and bands were visible within 40 minutes.