CHAPTER 5
INVIVO STUDIES - ANIMAL STUDY

5.1 MATERIALS AND METHODS

5.1.1 ALBINO RATS

- Ethical committee clearance, IAEC-46/IAEC/2011 was obtained
- Purchase and housing of animals
- Acclimatization of animals
- Selection of pathogen and infection free animals for the study

Male albino wistar rats weighing about 150-250gms were selected for this study.

5.1.2 MERCURIC CHLORIDE

Mercury in the form of HgCl2 was purchased from Fisher Scientifics (Mumbai), (Product no. 15564), DOSE: 0.5mg/kg/bw and 1mg/kg/bw [94]

5.1.3 Smilax china

The Methanolic extract of Smilax china prepared after preliminary phytochemical evaluation and the dose of the drug was fixed as 400mg/kg/Bw (1/5th of the LD50). The acute toxicity of the Methanolic extract of Smilax china was observed that the test extract was not lethal to the rats even at 2000mg/kg/Bw (LD50).[151].
5.1.4 EXPERIMENTAL DESIGN

PHASE I

- Animals were divided into 5 groups as follows containing 12 animals in each Group:
  - Group I - control Rats were fed with the standard diet
  - Group II - Receiving Mercuric chloride 1mg/kg/Bw., orally
  - Group III - Receiving Mercuric chloride 0.5mg/kg/Bw., orally
  - Group IV - Mercuric chloride 1mg/kg/Bw + *Smilax china* 400mg/kg/Bw., orally.
  - Group V - Mercuric chloride 0.5mg/kg/Bw + *Smilax china* 400mg/kg/Bw., orally.

Out of 12 animals in Group 2 and 3 only 4 animals were sacrificed, and other 8 animals in each of those group is taken for phase two of the study for a period of 15 days.

PHASE II

- Group VI - Post treatment with *Smilax china* 400mg/kg/BW orally (15 days) after receiving Mercuric chloride 1mg/kg/BW orally for 30 days (from Group - 2).
- Group VII - Natural recovery (15 days) after receiving Mercuric chloride 1mg/kg/BW orally for 30 days (from Group - 2).
- Group VIII - Post treatment with *Smilax china* 400mg/kg/BW orally (15 days) after receiving Mercuric chloride 0.5mg/kg/BW orally for 30 days (from Group - 3).
- Group IX - Natural recovery (15 days) after receiving Mercuric chloride 0.5mg/kg/BW orally for 30 days (from Group - 3).
5.1.5 Gravimetry

- Physical – body weight of the albino rats in all 9 groups will be assessed in regular intervals and compared using weighing machine.

- 1st, 7th, 15th, 30th and 45th day the body weight are recorded and compared.

5.1.6 Biochemical/ Hormonal /Toxicological Assay

- The following analysis are done under regular intervals, 2nd, 7th, 15th and 30th day for phase 1 animals (Group I, II, III, IV, V) and 45th day for phase 2 animals (Group VI, VII, VIII & IX) were analyzed.

  For Testicular Changes – Testosterone Assay

  For Liver Tissue – SGPT, SGOT, Albumin, Cholesterol

  For Renal Tissue – Total protein, Creatinine, Potassium, Urea

  For Toxicological & Stress Assessment – Cortisol, Hemoglobin, Total RBC Count, Packed Cell Volume, Total WBC Count and Random Blood Glucose Levels were analyzed.

  Periorbital Blood was collected from the animals and analysed in Autoanalyzer. Since the amount of the blood needed for the analysis for each time around 4.5 ml. Milking of the tail vein gives only 1 – 1.5ml of blood, which is not sufficient for the study hence Periorbital bleeding is done.

