CHAPTER - II

Materials and Methods
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Animals
Healthy, intact, adult male albino rats (*Rattus norvegicus*) of Wistar strain weighing around 200 g were obtained from the breeding facility of Jai Research Foundation, Valvada and were used for the experiments.

Acclimatisation
All animals were acclimatised in the experimental room for seven days prior to the commencement of the treatment.

Environment
During experimentation, all animals were maintained in an air conditioned room at a temperature of 22 ± 3°C with 12 hours artificial light and 12 hours darkness and relative humidity of 30 to 70 per cent at all times (Poole, 1989).

Housing
All animals were housed in solid floor polypropylene cages. Clean autoclaved paddy husk was used as bedding material throughout the study. Animals under different groups of experiments were caged separately.

Diet and Water
The animals had free access to pure drinking water filtered through aqua guard water filter system and commercial rat feed pellet feed (Amrut
brand manufactured by Maharashtra Chakan Oil Mills Limited, Pune - 400 030, India) before, during and after treatment.

**Chemicals**

A commercial sample of combination fungicide, Metalaxyl + Mancozeb (M + M) 72% wettable powder (WP) was procured from the market. Analytical reagents were obtained from Loba Chemicals, S.D. Fine Chemicals and Qualigen Fine Chemicals (Glaxo), Mumbai. For clinical chemistry, reagents were obtained from Transasia Bio-medicals Ltd., Mumbai and for hormonal assays, radioimmunoassay kits were procured from Sereno Laboratories, Italy, Orion diagnostics, Finland and CIS Bio international, France, Amersham Pharmacia, U.K.

**Dose Determination**

An acute study was conducted to determine the LD$_{50}$ value of M + M and was found to be 5150 mg/kg body weight (Finney, 1977). A short term oral toxicity study of 28 days was conducted at the dose levels of 1000, 750, 500 and 250 mg/kg body weight with special emphasis on observations of mortality, body weight gain, organ weight and histopathological findings of target organs. Since minor effects were observed at the dose level of 500 mg/kg body weight on above parameters, the same dose was selected for sub chronic study. Moreover, 1/10$^{th}$ LD$_{50}$ value was also approximately the same.

**Duration and Mode of Administration**

After acclimatization period, the rats were treated orally with M + M suspended in distilled water. The dose volume was adjusted weekly
throughout the experimental period to maintain the ratio with body weight i.e., 10 ml/kg body weight. The respective control animals received distilled water as vehicle. The duration of treatment was 30, 60 and 90 days on a 7-days/week basis and to study the effect on sperm, fertility, implantation and hormonal functions of thyroid gland and testis, the dosing period was restricted to 60 days. For withdrawal study, animals after oral feeding were kept for a further period of sixty days to detect recovery or persistence of toxic effects. The details of the experimental animals are as follows

Genotoxicity Studies *In vivo*

**Chromosomal aberration Study**

This study was performed with five groups with ten animals in each group.

- **Group I**: Treated with distilled water for 90 days
- **Group II**: Treated with M + M for 30 days
- **Group III**: Treated with M + M for 60 days
- **Group IV**: Treated with M + M for 90 days
- **Group V**: Intraperitoneal injection of Mitomycin C for one day (4 mg/kg body weight)

**Micronucleus Assay**

This study was performed with five groups with ten animals in each group.

- **Group I**: Treated with distilled water for 90 days
- **Group II**: Treated with M + M for 30 days
Group III : Treated with M + M for 60 days
Group IV : Treated with M + M for 90 days
Group V : Intraperitoneal injection of Mitomycin C for one day
          (4 mg/kg body weight)

**Dominant Lethal Assay**
This study was performed with four groups with ten animals in each group.

Group I : Treated with distilled water for 60 days
Group II : Treated with M + M for 60 days
Group III : Intraperitoneal injection of Ethyl methane sulphonate for 10 days (10 mg/kg body weight)
Group IV : Treated with M + M for 60 days with a recovery period of 60 days

From the above study, serum was collected from Group I, II and IV animals for hormonal estimation of thyroid gland and sacrificed for steroid enzyme estimations in testis.

**Culture studies (in vitro)**
Mutagenic potential of this fungicide combination was tested in cultured human lymphocytes at different concentrations (Appendix I)

**Clinical Chemistry**
Haematological and serum parameters were analysed immediately after 30, 60 and 90 days of treatment and at the end of 60 days of recovery period. All animals were fasted overnight before blood collection. Each group comprised 10 animals.
Group I : Treated with distilled water for 90 days with a recovery period of 60 days

Group II : Treated with M + M for 90 days with a recovery period of 60 days

Biochemical Studies of Tissues

Group I : Treated with distilled water for 60 days

Group II : Treated with M + M for 60 days

Group III : Treated with distilled water for 90 days

Group IV : Treated with M + M for 90 days

Group V : Treated with distilled water for 90 days with a recovery period of 60 days

Group VI : Treated with M + M for 90 days with a recovery period of 60 days

Sperm Parameters and Fertility

Group I : Treated with distilled water for 60 days

Group II : Treated with M + M for 60 days

Group III : M + M for 60 days with a recovery period of 60 days

1. GRAVIMETRIC STUDIES

Body Weights

The body weights of all the animals were recorded to the nearest gram at weekly intervals from the start of the treatment and till the cessation of treatment and during the recovery period.
Organ Weights
On the day of sacrifice, kidney, testis and liver were quickly excised, cleared off from the adhering fat and blotted free of blood before determining their absolute weight to the nearest gram (g). Then the same was processed for various experimental studies.

