MATERIALS AND METHODS
CHAPTER II
MATERIALS AND METHODS

PART I

ANIMALS

Healthy, adult male albino rats \textit{(Rattus novergicus)} of Charles Foster strain with proven fertility weighing 200-250 gm were used as the experimental animals. These animals were kept in air-conditioned animal house at a temperature of $26\pm2^\circ\text{C}$ and exposed to 12-14 hours of day light. Animals of different experimental groups were caged separately and a maximum of four animals per cage was maintained. The control as well as the other experimental animals were given free access to standard diet and water \textit{ad libitum}.

CHEMICALS

Depot medroxy progesterone acetate (DMPA) was procured as Depo provera from Up John Co., in microcrystalline form.

The non-aromatizable androgen, dihydrotestosterone (DHT) was purchased from Sigma Chemical Co., U.S.A. Pure Italian Olive oil was used as vehicle for administration. The analytical reagents were obtained from Hi Media Laboratories Private Limited, Mumbai.
PREPARATION OF HORMONES FOR INJECTION

Medroxyprogesterone acetate was dissolved in few drops of alcohol and made as microcrystalline suspension upto desired volume of olive oil. Same way dihydrotestosterone was also prepared.

MODE OF ADMINISTRATION

The treatments were given as bimonthly intramuscular injections using olive oil as the vehicle. A tuberculin syringe fitted with a 24 gauge needle was used for the injections. As the combination regimen of MPA and DHT care was taken not to inject both the drugs on the same thigh as it could cause a masking effect.

DOSE AND DURATION OF THE TREATMENT

The initial selection of the dose was according to the earlier published reports which was further standardized according to the laboratory conditions. The MPA + DHT treated and the recovery groups received injections of MPA (20 mg.kg⁻¹.d⁻¹) and DHT (1000 μg.kg⁻¹.d⁻¹) for 90 days. The recovery study in this group was carried out after 90 days of the cessation of the treatment. A minimum of 20 rats were used for each experimental group.

BODY WEIGHT

The body weights of all the experimental groups of animals were recorded to the nearest gm before and after the cessation of the treatment.
FERTILITY TEST

The fertility rate of control and other experimental animals was assessed according to the WHO Protocol MB.50 (1983). Experimental male rats were paired randomly for mating with proestrous or estrous female rats of proven fertility in a ratio of 1:2. The cyclicity of the females were determined by vaginal smear. The vagina was aspirated with normal saline. The aspirated fluid was smeared on a clean slide and observed under the microscope. Intromission was observed by a positive smear or copulation plug and the day was designated as the day one of the onset of pregnancy. Fertile males were those which caused pregnancy and in whose case healthy litter was born. Failure to give birth to a healthy litter was considered as a parameter to indicate fertility arrest. Mating rate was also evaluated.

SAMPLING

On the completion of the respective experimental periods the animals were sacrificed under light ether anesthesia. The blood was collected by cardiac puncture and kept in cool temperature for 2-4 hours from which the serum was separated by centrifugation and stored at -9°C for further analysis. The fresh unclotted blood was used for hematological parameters.

ABSOLUTE ORGAN WEIGHTS

The organs were quickly excised, cleared off from the adhering fat and blotted free of blood. The absolute weights of the testis, cauda and caput epididymides and the vas
deferens of different experimental groups were recorded to the nearest milligram on Roller Smith Torsion Balance. Then the tissue were processed for various experimental studies.

SPERM PARAMETERS

SPERM MOTILITY

The cauda epididymal sperm count 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 Neubauer chamber under low magnification (10x). The quantitative motility was determined by counting the number of motile and total number of spermatozoa in 20 separate fields. The percentage of motile spermatozoa was calculated from the mean of percent motility of all the fields observed.

SPERM COUNT

The cauda epididymal sperm count was carried out according to the method of Prasad et al. (1972) using Neubauer chamber of haemocytometer. Freshly prepared sperm suspension from cauda epididymidis in normal saline (100 mg/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 on the Neubauer chamber and gently covered with a cover slip. Spermatozoa was then counted in 64 sub squares of the white blood cell counting regions.
Sperm concentration was calculated as

\[ X = \frac{N \times \text{dilution} \times 1000}{\text{Volume of 64 subsquares}} \]

where, \( N \) = total number of sperm counted in 64 subsquares.

\( \text{Dilution} = 20 \) times

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

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

The sperm count was expressed as million sperm per ml.

**SPERM VIABILITY**

The percentage of the live spermatozoa was determined using 1% trypan blue (supravital stain) 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 0.1% trypan blue stain prepared in (0.87%) 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 40x magnification of a Nikon binocular microscope. The number of stained/total number of spermatozoa were scored in 10-12 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) spermatozoa was calculated as follows:

\[
\text{% viable} = \frac{\text{Total no. of viable sperm observed}}{\text{Total no. of sperm observed}} \times 100
\]

**DIFFERENTIAL STAINING OF SPERMATOZOA (ACROSOME INTEGRITY)**

The modified alcoholic acidic silver nitrate (AgNO₃) staining technique of Chinoy et al. (1992) was used to evaluate differential staining patterns of acrosomal, sub-acrosomal and post-acrosomal regions in spermatozoa from the experimental animals. The silver nitrate properties of the sperm are attributed to the presence of protein bound sulphhydryl and disulphied moieties which are richly distributed in the sperm membranes, particularly those of the post-acrosomal region. Freshly prepared 0.2 ml cauda epididymal sperm suspension was washed with balanced Hanks salt solution (Ca⁺² and Mg⁺² free) and finally expressed in 0.2 ml of the same solution. This suspension was smeared uniformly on a clean glass slide, air dried and fixed in 70% and 90% ethanol for 2 minutes each. The slides were then stained with 1-2 drops of 5% alcoholic acidic silver nitrate reagent (5 gm silver nitrate, AgNO₃) in 34 ml of distilled water and added to 66 ml of absolute alcohol and 5 ml glacial acetic acid. To each slide, 1-2 drops of 1% gelatin solution containing 10 drops of formic acid was added. The slides were covered with coverslip and placed at 4°C overnight in a moist air tight chamber. After incubation, the studies were differentiated in 5% alcoholic ammonia, dehydrated in 90% and absolute
alcohol and cleared in xylene. The spermatozoa were observed under 40X magnification on a Nikon binocular microscope and photographed.