ESTIMATION OF SGPT

Principle

SGPT transfers the amino group from alanine to 2-oxoglutarate to form pyruvate and glutamate. The addition of pyridoxal phosphate to the reaction mixture ensures maximum catalytic activity of ALT. The pyruvate enters a lactate dehydrogenase (LDH) catalysed reaction with NADH to produce lactate and NAD+. The decrease in absorbance
due to the consumption of NADH is measured at 340 nm and is proportional to the ALT activity in the sample. Endogenous pyruvate is removed during the incubation period.

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

\[
\text{Pyruvate + NADH + H+} \xrightarrow{\text{LDH}} \text{L-lactate + NAD}
\]

**Procedure:**

- **Sample volume:** 5 µl
- **Reagent volume:** R1 - 125 µl, R2 - 25 µl
- **Method:** RATE
- **Wavelength:** 340 nm
- **Calibrator/standard:** 85 mg/dL
- **Reaction slope:** Increasing
- **OD Value:** 0.0165

**ESTIMATION OF SGOT**

**Principle**

Serum glutamic oxaloacetic transaminase (SGOT) catalyses the transamination of aspartate and 2-oxoglutarate, forming L-glutamate and oxalacetate. The addition of pyridoxal phosphate to the reaction mixture ensures maximum catalytic activity of AST. The oxalacetate is reduced to L-malate by malate dehydrogenase (MDH), while NADH is simultaneously converted to NAD+. The decrease in absorbance due to the consumption of NADH is measured at 340 nm and is proportional to the AST activity in the sample. Endogenous pyruvate is removed by the LDH-reaction during the incubation period.

\[
\text{2-Oxoglutarate + L-Aspartate} \xrightarrow{\text{AST}} \text{L-Glutamate + Oxalacetate}
\]

\[
\text{Oxalacetate + NADH + H+} \xrightarrow{\text{MDH}} \text{L-Malate + NAD+}
\]
**Procedure:**

Sample volume : 5 µl  
Reagent volume : R1-125µl, R2-25µl  
Method : rate  
Wavelength : 340 nm  
Calibrator/standard : 91mg/dL  
Reaction slope : Increasing  
OD Value : 0.0168

**ESTIMATION OF TOTAL CHOLESTEROL [CHOLESTEROL – OXIDASE METHOD]**

**Principle:**

Cholesterol esterase hydrolyses cholesterol esters into free cholesterol and fatty acids. In the second reaction, cholesterol oxidase converts cholesterol to cholest-4-en-3-one and hydrogen peroxide. In the presence of peroxidase, hydrogen peroxide oxidatively couples with 4-amino antipyrine and phenol red quinoneimine dye which has absorbance maximum at 540/600 nm. The intensity of the red color is proportional to the amount of total cholesterol in the sample.

\[
\text{Cholesterol ester} \xrightarrow{\text{Cholesterol esterase}} \text{cholesterol + fatty acid.}
\]

\[
\text{Cholesterol + oxygen} \xrightarrow{\text{Cholesterol oxidase}} \text{hydrogen peroxide + Cholest-4-en-3-one}
\]

\[
2 \text{H}_2\text{O}_2 + 4\text{-aminoantipyrine} \xrightarrow{\text{Peroxidase}} \text{red quinoneimine dye + water}
\]

**Procedure:**

Sample volume: 3µl  
Reagent volume: 45µl
Method: end point

Wavelength: 540 nm

Calibrator/standard: 191mg/dL

Reaction slope: Increasing

Measuring point: 0-2

**ESTIMATION OF CREATININE**

**Principle:**

Creatinine reacts with picric acid at alkaline pH to form a yellow-orange complex. The rate of change in absorbance at 520/800nm is proportional to the creatinine concentration in the sample.

\[
\text{Creatinine} + \text{picric acid} \rightarrow \text{creatinine picrate complex}
\]