2. GENERAL TOXICITY STUDIES
Rats were observed for all visible signs of toxicity such as skin and fur changes, eye and mucous membrane changes, respiratory, circulatory, autonomic and somatomotor activities, behavioral patterns and general changes once a day. Physical examination was carried out on all rats prior to treatment and at weekly intervals thereafter. Ophthalmological examination was carried out on all rats on the day of commencement of M + M administration and prior to terminal and recovery sacrifices using Heine Beta-200M2 ophthalmoscope (manufactured by Heine Optotechnik, Germany).

3. GENOTOXICITY STUDIES
Chromosomal Aberration Study *In vivo*
Bone marrow contains large number of dividing cells which are suitable for chromosome analysis. The procedure described by Evans (1984) was followed with few modifications. Cell division was arrested *in vivo* by colchicine at the metaphase and collected cells were then allowed to swell by hypotonic treatment with 0.075 M potassium chloride. The cells in metaphase were fixed on slides following Carnoy’s fixative treatment. Slides were stained with 4 % Geimsa in phosphate buffer (pH 7.2). A minimum of 100 metaphase spreads per animal was analyzed.
Preparation of Phosphate Buffer Saline (PBS)
To 8.0 g of sodium chloride, 2.8 g of di sodium orthophosphate and 0.19 g of potassium hydrogen orthophosphate were dissolved in triple distilled water and made up to 1 litre. The pH of prepared solution was adjusted to 7.4 and stored at 4°C.

Preparation of Phosphate Buffer
Citric acid (10.5 g) was dissolved in 500 ml of distilled water and 35.8 g of sodium phosphate dibasic was dissolved in 500 ml distilled water to 193.2 ml of sodium phosphate dibasic solution, 56.8 ml of citric acid solution was added and the pH was adjusted to 7.4 and stored at 4°C.

0.075 M Potassium Chloride
An amount of 2.956 g of potassium chloride was weighed and dissolved in 500 ml of distilled water. Fresh solution was prepared each time.

Colchicine (Sigma, USA)
Colchicine (1 mg/ml) was prepared in distilled water and 4 mg/kg body weight was considered.

Fixative
Methanol: Glacial acetic acid in the ratio of 3:1 and stored at 4°C.

Staining of Chromosome Slides
To 4 ml of Giemsa solution, 4 ml of phosphate buffer (7.4 pH) was added and made up to 100 ml with distilled water.
Procedure

Rats of both control and treated groups were injected with colchicine intraperitoneally. The animals were sacrificed by cervical dislocation, 2 to 3 h after injecting colchicine. Femur bones were quickly removed, muscle was cleaned away from the bone and both femurs were placed on the edge of a pre-numbered plastic centrifuge tubes, which corresponds to the animal number. The bone marrow was expelled by aspiration with 5 ml of phosphate buffer saline through 22 gauze needle. The cell suspension was mixed vigorously to assure dissociation of the cells and centrifuged at 2000 rpm for 10 minutes. The supernatant was removed by gentle aspiration until a small volume remains above the pellet. The pellet is re-suspended in the remaining volume. A volume of 5 ml hypotonic solution of (0.075 M) potassium chloride was added to the residual cell pellet and mixed thoroughly, the cells were incubated in water bath maintained at 37 °C for 20 minutes. Freezer chilled Carnoy’s fixative (methanol + acetic acid 3:1) was added and the resuspended cells were refrigerated at 4 °C for 12 h. The cells were centrifuged on the following day for 10 minutes at 2000 rpm and the supernatant was discarded leaving about 0.5 ml of fixative along with the cell pellet.

Preparation of slides

On a clean wet slide, 1 or 2 drops of the suspension was placed and it was blotted free of moisture. The slides were kept on a slide warmer to dry and checked under a low power phase contrast objective for proper density and cell spreading. Air dried slides were stained with Giemsa (1 ml of Giemsa in 40 ml of water) for 3 minutes and dried. The slides were made permanent by mounting a cover slip with DPX.
Scoring Chromosomal Aberrations (CAS)

A minimum number of 500 cells were counted in different fields of slide per animal to determine the mitotic index by the following formula

\[
\text{Mitotic index} = \frac{\text{Number of metaphase}}{\text{Number of cells counted}} \times 100
\]

A minimum of 100 consecutive metaphase were scored under 100 X oil immersion objective. Aberrations are divided into chromatid type and chromosome type. The former involving only one chromatid, the latter involves both chromatids at identical sites. Chromatid type aberrations like gaps, breaks, chromatid exchanges and chromosome type aberrations like chromosome gaps and breaks were scored. Numerical aberrations were also counted.

Micronucleus Assay In vivo

The chromatin fragments, which may be produced by the clastogenic agents or spindle poisons lag behind during anaphase due to chromosomal breakage and spindle malformation, are not included into the nucleus of the daughter cells. These small fragments of chromatin give rise to micronuclei, which are present in the cytoplasm of the daughter cells. The procedure described by Schmid (1976) and modified by Salamone and Heddle (1983) was followed with few modifications.

Chemicals and Reagents

Fetal calf serum and Giemsa stain.
Procedure

Control and treated animals were sacrificed by cervical dislocation on day 30, 60 and 90 after treatment with M + M. In order to assess the sensitivity of the test system a separate experimental group was maintained in which rats received a single intraperitoneal dose of Mitomycin C at the dosage of 4 mg/kg body weight, a known genotoxic agent. Femur bones were dissected out and freed of adherent tissue. A small hole was made in the neck of the femur and the marrow content was flushed into a centrifuge tube along with 3 ml of fetal calf serum using 1 ml syringe and 22 gauge needle. The tube contents were mixed well and centrifuged at 2000 rpm for 10 minutes. The supernatant was discarded and the pellet was dissociated using a siliconised Pasteur pipette and a drop of material was placed on a clean, dry slide and smear was prepared. The slides were allowed to dry and fixed with methanol followed by staining with 5% Giemsa for 10 minutes. Excess stain was removed from the slides by successive rinsing in distilled water and allowed to air dry. The stained slides were covered with cover slips using DPX mountant.