**BIOCHEMICAL PARAMETERS**

**3 β HYDROXYSTEROID DEHYDROGENASE (3β HSD; E.C.1.1.1.51)**

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

\[
3 \beta \text{ HSD} \\
\text{Epiandrosterone} + \text{NAD} \rightarrow 5, \text{androstane} 3, 17 \text{ dione} + \text{NADH}
\]

A known amount of tissue was homogenized 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 substrate [(3 mg epiandrosterone; Sigma Chemical Co., U.S.A.), in 2 ml of N, N-dimethyl formamide)] and 0.2 ml of homogenate were added. Then 0.2 ml of NAD and 0.4 ml of glass 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 volume of the assay was 3 ml. The reduction in absorbance of NAD was read at every 15 second intervals against controls of 340 nm on a Systronics UV-visible spectrophotometer (Model 118). The protein content in the tissue was
estimated by the method of Lowry et al. (1951). The standard cure was prepared using 5α-androstane 3,17 dione.

**CALCULATION**

<table>
<thead>
<tr>
<th>Sample O.D.</th>
<th>Standard Concentration</th>
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<tbody>
<tr>
<td>------------</td>
<td>------------------------</td>
</tr>
<tr>
<td>Standard O.D.</td>
<td>Enzyme volume</td>
</tr>
</tbody>
</table>

\[
\text{Sample O.D.} \times \frac{\text{Standard Concentration}}{\text{Standard O.D.}} \times \text{Total volume of assay} \times 10
\]

Under specific condition, the enzyme activity was expressed as nanomoles of 5α-dione formed/mg protein/hour.

**17ß HYDROXYSTEROID DEHYDROGENASE (17ß HSD; E.C.1.1.51)**

The testicular 17ß hydroxysteroid dehydrogenase activity was assayed by the method of Talalay (1962). The enzyme 17ß hydroxysteroid dehydrogenase acts on substrate, testosterone and reduces nicotinamide adenine dinucleotide (NAD) to NADH and the absorbance was measured.

\[
17 \beta \text{ HSD} \\
\text{Testosterone} + \beta - \text{NAD} \rightarrow \text{Androst-4-ene 3,7 dione} + \beta \text{ NADH}
\]

A known amount of tissue was homogenized in 0.02 M phosphate buffer (pH 7.5) along with Triton X-100 (2.5 mg/ml) (1:10 dilutions). 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 0.2 ml of NAD and 0.4 ml of glass 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 ml. The reduction in absorbance of NAD was read at every 15 second intervals against control at 340 nm on a Systronics UV-visible Spectrophotometer (Model 118). Protein estimation in the tissue was carried out by the method of Lowry et al. (1951). The enzyme activity was expressed as nanomoles of 5α dione formed/mg protein/30 minutes.

**CALCULATION**

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

The standard curve was prepared using testosterone as substrate.

**SUCCINATE DEHYDROGENASE (SDH; E.C.1.3.99.1)**

The succinate dehydrogenase (SDH) activity was assessed by the modified tetrazolium reduction method of Beatty et al. (1966). In the assay system, the electrons released by the action of the enzyme on the substrate sodium succinate was accepted by an electron acceptor (2-4 iodophenyl 3-4-nitrophenol) 5-phenyl tetrazolium chloride (INT) which is reduced to a red coloured formazan. This resultant formazan was extracted in ethyl acetate and the colour intensity was measured using a Systronics 103 colorimeter at 420 nm.
A known amount of tissue was homogenised in desired volume of cold distilled water. The reaction mixture contained 0.4 ml of tissue homogenate, 1 ml of 0.2 M phosphate buffer (pH 7.6), 1 ml of 0.1 M sodium succinate and 1 ml freshly prepared INT solution (1 mg/ml). The blank tube was substituted by 1 ml of distilled water instead of INT solution. Then the tubes were incubated for 15 minutes followed by the addition of 0.1 ml of 30% TCA to stop the reaction. The formazan formed was then extracted in 7 ml of ethyl acetate by shaking vigorously. The solution was centrifuged for 5 minutes at 2000 rpm and the supernatant was taken off for measuring the optical density.

The concentration of the formazan was calculated according to the standard regression formula.

\[ X = 1.866 + 152.21 \times (Y) \]

where,

- \( X \) = Concentration of formation in \( \mu \)g.
- \( Y \) = O.D. of unknown sample

The final SDH was calculated as

\[
\text{Activity} = \frac{\text{Mean concentration of OD} \times \text{Dilution} \times 100}{\text{Tissue wt.} \times \text{aliquot volume}}
\]

The SDH activity was expressed as micrograms formazan formed/15 min/mg protein.
ALKALINE PHOSPHATASE (ALKPASE) : (E.C.3.1.3.1)

Alkaline phosphatase in the tissues was estimated by Bessey et al. (1946). The enzyme alkaline phosphatase hydrolyses the substrate p-nitrophenyl phosphate into inorganic phosphate and p-nitrophenol. The quantity of p-nitrophenol released under standardized condition was measured at 410 nm.