**Procedure:**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample volume</td>
<td>20 μl</td>
</tr>
<tr>
<td>Reagent volume</td>
<td>120 μl</td>
</tr>
<tr>
<td>Method</td>
<td>Fixed</td>
</tr>
<tr>
<td>Wavelength</td>
<td>520 nm</td>
</tr>
<tr>
<td>Calibrator/standard</td>
<td>4.3 mg/dL</td>
</tr>
<tr>
<td>Reaction slope</td>
<td>Increasing</td>
</tr>
<tr>
<td>OD value</td>
<td>0.0364</td>
</tr>
</tbody>
</table>
ESTIMATION OF TOTAL PROTEIN

Principle

Cupric ions in an alkaline solution react with proteins and polypeptides containing at least two peptide bonds to produce a violet coloured complex. The absorbance of the complex at 540/660 nm is directly proportional to the concentration of protein in the sample.

\[
\text{OH}^- \\
\text{Protein} + \text{Cu}^{2+} \rightarrow \text{Blue violet complex}
\]

Procedure :

- Sample volume : 6 µl
- Reagent volume : 50 µl
- Method : end point
- Wavelength : 540 nm
- Calibrator/ standard : 5.1 g/dL
- Reaction slope : Increasing
- OD Value : 0.0632

ESTIMATION OF UREA

Principle

Urea is hydrolysed in the presence of water and urease to produce ammonia and carbon dioxide. The ammonia produced in the first reaction combines with 2-oxoglutarate and NADH in the presence of glutamate-dehydrogenase (GLDH) to yield glutamate and NAD+. The decrease in NADH absorbance per unit time is proportional to the urea concentration.
Urease

\[
\text{Urea} + 2 \text{H}_2\text{O}_2 \rightarrow 2 \text{NH}_4^+ + \text{CO}_3^{2-} + 2 \text{H}_2\text{O}
\]

GLDH

\[
2\text{-Oxoglutarate} + 2 \text{NH}_4^+ + 2 \text{NADH} \rightarrow 2 \text{L-Glutamate} + 2 \text{NAD}^+ + 2 \text{H}_2\text{O}
\]

**Procedure:**

- Sample volume: 2.5 µl
- Reagent volume: 50 µl
- Method: Rate
- Wavelength: 340 nm
- Calibrator/standard: 98mg/dL
- Reaction slope: Increasing
- OD Value: 0.0588

**ESTIMATION OF ALBUMIN (BCG Method)**

**Principle**

A coloured complex is formed when bromocresol green reacts with albumin. The absorbance of the albumin-BCG complex is measured bichromatically (600/800nm) and is proportional to the albumin concentration in the sample.

\[
\text{pH } 4.2
\]

\[
\text{Albumin} + \text{Bromocresol} \rightarrow \text{Green Green Complex}
\]
**Procedure** :

Sample volume : 2 μl

Reagent volume : 58 μl

Method : end point

Wavelength : 600 nm

Calibrator/ standard : 3.6 g/dL

Reaction slope : Increasing

OD Value : 0.1363

**ESTIMATION OF POTASSIUM (TURBDIOMETRIC METHOD)**

**Principle**

The extent of turbidity is proportional to the potassium concentration and is measured photometrically at 578nm (570 - 620nm)

**Procedure** :

Sample volume : 2.5 μl

Reagent volume : 1000 μl

Method : end point

Wavelength : 578nm nm

Calibrator/ standard : 14mmol/dL

Reaction slope : Increasing

OD Value : 0.05
GLUCOSE (GOD/POD METHOD):

PRINCIPLE:

Glucose is determined after enzymatic oxidation in the presence of glucose oxidase. The hydrogen peroxide formed reacts, under catalysis of peroxidase, with phenol and 4-aminophenazone to form a red-violet quinoneimine dye as indicator.

\[
\begin{align*}
\text{Glucose oxidase} & \quad \text{Glucose + O}_2 + \text{H}_2\text{O} \quad \rightarrow \quad \text{gluconic acid + H}_2\text{O}_2 \\
\text{Peroxidase} & \quad \text{H}_2\text{O}_2 + 4\text{-Aminophenazone + Phenol} \quad \rightarrow \quad \text{Quinoneimine + 4 H}_2\text{O}
\end{align*}
\]