Scoring of slides

A total of 1000 polychromatic erythrocytes (PCE) with and without micronuclei were recorded from each prepared slide. The corresponding number of normochromatic erythrocytes (NCE) with and without micronuclei was also recorded to determine the PCE/NCE ratio. Analysis of the per cent micronucleated erythrocytes (% MNE) and PCE/NCE ratio were also recorded (Gad and Weil, 1994).
Dominant Lethal Assay

This study was designed in order to evaluate mutagenic potential of M + M in male germ cells thereby affecting the viability of progeny in untreated female rats at the time of implantation. To understand the anti-implantation effect of this combination fungicide through genetic alteration in male germinal cells, pre-implantation, post-implantation and total implantation loss in female rats mated with treated males were evaluated by considering the number of live, dead implants which includes early and late resorptions at the implantation site. This study reveals the chromosomal alterations in sperm through the early and late deaths of developing embryo. The procedure described by Bateman and Epstein (1971) and Green et al. (1987) was followed with few modifications.

Procedure

Male rats were treated with this combination fungicide for 60 days to ensure the complete duration of one spermatogenic cycle. Control group animals received vehicle for 60 days. Positive control group was treated for 10 days to check the sensitivity of the test system. At the end of treatment every treated male was kept in cohabitation with two untreated virgin, nulliparous female rats for one week. Intromission was observed by a sperm positive smear and the day was designated as the day one of the onset of pregnancy. Female rats were sacrificed on day 16 from day 1 of cohabitation and uterine analysis was performed.
Every pregnant rat was counted for total number of implants, which include live and dead implants. Dead implantations include early and late fetal deaths. Finally results were computed to study the pre implantation loss, post implantation loss and dominant lethal effect.

Pre implantation loss was estimated by comparing the total implants per female in treated with control group. Post implantation loss was estimated by determining the ratio of dead to total implants from the treated group to the ratio of dead to total implants from the control group. The calculation of the dominant lethal effect was based on comparison of the live implants per female in the treated groups to the live implants per female in the control groups.

Recovery
To determine the period of recovery in the treated animals, on the last day of recovery, males from treated recovery group were cohabited with female rats. Female rats were sacrificed on day 16 from day 1 of cohabitation and uterine analysis was performed for studying the recovery of implantation loss induced by this combination fungicide as genetic alterations in male germinal cells.

4. HAEMATOLOGICAL STUDIES

Blood Sampling
Blood samples, for haematological studies were collected in Ethylenediaminetetracetic acid (EDTA) tubes by puncturing the orbital sinus of rats with the help of a fine capillary tube (Hodgson and Guthrie, 1984). All collected samples were analysed for total WBC, RBC and
platelet counts, haematocrit value and haemoglobin content using Sysmex K-1000 instrument (manufactured by Tao Medical Electronics Co. Ltd., Kobe, Japan.). For differential leucocyte cell count (DLC), one drop of anticoagulant was added, free flowing blood sample was taken on clean glass slide, spread and stained with Leishman's stain (Jain, 1986).

**Haemoglobin Content**

Around 6 μl of the whole blood sample measured by the sample rotor valve was diluted into 1:500 by adding approximately 2 ml of the diluent and 1 ml of haemoglobin lysing reagent in the haemoglobin flow cell. The intensity of colour change produced by cyanomethemoglobin was measured as absorbance in the haemoglobin flow cell. The estimated haemoglobin content is expressed as g/dl.

**Principle of Cell Counting and Sizing**

The blood cells and platelets were counted by detecting a difference in conductivity between the particles and the diluent in which they are suspended. Blood cells are diluted in an electrically conductive diluent. As there are large differences between conductivity and resistance of cells and diluent, the cells can be enumerated in both quantity and volumetric size by detecting and measuring the difference in conductivity.

**WBC Sample Flow**

Blood samples, with EDTA as an anti-coagulant was aspirated through an aspiration pipette into the sample rotor valve. Twelve microlitre of
the whole blood sample was measured and diluted into 1 : 250 by adding approximately 2 ml of diluent and 1 ml of WBC lysing reagent in the BC transducer chamber and was aspirated through the aperture and counted. Finally 0.4 ml of diluted volume was used for counting by the electric resistance detection method. The estimated WBC count is expressed as $10^3 \mu l$.

**RBC Sample Flow**

About 4 $\mu l$ of the whole blood sample, measured by the sample rotor valve, was diluted into 1:500 by adding approximately 2 ml of diluent in the mixing chamber and aspirated into the sampler rotor valve again and 40 $\mu l$ of diluted sample was further diluted into 1:50 by adding approximately 2 ml of diluent in the RBC transducer chamber and was aspirated through the aperture and counted. RBC and platelets were counted simultaneously by the electric resistance detection method. RBC count is expressed as $10^6 \mu l$ and platelet count as $10^3 \mu l$. Haematocrit value was computed by cumulative pulse height detection method and its percentage was calculated.

**Differential Leucocyte Cell (DLC) Counts**

For differential leucocyte count, thin blood film was examined systematically, by observing three fields along the edge and two fields above and down. This sequence was continued until a minimum of 100 cells has been enumerated and percentage was calculated.
5. BIOCHEMICAL STUDIES OF SERUM

During blood sampling an addition of 4 ml blood was collected separately in a clean test tube to obtain serum. Serum parameters were assayed in all experimental groups using Serological Kits (ERBA reagents) from Transasia Bio-medicals Ltd., Mumbai - 400 093, on Erba-Chem instrument.