One ml of mixture of equal volume of alkaline buffer and substrate is pipetted in a test tube and incubate at 37°C for 5 minutes and then add 0.2 ml of the homogenized tissue in sample tubes and 0.2 ml of distilled water in the blank test tube. After incubation period of 30 minutes, 10 ml of 0.02 N NaOH is added in all the tube. Read the absorbance at 410 nm for calculating enzyme activity.

The enzyme activity was calculated using standard curve as following.

\[
\text{Activity} = \frac{\text{Concentration of sample}}{\text{Tissue weight}} \times \frac{\text{Conversion factor}}{\text{Aliquot vol.}} \times 100
\]

Conversion factor = 0.741

The enzyme activity was expressed as \(\mu\) moles p-nitrophenol released/30 min/mg protein.

ACID PHOSPHATASE (ACPASE; E.C.3.1.3.2)

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

A known amount of tissue was homogenised in desired amount of distilled solution (16.5 mg of 4-nitrophenyl phosphate dissolved in 10 ml of citrate buffer). The blank tubes are run with 0.2 ml of distilled water. All the tubes are kept for 30 minutes at 37°C. Then 4 ml of NaOH was added to all the tubes to terminate the reaction. The activity of ACPase was measured at 410 nm wavelength, on a Systronics 103 Colorimeter and calculated according to the following formula.

\[
\text{Enzyme activity} = \frac{\text{Conc. of unknown sample} \times \text{Conversion factor}}{\text{Tissue weight} \times \text{aliquot volume}} \times \text{Dilution} \times 100
\]

Conversion factor = 0.741 (obtained from standard graph)

The ACPase enzyme activity was expressed as \( \mu \) moles of paranitrophenol released/mg protein/30 minutes.

**PHOSPHORYLASE ACTIVITY (E.C.; 2.4.1.1)**

The activity of phosphorylase enzyme in the liver was assayed by the method of Cori et al. (1943). A known amount of tissue (100 mg) was homogenized in 2 ml of fresh cold distilled water. To a cold incubation mixture containing 0.2 ml citrate buffer (0.1 M; pH 5.9), 0.3 ml of potassium fluoride (0.15 M) and 1 ml of glucose-1-phosphate (0.2 M disodium salt in distilled water), 0.1 ml of the tissue homogenate was added. The blank tube was run by the incubation medium devoid of the substrate. The solutions were then mixed and incubated for 20 minutes at 30°C, after which 0.5 ml of 10% TCA was added
to stop the reaction. The incubated solutions were kept at 4°C for 10 minutes and then centrifuged at 3000 rpm for 10 minutes and the supernatant was made up to approximately 8 ml with distilled water and the tubes were placed in ice cold water bath at 20°C. For blank 0.1 ml TCA was added instead of the homogenate. One ml of ammonium molybdate was added to all the tubes followed by 0.5 ml of 2,4, amino naphthol sulphonic acid (ANSA) reagent to make the total volume to 10 ml with distilled water. The solution was mixed and allowed to stand for 6 minutes and the optical density of the blue colour developed was read at 660 nm to calculate inorganic phosphate (Fiske and Subba Row (1925)). The enzyme activity was expressed as μ moles of i.p. released/15 min/mg protein.

**CALCULATION**

The phosphorylase activity is determined by the following regression formula.

\[ X = 2.5063 + 85.76Y \]

where, \( X \) = concentration of phosphorylase

\( Y \) = OD of unknown sample

\[
\text{Phosphorylase activity} = \frac{\text{‘}X\text{’ x dilution}}{\text{Aliquot volume}} \times \frac{\text{tissue wt. in mg x 100}}{\text{Aliquot volume}}
\]

The enzyme activity was determined by substituting the value of ‘X’ in the above formula.
The protein content was done according to the method of Lowry et al. (1951). The protein containing sample when treated with phenol reagent of Folin-Ciocalteu, a deep blue colour develops. The colour development is due to two reactions occurring viz., 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 is measured colorimetrically.

To the sample tubes 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 (0.1N 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 physiological saline 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-Ciocalteu solution (2N) diluted with 2 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 on a Systronics 103 Colorimeter against the blank. The calculations were done using standardized regression formula.

\[
X = 724.166Y + 6.26
\]

where, \( X \) = concentration of sample

\( Y \) = OD of sample
The regression formula used is follows.

\[
\text{Conc. of protein} = \frac{X \times \text{dilution}}{\text{Tissue weight} \times \text{Aliquot Volume}} x 100
\]

The concentration was expressed as mg protein/100 mg tissue wt.

**SIALIC ACID**

A periodate resorcinol method (Jourdian et al., 1971) was used for the quantitative determination of free and glycosidically bound sialic acids.

This method involves the oxidation of total sialic acid i.e., the free and glycosidically bound sialic acid by the treatment with periodic acid which forms a chromogen with resorcinol reagent. The chromogen was extracted in an organic solvent and compared with standard at 630 nm.

To the sample tube containing not more than 0.2 µ mole of N-acetyl neuraminic acid in a total of 0.5 ml and to it was added 0.1 ml of 0.04 M periodic acid solution, mixed and allowed to stand in an ice bath for 20 minutes. Then add 1.5 ml resorcinol reagent (0.6%). The solutions were again mixed and placed in an ice bath for 5 minutes, then heated at 100°C for 15 minutes, cooled under tap water and 1.25 ml of tertiary butyl alcohol was added and vigorously mixed. For the colour stabilization, the tubes were kept in water bath at 37°C for 3 minutes. Then cooled the tubes at room temperature and the absorbance was read at 630 nm on a Systronics 103 colorimeter. Standard (0.1 mg/ml) concentration was run alongwith the sample for each estimation. The concentration of
sialic acid was calculated as follows.

\[
\text{O.D. of sample} \times \text{Conc of Std.} \times \text{Dilution} \times \frac{\text{O.D. of Std.}}{\text{Tissue wt.} \times \text{Aliquot volume}} \times 1000
\]

The concentration was expressed as \(\mu\text{g/mg}\) fresh tissue weight.

**GLYCOGEN**

The concentration of glycogen in the vas deferens was estimated by the method of Seifert et al. (1950). The tissue was digested in boiling 30% KOH and glycogen was precipitated in alcohol which was then dissolved in water and then heated with anthrone reagent made in sulfuric acid (85%) so as to convert glycogen into glucose. The latter gives colour reaction with anthrone reagent which is measured calorimetrically. In each 15 ml centrifuge tube, 2 ml of 30% KOH was taken and the tubes were kept in a boiling water bath. Accurately weighed fresh tissue (20 mg) was transferred to hot KOH and the tubes were heated for 10 minutes to completely digest the material. After digestion the material was cooled and 3 ml 95% ethyl alcohol was added to each tube and their contents mixed thoroughly by shaking. The tubes were then placed in a boiling water bath till alcohol was boiled. After cooling, the tubes were centrifuged for 15 minutes at 3000 rpm. The supernatant containing KOH-alcohol mixture was decanted off and again 3 ml of ethyl alcohol was added to the tubes and kept in refrigerator for 30 minutes in order to precipitate the glycogen and then the solutions were centrifuged. After discarding the supernatant, the test tubes containing the residue were inverted at an angle to drain off the liquid completely.
The precipitates were then dissolved in a known volume of distilled water (5 ml) and 1 ml aliquot of the same was used for estimation. The sample test tube contained 1 ml aliquot whereas in the blank and standard tubes 1 ml of distilled water and 1 ml of standard glucose solution (40 µg/ml) were added respectively.

All the tubes were transferred to an ice bath and 4 ml of anthrone reagent was added (0.2% anthrone prepared freshly in 95% sulfuric acid). The test tubes were shaken thoroughly and heated in boiling water bath for exactly 4 minutes. Once again the tubes were placed in an ice bath to cool. The intensity of the green colour developed was read on a Systronics 103 Colorimeter at 620 nm as % transmission after setting the instrument at 100% transmission with the blank.

The glycogen content was calculated as

\[
\frac{\% \text{ transmission of sample} \times \text{Conc. of Std.} \times \text{Dilution} \times 100}{\% \text{ transmission of Std.} \times \text{Tissue wt.} \times \text{Aliquot vol.} \times \text{Conc. factor}}
\]

where,

- Concentration of the standard = 40 µg
- Conversion factor = 1.11
- Dilution factor = 5

The concentration was expressed as µg glycogen/100 mg fresh tissue weight.
TOXICOLOGY PARAMETERS

(A) HAEMATOLOGICAL PARAMETERS

HAEMOGLOBIN CONTENT

The haemoglobin content of the blood from the control and the experimental group of animal was estimated using standard haemoglobinometer. To the calibrated tube of the haemoglobinometer containing 0.2 ml of 1 N HCl, 20 μl of fresh non-clotted blood was added. Kept for 5 minutes and mixed thoroughly and diluted with distilled water and compared with reference medium. The haemoglobin content was expressed in gm %.

BLOOD CELL COUNTS

The Red Blood Cell (RBC) and White Blood Cell (WBC) counts from control and experimental group of animals were carried out using the Neubauer chamber of a Haemocytometer. For RBC, fresh unclotted blood was sucked upto 0.5 mark of the RBC pipette and was diluted upto 101 mark (200 times dilution) with RBC diluting fluid (0.25 gm of MgCl₂, 2.5 gm of Na₂SO₄ and 0.5 gm of NaCl in 100 ml of distilled water and mixed vigorously. One drop of this suspension was placed on the Neubauer chamber with a coverslip on it. Counting was carried out in RBC squares with the help of a binocular microscope under 10X magnification.

For WBC counts, the fresh unclotted blood was diluted upto 20 times in the WBC pipette of the haemocytometer using the WBC diluting fluid (0.5 ml of glacial acetic acid in 100 ml of distilled water with a drop of gentian violet). The count was carried out in the WBC counting columns of the Neubauer chamber. The RBC count was expressed as
million/cubic mm and WBC count was thousand per cubic mm.

(B) SERUM PARAMETERS

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). An unlabelled hormone (Ag) of unknown concentration in the standard or sample competes with a known concentration of radiolabelled hormone (Ag*) for the limited sites of the specific antibody (Ab₁). At the end of incubation, the antibody bound and free hormones are separated by the addition of the second antibody (Ab₂) and a suitable precipitating agent. The hormone concentrations of the samples are quantitated by measuring radioactivity associated with the bound fractions of the samples or standard. The percentage of bound radiolabelled antigen decreases as a function of increase in concentration of unlabelled antigen in the sample.