Creatinine

Serum creatinine was estimated by following the method of modified Jaffe reaction (Jendrassik and Grof, 1938).

Creatinine reacts with alkaline picrate to produce a reddish colour. The orange yellow colour formed is directly proportional to creatinine concentration.

To 100 μl unhaemolysed serum, 1000 μl working reagent containing 5.8 mmol/l picric acid and 95 mmol/l sodium hydroxide was added. For standard sample, 100 μl of creatinine standard (0.166 mmol/l) was used. The content of the tubes was thoroughly mixed and was immediately read at 510 nm. The estimated creatinine is expressed as mg/dl.

Total Bilirubin

Serum bilirubin was estimated by following the method of Walter and Gerarde (1970) with dimethyl sulfoxide (DMSO) as accelerator.

Bilirubin reacts with diazotised sulphanilic acid to form a coloured azobilirubin in strongly acidic or alkaline solution Conjugated and
solubilised unconjugated bilirubin react with diazotised sulphanilic acid to produce an acid azobilirubin. To 100 μl of unhaemolysed serum sample, 1000 μl working reagent containing 2.0% surfactant, 300 mmol/l HCl, 100 mmol/l sulphanilic acid and 72 mmol/l sodium nitrate were added. For blank sample 100 μl of distilled water and for standard sample 100 μl of bilirubin standard was used.

Reaction mixtures were mixed well and incubated for 10 minutes in the dark at room temperature and read at 546 nm. The estimated bilirubin is expressed as mg/dl.

Glucose
Serum glucose was estimated by Glucose Oxidase-Peroxidase (GOP) method (Trinder, 1969).

Glucose Oxidase

\[
\text{Glucose} + \text{O}_2 + \text{H}_2 \rightarrow \text{Gluconic acid} + \text{H}_2\text{O}_2
\]

Peroxidase

\[
\text{H}_2\text{O}_2 + \text{Phenol} + 4\text{-Aminoantipyrine} \rightarrow \text{Red dye} + 2\text{H}_2\text{O}
\]

To 100 μl of unhaemolysed serum sample, 1000 μl working reagent containing 20 000 IU/l glucose oxidase, 250 IU/l peroxidase, 0.52 mmol/l 4-aminoantipyrine, 10 mmol/l 4-hydroxy benzoic acid and 110 mmol/l phosphate buffer were added. For blank sample 10 μl of distilled water and for standard sample 10 μl of glucose standard were used. Then reaction mixture was mixed well and incubated for 15 minutes in the
dark at room temperature and the intensity of the pink colour formed is proportional to the glucose concentration and was measured photometrically at 510 nm. The estimated glucose is expressed as mg/dl.

**Blood Urea Nitrogen (BUN)**

BUN was estimated by the method of Talke and Schubert (1965).

**Urease**

\[
\text{Urea} + H_2O \rightarrow 2 \text{NH}_3 + \text{CO}_2
\]

**Glutamate dehydrogenase**

\[
\text{NH}_3 + \alpha\text{-kétoglutarate} + \text{NADH} \rightarrow \text{L-Glutamate} + \text{NAD}
\]

To 20 μl of unhaemolysed serum sample, 1000 μl working reagent containing 7.5 mm/l 2-Oxoglutarate, 0.32 mm/l NADH (yeast), 8 IU/l urease, 1 IU/l glutamate dehydrogenase (GLDH), 1.2 mm/l ADP and 100 mmol/l tris buffer (pH 7.9 ± 0.1 at 25° C) were added. A volume of 20μl of standard reagent containing 50 mg/dl urea was used. Reaction mixture was mixed well and aspirated for standard followed by samples. The absorbance was read at 340 nm and the estimated blood urea nitrogen is expressed as mg/dl.

**Aspartate Aminotransferase (AST) (E.C. 2.6.1.1)**

The methodology of International Federation of Clinical Chemistry was adopted (IFCC. 1980).

**AST**

\[
\text{L-Aspartate} + 2\text{- Oxoglutarate} \rightarrow \text{Oxaloacetate} + \text{L- Glutamate}
\]
Malate dehydrogenase

\[ \text{Oxaloacetate} + \text{NADH} \rightarrow \text{Malate} + \text{NAD} \]

Lactate dehydrogenase

\[ \text{Sample Pyruvate} + \text{NADH} \rightarrow \text{L-Lactate} + \text{NAD} \]

Working reagent was maintained at 37°C before performing the test. To 100 \( \mu l \) of unhaemolysed serum sample, 1000 \( \mu l \) working reagent containing 12 mmol/l 2-oxoglutarate, 200 mmol/l L-aspartate, 545 IU/l malate dehydrogenase, 909 IU/l lactate dehydrogenase, 0.18 mmol/l NADH (yeast), 5 mmol/l EDTA and 80 mmol/l tris buffer (pH 7.8 ± 0.1 at 25°C) were added. After mixing, it was aspirated and the absorbance was read at 340 nm. The activity of aspartate aminotransferase is expressed as IU/l.

Alanine Aminotransferase (ALT) (E.C. 2.6.1.2)

The methodology of International Federation of Clinical Chemistry was adopted (IFCC, 1956).

\[ \text{ALT} \]

\[ \text{L-Alanine} + \text{2-Oxoglutarate} \rightarrow \text{Pyruvate} + \text{L-Glutamate} \]

Lactate dehydrogenase

\[ \text{Pyruvate} + \text{NADH} \rightarrow \text{L-Lactate} + \text{NAD} \]

Working reagent was maintained at 37°C before performing the test. To 100 \( \mu l \) of unhaemolysed serum sample, 1000 \( \mu l \) working reagent containing 400 mmol/l L-alanine, 1820 IU/L lactate dehydrogenase, 0.18
mmol/l NADH (Yeast), 12 mmol/l 2-oxoglutarate and 80 mmol/l tris buffer (pH 7.5 ± 0.1 at 25° C) were added. After mixing solution was aspirated and the absorbance was read at 340 nm. The activity of aspartate aminotransferase is expressed as IU/l.