Blood from the control and treated rats was collected, allowed to clot at room temperature and the tubes were centrifuged at 1500 rpm for 15 minutes. The supernatant was collected using a pasteur pipette. The serum was stored in 5 ml vials of -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 mg/ml to 20.0 mg/ml was used as the standard. Specificity of the antibody to testosterone was 100% with 17% cross reactivity to dihydrotestosterone.
METHOD

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

1. Two tubes contained 100 µl labelled antigen (Ag*) to determine the total activity. The purified hormone, labelled with I\(^{125}\) (half life of 60 days) of suitable litre was used for each assay as tracer hormone.

2. 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.

3. The total binding (Bo) was determined by addition of 100 µl antiserum (Ab) to 100 µl labelled antigen (Ag*). The antiserum was raised in rabbit and kept in cool temperature as prescribed by kit protocol.

4. A series of 5 standard tubes of different concentrations were run in each assay. 100 µl of the pure standard antigen (Ag) was incubated with 100 µl of antiserum (Ab) and 100 µl of antigen tracer (Ag*).

5. 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 one minute in a Beckman automatic gamma counter (Model 5500).

CALCULATION

The non specific binding (NSB) counts were substrated 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 samples were calculated using the following formula.

\[
\frac{\text{Mean counts of sample or Std/Min (CPM)} - \text{Non-specific binding (NSB)} \times 100}{\text{Maximum binding (Bo)}}
\]

The dose response curve was plotted as the percent relative concentration of each standard (X-axis) on a logit log graph paper. The percent relative binding of each sample was interplotted on the standard curve and the corresponding sample antigen concentration was determined and expressed as ng/ml of serum.

SERUM PROTEIN

The serum protein was assayed by the method of Lowry et al. (1951) as mentioned earlier. A volume of 0.2 ml of serum was used in this assay and units were expressed as mg protein/100 ml serum.
SERUM CHOLESTEROL

The serum cholesterol was assayed by the method of Zlatki et al. (1953) as mentioned in Part-II.A. A volume of 0.2 ml serum was used in the assay and units were expressed as mg/mg serum.

SERUM GLUTAMATE OXALOACETATE TRANSAMINASE OR SERUM ASPARTATE AMINOTRANSFERASE (SGOT OR AAT : E.C.; 2.6.1.1.)

The photometric assay of SGOT was carried out by the method of Reitman and Frankel (1957). The serum was allowed to act in a buffered solution of Ketoglutaric acid and L-asparatate. SGOT catalyses the reaction of

\[ \alpha\text{-Ketoglutaric acid} + \text{aspartic acid} \rightarrow \text{oxaloacetic acid} + \text{glutamic acid} \]

The oxaloacetate produced forms a coloured complex i.e., a formazan with 2-4 dinitrophenyl hydrazine in alkaline medium. As the \(\alpha\)-ketoglutarate present in the sample also forms a hydrazine the measurements were counted in 500-550 range where the absorbance of different hydrozons differ maximally.

The serum diluted with physiological saline (1:2) was used for the estimation. The assay medium consisted one 1 ml substrate buffer (0.1 M \(K_2HPO_4\); 0.2 M L-aspartate and 0.002 M \(\alpha\)-Ketoglutarate in distilled water. The pH was adjusted (using 1 M \(NaOH\)), which was stabilized for 5 minutes at 37\(^\circ\)C for exactly one hour. The blank was run with 0.2 ml normal saline instead of the serum. Then 1 ml of colouring reagent (0.001 M, 2,4 dinitrophenol hydrazine) in 1 N \(HCl\) was added and kept exactly for 20 minutes at room temperature and then 10 ml of 0.4 M \(NaOH\) was added, mixed thoroughly and allowed
to stand for 5 minutes. The measurement of the absorbance of sample against the blank was taken at 545 nm on a Systronics 103 Spectrophotometer. The concentration was read off from the measured absorbance of known standard as given below.

SERUM GLUTAMATE PYRUVIC TRANSAMINASE OR ALANINE AMINOTRANSFERASE (SGPT OR ALT: E.C.; 2.6.1.2.)

SGPT catalyses the reaction of

\[ \alpha\text{-Ketoglutaric acid} + \text{alanine} \rightarrow \text{Pyruvic acid} + \text{glutamic acid} \]

The assay was carried out according to the method of Reitman and Frankel (1957) as described earlier for SGOT. Here the pyruvate produced forms a coloured complex i.e. formazan with 2-4 dimtrophenyl hydrazine. The assay medium consisted of D-4 alanine instead of aspartate. The serum (diluted with normal saline 1:2) was incubated with the substrate buffer of pH 7.4 (0.1 M KH₂PO₄; 0.1 M K₂HPO₄; 0.2 M D-L alanine and 0.002 M \(\alpha\)-ketoglutarate) in a test tube and incubated at 37°C for exactly 30 minutes. The rest of the assay was same as that of SGOT.

The concentration was determined from the measured absorbance by reading them off from the calibration table and expressed in mIU/ml serum.
<table>
<thead>
<tr>
<th>O.D.</th>
<th>SGOT Value</th>
<th>O.D.</th>
<th>SGPT Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.02</td>
<td>3</td>
<td>0.02</td>
<td>3</td>
</tr>
<tr>
<td>0.04</td>
<td>6</td>
<td>0.04</td>
<td>5</td>
</tr>
<tr>
<td>0.06</td>
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<tr>
<td>0.08</td>
<td>14</td>
<td>0.08</td>
<td>12</td>
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<tr>
<td>0.10</td>
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<td>0.10</td>
<td>15</td>
</tr>
<tr>
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<td>23</td>
<td>0.12</td>
<td>19</td>
</tr>
<tr>
<td>0.14</td>
<td>28</td>
<td>0.14</td>
<td>23</td>
</tr>
<tr>
<td>0.16</td>
<td>34</td>
<td>0.16</td>
<td>27</td>
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<td>41</td>
<td>0.18</td>
<td>31</td>
</tr>
<tr>
<td>0.20</td>
<td>50</td>
<td>0.20</td>
<td>35</td>
</tr>
<tr>
<td>0.22</td>
<td>60</td>
<td>0.22</td>
<td>40</td>
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<tr>
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<td>72</td>
<td>0.24</td>
<td>45</td>
</tr>
<tr>
<td>0.26</td>
<td>86</td>
<td>0.26</td>
<td>50</td>
</tr>
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<td></td>
<td></td>
<td>0.28</td>
<td>55</td>
</tr>
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<td></td>
<td></td>
<td>0.30</td>
<td>61</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.32</td>
<td>68</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.34</td>
<td>75</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.36</td>
<td>83</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.38</td>
<td>93</td>
</tr>
</tbody>
</table>
PHYSIOLOGICAL CONTRACTIONS OF VAS DEFERENS