**Total Protein**

Total protein estimation was followed as per method of Tietz (1986). The peptide bonds of protein react with copper (II) ions in alkaline solution to form a blue-violet complex (Biuret reaction). Each copper ion complexing with 5 or 6 peptide bonds. Tartarate was added as a stabiliser while iodide is used to prevent auto-reaction of alkaline copper complex. To 100 µl of unhaemolysed serum sample, 1000 µl working reagent containing 19 mmol/l Copper II sulphate, 43 mmol/l potassium sodium tartarate, 30 mmol/l potassium iodide and 600 mmol/l sodium hydroxide were added. For blank sample 100 µl of distilled water and for standard sample 20 µl of total protein standard was used. After mixing, incubation was done at room temperature for 10 minutes and the absorbance was read at 546 nm and total protein is expressed as g/dl.

**Cholesterol**

Serum cholesterol was estimated by modified method of Roeschlau (1974).

**Cholesterol esterase**

Cholesterol ester -------------------------------→ Cholesterol + Fatty acid
Cholesterol oxidase

\[
\text{Cholesterol + O}_2 \rightarrow \text{Choles-4-en-3-one + H}_2\text{O}_2
\]

Peroxidase

\[
2\text{H}_2\text{O}_2 + 4\text{Aminoantipyrine + Phenol} \rightarrow 4\text{H}_2\text{O}_2 + \text{Quinoneimine}
\]

To 100 µl of unhaemolysed serum sample, 1000 µl working reagent containing 200 IU/l cholesterol esterase, 150 IU/l cholesterol oxidase, 2000 IU/l peroxidase, 20 mmol/l sodium phenolate, 0.5 mmol/l 4-aminoantipyrine and 68 mmol/l phosphate buffer (pH 6.5 ± 0.1) were added. For blank sample 20 µl of distilled water and for standard sample 20 µl of cholesterol standard were used. Absorbance was read at 510 nm. The estimated cholesterol is expressed as mg/dl.

Radioimmunoassay of Testosterone

The levels of serum testosterone (T) were assayed using the double antibody radioimmunoassay technique of Peterson and Swerdlof (1979). It is based on the principle of competitive binding (Odell and Franchimont, 1983) of an unlabelled hormone (Ag) with unknown concentration of radiolabelled hormone (Ag*) for limited binding sites of the specific antibody (Ab1). At the end of the incubation, the antibody bound and free hormones are separated by the addition of the second antibody (Ab2) and a suitable precipitating agent. The hormone concentration of the sample is estimated by measuring radioactivity associated with bound antigen which decreases as a function of increased concentration of unlabelled antigen (hormone) in the sample.
Blood from the control and treated rats was collected, allowed to clot at room temperature and the tube were centrifuged at 1500 rpm for 15 minutes. The supernatant was collected using a Pasteur pipette. The serum was stored in 5 ml vial at -9°C till analysis. The samples were assayed using radioimmunoassay kits obtained from Sereno Laboratories, Italy. Human testosterone of high purity ranging from 0.15 ng/ml to 20 ng/ml was used as the standard. Specificity of the antibody to testosterone was 100% with 17% cross reactivity to dihydrotestosterone.

Each sample was assayed in duplicate and the assay was prepared as follows:

- Two tubes contained 100 µl labelled antigen (Ag*) to determine the total activity. The purified hormone, labelled with I¹³¹ (half-life of 60 days) of suitable titre was used for each assay as tracer hormone.
- Two tubes, each having 100 µl labelled antigen (Ag*) and 100 µl hormone free serum were used to determine the non-specific binding (NSB) of the labelled antigen.
- The total binding (Bo) was determined by addition of 100 µl antiserum (Ab) to 100 µl labelled antigen (Ag*).
- A series of 5 standards were run in each assay. 100 µl of pure standard antigen (Ab) was incubated with 100 µl of antiserum (Ag) and 100 µl of antigen tracer (Ag*).
- Each sample (100 µl) was incubated along with 100 µl of labelled antigen and 100 µl of purified antiserum.
The reaction mixture was gently vortexed and incubated at room temperature for 30 minutes to 4 hours as per kit protocol. At the end of incubation 100 µl of anti-rabbit gamma globulin (RGG to second antibody) and 0.5 to 1 ml of precipitating agent (8% polyethylene glycol) was added. The tubes were mixed and centrifuged on a cooling Remi C-23 centrifuge at -8°C at a speed of 3000 rpm for 20 minutes to precipitate the bound complexes.

The supernatant was carefully decanted without disturbing the pellet and each tube was blotted free of liquid droplets. The pellet was then counted, keeping each tube for 1 minute in a Beckman automatic gamma counter (Model 5500).

**Calculation**

The Non-specific Binding (NSB) counts were subtracted from all mean counts of sample and standard tubes. The total binding or maximum binding (Bo) was determined and percentage relative binding of the standard and the samples were calculated. The estimation of testosterone is expressed as ng/ml.

\[
\% = \frac{\text{CPM} - \text{NSB}}{\text{Bo}} \times 100
\]

Where,

- CPM is mean counts of sample or standard per minute
Radioimmunoassay of Thyroid Hormones

Thyroid evaluation was carried out by estimating thyroid hormone profiles. Thyroid hormones in the serum were assayed radioimmunologically using kits from Orion diagnostics (Finland) for T₃, T₄ and from Cis Bio International (France) for free T₃ (fT₃) and free T₄ (fT₄). Plasma TSH was done using radioimmunoassay kits from Amersham Pharmacia (U.K.). The principle of the assays are same as mentioned above in estimation of androgen levels. Thyroid stimulating hormone (TSH) levels were expressed as ng/ml, total triiodothyronine (T₃) mentioned as ng/dl and total thyroxine (T₄) as μg/dl whereas the units for free triiodothyronine (fT₃) mentioned as pg/dl and free thyroxine (fT₄) expressed as ng/dl.

6. BIOCHEMICAL STUDIES OF TISSUES

Protein

The protein content in various tissues was estimated according to the method of Lowry et al. (1951). The protein containing sample when treated with phenol reagent of Folin - Ciocalteau, a deep blue colouration develops. This colour development is due to the reaction of alkaline copper sulphate solution with peptide bonds and the reduction of phosphomolybdic acid and phosphotungstic acid by the aromatic amino acids present in the protein. The blue colour developed is quantitatively proportional to the total protein in the medium, which was measured colorimetrically.
To the sample containing 0.2 ml of tissue homogenate, 0.6 ml of distilled water and 4 ml of alkaline copper sulphate solution [a mixture of 100 ml of alkaline Na-K-tartarate (0.1 N NaOH, 2% Sodium carbonate, 0.01% Na-K-tartarate) and 2 ml of 0.5% copper sulphate] were added. In the blank tube, instead of the sample 0.2 ml of distilled water was taken. The tubes were kept for incubation at 37° C for 20 minutes. Then 0.4 ml of Folin Phenol (one part of Folin -Ciocalteau solution diluted with two parts of distilled water) was added to each tube and was mixed thoroughly. Then the tubes were allowed to stand at room temperature for 20 minutes. The optical density was read at 540 nm against the blank. The calculations were done using standardized regression formula. The concentration is expressed as mg protein/100 mg of tissue weight.

**Calculations**

The regression formula was \( Y = 0.109 + 3.106 \times X \)

\[
X = \frac{Y \times \text{Dilution}}{100} \times \frac{100}{\text{Tissue weight} \times \text{Aliquot volume}}
\]

Where,

\( Y = \text{Concentration of optical density} \)

\( X = \text{Optical density of the sample} \)
Glycogen

Glycogen was estimated by the method described by Hassid and Abraham (1957). After digestion of the tissue with potassium hydroxide, glycogen was precipitated with ethanol. The precipitate was reacted with anthrone reagent, the glucose in the hydrolysate was determined colorimetrically.

A known amount of tissue was digested with 1 ml of 30% potassium hydroxide (30 g of KOH was dissolved in 100 ml of distilled water) for few minutes in a boiling water bath. The digest was cooled in an ice bath and 1.25 ml of 95% ethanol (95 ml of 100% ethanol was made upto 100 ml with distilled water) was added. The content was thoroughly mixed and gently allowed to boil in a hot water bath. This was cooled and centrifuged for 15 minutes, at 3000 rpm. The supernatant was discarded and the tubes were allowed to drain on a filter paper for a few minutes, The precipitate was redissolved in 1 ml of distilled water, reprecipitated with 1 ml of 95% ethanol, centrifuged and drained as before. The precipitate was dissolved in 5 ml of distilled water and 10 ml of 0.2% anthrone reagent [0.2 g of anthrone was dissolved in 100 ml of 95% sulphuric acid (95 ml of concentrated sulphuric acid was made upto 100 ml with distilled water)] was added under ice cold conditions. The tubes were covered with glass marbles and heated for 10 minutes in a boiling waterbath. The contents were cooled immediately and the colour was read at 620 nm. To separate tubes, 5 ml of water and 5 ml of standard solution containing 0.1 mg of glucose (100 mg of D-glucose was dissolved in 100 ml of distilled water) were added and treated with anthrone reagent and subjected to the same procedure to serve as blank
and standard respectively. Concentration of glycogen is expressed as mg glucose/g wet tissue.

**Calculations**

The amount of glycogen was calculated from the following equation,

\[
\frac{\text{Optical density of unknown} \times 1000}{1.11 \times \text{optical density of 100 mg of glycogen standard}}
\]

Where,

1.11 = Morris factor for the conversion of glucose to glycogen with this equation.

**Alkaline Phosphatase: (ALPase : E.C. 3.1.3.1)**

ALPase was estimated by the method described by Bessey et al. (1946). The enzyme alkaline phosphatase hydrolyses the \( p \)-nitrophenyl phosphate substrate to inorganic phosphate and \( p \)-nitrophenol. The quantity of \( p \)-nitrophenol released in alkaline medium was measured colorimetrically at 410 nm.

To 1 ml of mixture, equal volume of alkaline buffer and substrate was pipetted in a test tube and incubated at 37\(^0\) C for five minutes and then 0.2 ml of the homogenized tissue (prepared in 5 ml of cold distilled water) was taken in sample tubes and 0.2 ml of distilled water in the blank. After incubation period of 30 minutes, 10 ml of 0.02 M NaOH was added to all the test tubes and optical density was read. To this, 0.1 ml of concentrated HCl was added to all the test tubes. The optical
density was read again and second reading was subtracted from the first one for calculating the enzyme activity. This removes bilirubin contamination and turbidity. The activity of ALPase is expressed as $\mu$ moles of $p$-nitrophenol released/100 mg fresh tissue weight/30 minutes.