The vas deferens from different groups of animals were dissected out immediately after cervical dislocation. The isolated tissue was cleaned and flushed free of seminal materials, and mounted in an isolated double organ bath containing modified Kreb's bicarbonate solution of a pH 7.4 at 37°C. The composition of the Kreb's solution is NaCl 94.01 mM; KC1 4.69 mM; 9 mM; MgSO4 25 mM; NaH2PO4 mM 1.17 mM and glucose 10.75 mM. The physiological salt solution (PSS) was continuously bubbled with air. The stabilizatin period was 30 minutes. During the period the physiological saline solution (PSS) was changed at an interval of every five minutes. The responses were recorded on electric physiograph. A tension of 0.5 gm was exerted on the vas deferens. After the stabilization, the organ was exposed separately to gaded dosea of (50 to 500 μm) of adrenalin (epinephrine; Sigma Chemicals Co., USA) and recordings were done with each dose. The conduct time of each dose was 45 seconds. The dose response curve was recorded on electric physiograph and then the 5% dose response was calculated with respect to each peak height against the maximum dose response and compared with the different groups of animals. The graphic representation of each group has been given.

HISTOLOGICAL AND MORPHOMETRIC STUDIES .

The histology of testis, epididymis and vas deferens was carried out by the standard haematoxylon-eosin (HE) staining technique (Gurr, 1962). The tissues were blotted free of blood and fixed in the Bouins Fixative. The tissues were then transferred to 70% alcohol till the yellow colour disappeared. The tissues were dehydrated through
90% and absolute alcohol cleared in xylene (5-15 minutes) and then transferred to molten wax (58-62°C) in an oven for 2-3 hours, to prepare the blocks. The tissues in the wax blocks were microtomed at a thickness of 4-5 µm, overnight and stained with haematoxylin and eosin Clear, stained sections were photographed using Nikon Microscope. Histocytometry was done using ocular lens.

STATISTICAL ANALYSIS

A minimum of 8-10 replicates were done for each biochemical parameter and tissue and the data were statistically analysed using Student’s ‘t’ test. A value of P<0.05 is considered to be significant.

PART - II

ANIMALS

Healthy, colony bred adult female albino mice (Mus musculus) of Swiss inbred strain were used for the experiments. The mice weighed 30-40 gms. The animals were housed in an air conditioned animal house at a temperature of 26±2°C and exposed to 12-14 hours of day light and maintained on standard chow and water ad libitum. A maximum of 5-6 animals per cage were maintained. Animals under different groups of experiments were caged separately.

PREPARATION OF ALCOHOLIC EXTRACTS

Alcoholic extracts (70%) from Pericarp of unripe fruits of Balanites roxburghii
and whole plant of *Phyllanthus amarus* were prepared by using following WHO protocols.

Mature green fruits of *Balanites roxburghii* were collected during March and September in our University Campus. The green pericarp of these fruits was separated and dried at 55-60°C in an oven and powdered to 40 mesh. Plants of *Phyllanthus amarus* were collected during rainy season and dried at 55-60°C in an oven and powdered to 40 mesh.

About 50 gms of the powder was taken in a conical flask and was covered with sufficient volume (1 litre) of 70% alcohol. The contents of the flask were heated on a water bath intermittently and macerated overnight. The extract was filtered, returned to the flask and extraction process was repeated two more times with additional amount of 70% alcohol. The extracts were pooled and concentrated under vacuum using rotavaporation. The syrup extract left was completely dried in a vacuum oven. The extracts were stored in air tight bottles in the refrigerator. This extract was used by dissolving it in double distilled water in a required manner.

**DOSAGE AND DURATION OF THE TREATMENT**

A dose of 100 mg.kg⁻¹.d⁻¹ body weight was fed orally to mice using a feeding metal canula. The feeding was done at the same time of the day daily. The animals of each group received 100 mg/kg of *Phyllanthus amarus* and *Balanites roxburghii* respectively. The recovery study in these groups was carried out after 45 days of the cessation of the treatment. A minimum of 20 animals were used for each group. The extract was fed for 30 days and the experimental animals were grouped as follows.
<table>
<thead>
<tr>
<th>Group</th>
<th>Extract feeding</th>
<th>No. of animals used</th>
<th>Autopsy Day</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Control</td>
<td>25</td>
<td>Sacrificed along with treated</td>
</tr>
<tr>
<td>II</td>
<td><em>B. roxburghii</em> fed 100 mg/kg body wt./mice for 30 days</td>
<td>20</td>
<td>31st day</td>
</tr>
<tr>
<td>III</td>
<td><em>P. amarus</em> fed 100 mg/kg body wt./mice for 30 days</td>
<td>20</td>
<td>31st day</td>
</tr>
<tr>
<td>IV</td>
<td>45 days withdrawal of <em>B. roxburghii</em> fed mice for 30 days</td>
<td>20</td>
<td>76th day</td>
</tr>
<tr>
<td>V</td>
<td>45 days withdrawal of <em>P. amarus</em> fed mice for 30 days</td>
<td>20</td>
<td>76th day</td>
</tr>
</tbody>
</table>

After the last day of the respective treatments, the body weights of each animal was recorded and were then sacrificed along with normal animals. Blood was collected in sterile tubes by cardiac puncture. Ovary and uterus of each animal were excised from the surrounding tissue and blotted free of blood. The tissue were weighted on a torsion balance. The blood was collected by cardiac puncture to obtain serum.