**Calculations**

$$\text{O.D. of sample} \times \text{Conversion factor (1.88)} \times \text{Dilution} \times 100$$

$$\text{ALPase activity} = \frac{\text{Tissue weight (mg)} \times \text{Aliquot volume}}{\text{ blank}}$$

**Acid Phosphatase : (ACPase; E. C. 3.1.3.2)**

ACPase activity was estimated by the method of Bessey et al. (1946). ACPase, orthophosphoric monoester phosphohydrolase, catalyses the hydrolysis of $p$-nitrophenyl phosphate at pH 4.8 and liberates $p$-nitrophenol and inorganic phosphate. The liberated $p$-nitrophenol combines with NaOH to form a yellow coloured complex, which was measured directly proportional to the acid phosphatase activity.

A known amount of tissue was homogenized in 5 ml distilled water. To 0.2 ml of homogenate, 0.6 ml substrate (16.5 mg 4-nitrophenyl phosphate dissolved in 10 ml of citrate buffer) was added. The blank contained 0.2 ml of distilled water instead of homogenate. After an incubation period of 30 minutes at $37^0$ C, 4 ml of 0.1 N NaOH was added to all the tubes to terminate the reaction. The activity of the ACPase was measured at 410 nm and is expressed as $\mu$ moles of $p$-nitrophenol released/100 mg fresh tissue weight/30 minutes.
Calculations

O..D. of sample x Conversion factor (0.741) x Dilution x 100

ACPase activity = \frac{\text{O.D. of sample} \times \text{Conversion factor (0.741)} \times \text{Dilution} \times 100}{\text{Tissue weight (mg) \times \text{Aliquot volume}}}

Cholesterol

Estimation of cholesterol in tissues was done by the method of Zlatki et al. (1953) using ferric chloride as the colouring reagent. Cholesterol present in the tissue homogenate reacts with the ferric chloride in the presence of acetic acid to give a colour complex, which is measured colorimetrically.

A known amount of tissue was homogenized in 2 ml of glacial acetic acid. 200 \mu l (0.2 ml) of this homogenate was added to the test tubes containing 5 ml of FeCl\textsubscript{3} solution (0.05 N). In the standard tubes, 0.2 ml of standard cholesterol solution (100 \mu g/0.2 ml) was added. The blank tube was with 0.2 ml of glacial acetic acid instead of homogenate. A volume of 3 ml of concentrated sulphuric acid was added to all the tubes and kept at room temperature for 20 minutes. Optical density was read at 540 nm. The concentration is expressed as mg cholesterol/100 mg of tissue weight.

Calculations

Cholesterol concentration was calculated as follows

\frac{\text{O.D. of sample} \times \text{Concentration of standard [mg]}}{\text{O.D. of standard} \times \text{Aliquot volume} \times \text{Tissue weight}} \times 100 \times 5 \text{ as dilution factor}
3β hydroxy steroid dehydrogenase (3β - HSD)

The testicular 3β - HSD activity was assayed by the method of Talalay (1962). 3β - HSD acts on substrate 3β -hydroxy 5α - androstane -17 – one (epiandrosterone) and reduces nicotinamide adenine dinucleotide (NAD) and the absorbance was measured at 340 nm.

3β - HSD

Epiandrosterone + NAD -----------------> 5 androstane 3,17 dione + NADH

A known amount of tissue was homogenised in 0.02 M phosphate buffer (pH 7.5) along with Triton X-100 (2.5 mg/ml) (1:10 dilution). The homogenate was centrifuged at 8000 g for 30 minutes at 4°C. The supernatant was used for the assay. To 2 ml of 0.1 M sodium pyrophosphate buffer (pH 8.9), 0.2 ml of NAD and 0.4 ml of distilled water were added. The blank was prepared by adding 2 ml of buffer, 0.2 ml of substrate and 0.8 ml of distilled water. Final assay of the volume was 3.0 ml. The reduction in the absorbance of NAD was read at every 15 seconds intervals against controls at 340 nm. The standard curve was prepared using 5α - androstan 3,17 dione.

Calculations

3β - HSD concentration was calculated as follows

\[
\text{O.D. of sample} \times \frac{\text{Concentration of standard}}{\text{O.D. of standard} \times \text{Enzyme volume}} \times \frac{\text{Total volume of assay}}{10}
\]

Under specific condition, the enzyme activity was expressed as nanomoles of 5α dione formed/mg protein/ hour.
17β hydroxy steroid dehydrogenase (17β - HSD)

The testicular 17β - HSD activity was assayed by the method of Talalay (1962). 17β - HSD acts on substrate testosterone and reduces nicotinamide adenine dinucleotide (NAD) to NADH and the absorbance was measured.

\[
\text{17β - HSD} \\
\text{Testosterone + β NAD} \rightarrow \text{Androst-4-ene 3,17 dione + β NADH}
\]

A known amount of tissue was homogenised in 0.02 M phosphate buffer (pH 7.5) along with Triton X-100 (2.5 mg/ml) (1:10 dilution). The homogenate was centrifuged at 8000 g for 30 minutes at 4°C. The supernatant was used for the assay. To 2 ml of 0.1 M sodium phosphate buffer (pH 8.9), 0.2 ml of substrate buffer (1.5 mg testosterone dissolved in 2.0 ml of methyl alcohol) and 0.2 ml of homogenate were added. Then 0.2ml of NAD and 0.4 ml of distilled water were added. Blank was prepared by adding 2.0 ml of buffer, 0.2 ml substrate and 0.8 ml of distilled water. Final volume of the assay was made upto 3.0 ml. The reduction in the absorbance of NAD was read at every 15 seconds intervals against controls at 340 nm. The standard curve was prepared using testosterone as substrate.

Calculations

17β - HSD concentration was calculated as follows

\[
\text{O.D. of sample} \times \text{Concentration of standard} \\
\frac{\text{--------------------------------------------}}{\text{O.D. of standard} \times \text{Enzyme volume}} \times \text{Total volume of assay} \times 10
\]
Under specific condition, the enzyme activity was expressed as nanomoles of 5 α-dione formed/mg protein/hour.