**WHOLE BODY AND ORGAN WEIGHTS**

After the treatment, the animals were weighed and autopsied. The ovary and uterus were excised and weighed to the nearest milligram on a torsion balance.

**FERTILITY TEST**

The fertility rate of control and other experimental animals were assessed according to the WHO protocol MB50 (1983) as described earlier in Part-I.
BIOCHEMICAL PARAMETERS

3\Beta HYDROXY STEROID DEHYDROGENASE (3\Beta HSD) (E.C.1.1.1.51)

The ovarian 3\Beta hydroxysteroid dehydrogenase activity was assayed by the method of Talalay (1962) as mentioned earlier in Part-I.

The enzyme activity was expressed as nano moles of 5\alpha-dione formed/mg protein/hour.

17\Beta HYDROXY STEROID DEHYDROGENASE (17\Beta HSD) (E.C.1.1.1.51)

The ovarian 17\Beta hydroxysteroid dehydrogenase activity was assayed by the method of Talalay (1962) as described earlier in Part-I.

The enzyme activity was expressed as nano moles of 5\alpha-dione formed/mg protein/30 minutes.

PHOSPHORYLASE ACTIVITY (E.C.2.4.1.1)

The activity of phosphorylase enzyme in the uterus was assayed by the method of Cori et al. (1943) as described in Part-I.

CHOLESTEROL

Estimation of cholesterol 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 calorimetrically.

A known amount of tissue was homogenized in 2 ml of the glacial acetic acid. Two hundred µl (0.2 ml) of this homogenate was added to the test tubes containing 5 ml of FeCl₃ solution (0.05 N). In the standard tubes, 0.2 ml of Std. cholesterol solution (100 µg/0.1 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 H₂SO₄ was added to all the tubes. The tubes were kept at room temperature for 20 minutes. After colour development, the optical density was read at 540 nm on a Systronics 103 Spectrophotometer.

**Calculation**

Cholesterol concentration was calculated by the following formula

\[
\frac{\text{O.D. of sample} \times \text{conc. (0.2) of Std. in mg} \times 100}{\text{O.D. of Std.} \times \text{Aliquot vol.} \times \text{Tissue wt.}}
\]

The concentration was expressed as mg cholesterol/100 mg tissue wt.

**TOTAL ASCORBIC ACID**

Ascorbic acid levels in the ovary were estimated by the method of Roe and Kuether (1943). Total ascorbic acid (TAA) was oxidized to dehydro ascorbic acid (DHA) by Norit reagent in the presence of trichloro acetic acid (TCA). This couples with 2,4-dinitrophenyl hydrazine to yield a red coloured complex by the reaction of sulfuric acid which is measured colorimetrically.

One homogenate was prepared in 10 ml of Norit reagent (prepared by dissolving
2 gms of activated charcoal in 100 ml of 6% trichloro acetic acid). The mixture was shaken well and allowed to stand for 15 minutes and then filtered through Whatman No.42 filter paper. Another homogenate was prepared in 10 ml of 6% trichloro acetic acid for total and dehydro ascorbic acid estimations respectively to 4 ml of homogenate, 1 ml of 2,4 dinitrophenol hydrazine reagent (2 gms, in 100 ml of 50% ethanol) was added and then 1 drop of 10% thiourea (10 gms, thiourea in 100 ml of 50% ethanol) was added in order to activate the reaction. In the blank tube 4 ml of 6% trichloro acetic acid was taken instead of homogenate and in the standard tube, 4 ml of ascorbic acid solution (50 mg of ascorbic acid dissolved in 50 ml of 6% trichloro acetic acid. One ml of this solution was diluted upto 100 ml with 4% trichloroacetic acid). This contained 10 μg ascorbic acid per ml. The contents of the tubes were mixed well and kept in boiling water for 15 minutes and thereafter were collected in an ice bath. Then 5 ml of 85% H₂SO₄ was added along the sides of the tubes kept in an ice bath. After that, the tubes were allowed to stand for 30 minutes and the optical density was measured at 540 nm against blank on Systronics 106 Colorimeter.

The concentration of ascorbic acid was calculated by the formula.

\[
\frac{\text{O.D. of sample}}{\text{O.D. of Std.}} \times \frac{\text{Conc. of Std.}}{\text{Tissue wt. in mg}} \times \frac{\text{Dilution}}{\text{Aliquot vol.}} \times 100
\]

The concentrations were expressed as mg/100 mg fresh tissue wt.
GLUTATHIONE (GSH)

The concentration of glutathione was assayed by Grunert and Philips (1957). Glutathione (GSH) present in the tissue reacts with sodium nitro-prusside especially to give a red coloured complex in saturated alkaline medium. A mixture of Na$_2$CO$_3$ and NaCN was added to the contents to stabilize the reaction.