7. SPERM PARAMETERS AND FERTILITY

Preparation of the Sperm Suspension
The sperm suspension for the experimental analysis was prepared from the cauda epididymidis of the experimental animals. The distal end of the cauda epididymidis was cut with scissors, nicked and flushed gently in to normal saline (0.87 %) immediately after the necropsy.

Sperm Count
The cauda epididymal sperm count was carried out according to the method of Prasad et al. (1972) using the Neubauer chamber of a Haemocytometer. Freshly prepared sperm suspension in normal saline [100mg/2 ml] was well mixed and diluted (1:20) with a spermicide (5% NaHCO₃) in a WBC micropipette. The diluted sample was mixed thoroughly and a drop was placed onto the haemocytometer and covered gently with a cover slip. Spermatozoa were then counted in 64 sub-squares of white blood cell counting regions. The sperm count is expressed as million spermatozoa per ml.

Sperm concentration was calculated as

\[ X = \frac{N \times \text{Dilution} \times 1000}{\text{Volume of 64 sub-squares}} \]
Where, \( N \) = total number of sperms counted in 64 sub-squares.

Dilution = 20 times.

Volume of 64 sub-squares = \( \frac{1}{4} \times \frac{1}{4} \times \frac{1}{10} \times 64 = 0.4 \text{ cu. mm.} \)

\[
X = \frac{N \times 20 \times 1000}{0.4}
\]

Sperm Motility
The cauda epididymal sperm motility of the experimental animals was assessed according to the method of Prasad et al. (1972). Freshly prepared sperm suspension was diluted suitably with physiological saline and observed after placing a drop on a Neubauer chamber under low magnification (10 X). The quantitative motility was determined by counting motile and total number of spermatozoa in 20 separate random fields. The percentage of motile spermatozoa was calculated from the mean of per cent motility of all the fields counted.

Sperm Viability [Membrane Integrity]
Percentage of the live spermatozoa was determined using 1% trypan blue as described by the method of Talbot and Chacon (1981).

An aliquot of 0.2 ml of sperm suspension was incubated with 0.2 ml of 1% trypan blue (supravital stain) prepared in (0.88%) physiological saline. A drop of the suspension was placed on the Neubauer chamber under a cover slip and allowed to settle for one minute. Observations were carried out under 40 X magnification of a Nikon binocular
microscope. The number of stained/total number of spermatozoa were scored in 10-20 separate visual fields. The live viable spermatozoa remained unstained while dead sperm took up the supravital stain. This technique makes it possible to differentiate motile but live spermatozoa from motile non-viable ones. In each sample, the percentage of live/dead [non-viable] sperm was calculated as follows

\[
\% \text{ Viable} = \frac{\text{Total number of viable sperm observed}}{\text{Total number of sperm observed}} \times 100
\]

**Fertility Test**

The fertility rate was determined for control, M + M treated animals and 60 days after post treatment in M + M treated animals according to the WHO protocol MB - 50 (WHO, 1983). Male rats were paired for mating with pre-estrous or estrous female rats at the ratio of 1:2 after 60 days treatment with M + M. The cyclicity of the female rats was determined by vaginal smear. The vagina was aspirated with normal saline. The aspirated fluid was smeared onto a clean slide and observed under the microscope. Intromission was observed by a sperm positive smear and the day was designated as the day one of the onset of pregnancy. All the gestating females were allowed to carry their pups to term. Fertile males were those which caused pregnancy and in whose case a healthy litter was born. Failure to give birth to a healthy litter was considered as a parameter to indicate fertility arrest. Copulation rate was also evaluated.
Number of copulations

Copulation rate = \frac{\text{Number of copulations}}{\text{Number of matings}} \times 100

Number of pregnancies

Pregnancy rate = \frac{\text{Number of pregnancies}}{\text{Number of copulations}} \times 100

**Recovery**

To determine the period of recovery in the treated animals, on the last day of recovery, randomly selected males were cohabited with females of proven fertility in a ratio of 1:2. They were kept till the time, the females delivered. Thus, the recovery period was considered as the time in days taken from the day of the last dosing, till the day the litter was born, minus the gestation period of the female Wistar rats, which in this case was taken to 21 days. Of the born litters their survival rate was considered.

**8. HISTOLOGICAL STUDIES (LIGHT MICROSCOPY)**

After termination of an experiment the animals were sacrificed by cervical dislocation and subjected to thorough gross pathological examination of whole viscera and noted for the lesions manifested by the organs.

Immediately after the gross pathological examination, liver, testis, thyroid, kidney and cauda epididymis were cleaned and removed the extraneous material with blood tinges. The cleaned tissues with
appropriate thickness were collected and kept in 10% formalin about a week for fixation.

Well fixed tissues were subjected to routine histopathological processing as described by Culling (1974). The routine histopathological processing which includes dehydration by ascending grades of alcohol and clearing was carried out in xylene. Impregnation and embedding were done by using paraffin wax at 58 to 60 °C.

Transverse sections of 3 to 4 μ thickness were obtained from embedded tissues fixed on slides by using the egg albumin adhesive. The same slides were stained by Haemtoxylin and Eosin (HE) staining technique. The well stained slides were subjected to microscopic examination and were photographed using a Nikon binocular microscope.

9. STATISTICS
Minimum of 8 replicates were done to each haematological, serum and tissue biochemical parameters. The data were statistically analysed using Student’s ‘t’ test (Snedecor and Cochran, 1967). A significance level of P < 0.05 was accepted. Results were presented in tabular forms.