A known amount of tissue was homogenized in 3 ml of 3% HPO$_3$ and 1 ml of distilled water and saturated salt solution (1.5 gm NaCl (crystals) and centrifuged). Two ml of supernatant aliquot is added to the sample tube containing 6 ml of saturated NaCl solution and allowed to stand for 10 minutes at 20°C for equilibrium. The blank tube is run with 2 ml of 2% HPO$_3$ instead of aliquot. Then 1 ml each of solution nitro-prusside and sodium cyanide mixture is added to blank and sample tubes respectively. The coloured complex developed is measured at 520 nm on a colorimeter using blank tube.

Calculations

Regression formula : $X = 272.01 \ Y - 2.32$

$Y = \text{O.D. of sample}$

$X = \text{Concentration}$

Conc. of Glutathione (GSH) : $\frac{\text{Conc. of sample x dilution}}{\text{Tissue wt. (mg) x Aliquot Vol.}} \times 100$

The units were $\mu$g/100 mg fresh tissue weight.
PROTEIN

The protein content in the ovary and uterus was assayed by the method of Lowry et al. (1951) as described in Part-I. The concentration was expressed as mg protein/100 mg tissue weight.

GLYCOGEN

The concentration of glycogen in the uterus was estimated by the method of Siefert et al. (1950) as described in Part-I.

The concentration was expressed as µg glycogen/100 mg fresh tissue weight.

TOTAL LIPIDS

Total lipids was estimated by the method of Zollner and Kirsch (1962). Lipid containing sample is heated with concentrated sulfuric acid without prior deproteinization and then mixed with phosphoric acid/vanilline reagent. In this sulpho-phosphovanilline reaction, lipid forms a pink colour dye stuff which is measured photometrically. The intensity of the colour developed in sample is quantitatively proportional to the total lipid concentration in the tissue.

To the sample tubes containing 0.05 ml of homogenate, 2 ml of concentrated H$_2$SO$_4$ is added and kept in boiling water bath for 10 minutes cool and centrifuge for 5 minutes. Transfer the supernatant and add 0.1 ml to sample tubes. A volume of 0.1 ml of Std. solution (100 mg/1 ml) is added to Std. tubes. In blank 0.1 ml of concentration H$_2$SO$_4$ is added instead of homogenate. Add 2 ml of colouring reagent (11.9M,
phosphoric acid, 0.008 M/Vanilline) to all the tubes. Then the tubes are allowed to stand at room temperature for 40-50 minutes. Then measure the absorbance of the sample and the standard against that of the blank at 530 nm. The total lipid content was calculated using the following formula.

\[
\text{Total lipid concentration} = \frac{\text{As}}{\text{Ast}} \times \frac{\text{Std. Conc.}}{\text{Aliquot Vol.}} \times \frac{100}{\text{Tissue wt.}} \times \text{dilution}
\]

As = Absorbance of the sample

Ast = Absorbance of the Standard

Concentration was expressed as mg of total lipid content/100 mg tissue weight.

TOXICOLOGICAL STUDIES

HEMATOLOGICAL PARAMETERS

The haemoglobin content and the blood cell counts from control and experimental groups of animals were carried out by the methods described in Part-I.

SERUM PARAMETERS

SERUM CHOLESTEROL

Serum cholesterol was assayed by the method of Zlatki et al. (1953) as mentioned earlier and the units were expressed as mg/ml serum.
SERUM PROTEIN

The protein levels in the serum were assayed by the method of Lowry et al. (1957) as described in Part-I and the units were mg/ml serum.

SGOT (SERUM GLUTAMATE OXALOACETATE TRANSAMINASE) (E.C.2.6.1.2.)

The photometric assay of SGOT was carried out by the method of Reitman and Frankel (1957) as mentioned in Part-I and expressed as mIU/ml serum.

SGPT (SERUM GLUTAMATE PYRUVIC TRANSAMINASE (E.C. 2.6.1.2))

The assay was carried out in the similar manner described in Part-I and expressed in mIU/ml serum.

ELECTROPHYSIOLOGICAL STUDIES ON THE UTERUS

The uterus from the different groups of animals was dissected out immediately after cervical dislocation. The estrous phase of the mouse was confirmed before sacrificing it by microscopic examination of vaginal smear (the presence of nonnucleated scales). The abdomen wall was immediately opened and the uterine horns were quickly dissected out and placed in a petridish containing De-Jalon’s solution maintained at 37±1°C. The connective tissue attached to the uterine horns were removed. The composition of De-Jalon solution consists of NaCl, 112.0; CaCl₂ nM; 0.25; KCl, 4.69nM, glucose, 2.68 mm and sodium bicarbonate, 5.95 nM (PH 7.3). The preparations were then
mounted in the organ bath containing 20 ml Dc-Jalon solution maintained at 37±1°C and was continuously bubbled with air. The preparation was allowed to stabilize for 30 minutes during which the bathing solution was changed every 10 minutes. A tension of 1 gm was exerted on the uterus. After stabilization, the spontaneous contractions of the uterus was recorded on electric physiograph and then the maximum contraction was calculated with respect to the peak height of contractions and compared with different groups of animals.

HISTOLOGICAL AND MORPHOMETRIC STUDIES

The histology of ovary and uterus was carried out by the standard haematoxylon-eosin staining technique (Gurr, 1962) as described in Part-I while histocytometry was done using ocular lens.

STATISTICAL ANALYSIS

A minimum of 8-10 replicates were done for each biochemical parameters and tissue and the data were statistically analysed using Student’s ‘t’ test. A value of P<0.05 is considered to be significant as cited earlier under Part-I.