HORMONAL ASSAY

TESTOSTERONE:

(ALPCO KID- CatalogNumber: 55-TESMS E01)

PRINCIPLE

The Testosterone rat/mouse ELISA Kit is a solid phase enzyme-linked immunosorbent assay (ELISA), based on the principle of competitive binding. An unknown amount of testosterone present in the sample and a defined amount of testosterone conjugated to horseradish peroxidase compete for the binding sites of testosterone antiserum coated to the wells of a micro plate. After one-hour incubation on a shaker the micro plate is washed four times. After addition of the substrate solution the concentration of testosterone is inversely proportional to the optical density measured.

REAGENTS:

1. Microtitre plate, 12 x 8 (break apart) strips with 96 wells; Wells coated with antitestosterone antibody.

2. Calibrator 0, 1 vial, 0.3 ml, ready to use
3. Calibrator (Calibrator 1-5), 5 vials, 0.3 ml each, ready to use; Concentrations: 0.1 - 0.4 - 1.5 - 6.0 - 25.0 ng/ml

4. Incubation Buffer, 1 vial 11 ml, ready to use;

5. Enzyme Conjugate, 1 vial, 7 ml, ready to use; Testosterone conjugated to horseradish peroxidise.

6. Substrate Solution, 1 vial, 22 ml, ready to use; contains tetramethylbenzidine (TMB) and hydrogen peroxide in a buffered matrix.

7. Stop Solution, 1 vial, 7 ml, ready to use; contains 2 N Hydrochloric Acid solution.

8. Solution, 1 vial, 50 ml (10X concentrated).

**MATERIALS REQUIRED**

1. Centrifuge

2. A microtiter plate reader capable for endpoint measurement a 450nm

3. Microplate mixer operating more than 600 rpm

4. Vortex mixer

5. Calibrated variable precision micropipettes (10 µl, 50 µl, 100 µl 200 µl).

6. Absorbent paper

7. Distilled or deionized water

8. Timer

9. Semi logarithmic graph paper or software for data reduction
**ASSAY PROCEDURE**

Each run must include a standard curve.

1. Prepare a sufficient number of micro plate wells to accommodate calibrators and samples in duplicates.

2. Dispense 10 µl of each Calibrator, Sample and Control with new disposable tips into appropriate wells.

3. Dispense 100 µl of Incubation Buffer into each well.

4. Add 50 µl Enzyme Conjugate into each well.

5. Incubate for 60 minutes at room temperature on a Micro plate mixer.

6. Discard the content of the wells and rinse the wells 4 times with diluted Wash Solution (300 µl per well). Remove as much Wash Solution as possible by beating the microplate on absorbent paper.

7. Add 200 µl of Substrate Solution to each well.

8. Incubate without shaking for 30 minutes in the dark.

9. Stop the reaction by adding 50 µl of Stop Solution to each well.

10. Determine the absorbance of each well at 450 nm. It is recommended to read the wells within 15 minutes.

**CORTISOL ASSAY:**

*Cortisol EIA Kit* (Item No. 500360- CAYMAN CHEMICALS)

**PRINCIPLE OF THE TEST**

The procedure follows the basic principle of enzyme immunoassay where there is competition between an unlabeled antigen and an enzyme labeled antigen for a fixed
number of antibody binding sites. The amount of enzyme-labeled antigen bound to the antibody is inversely proportional to the concentration of the unlabeled analyte present. Unbound materials are removed by decanting and washing the wells.

REAGENTS

The DSL-10-2000 ACTIVE® Cortisol EIA Kit contains sufficient reagents for 96 wells. Each kit contains the following reagents:

A. GARG-Coated Microtitration Strips:

One strip holder, containing 96 polystyrene Microtitre wells with goat anti-rabbit globulin serum. Store at 2-8 °C until expiry date in the resealable pouch with a desiccant to protect from moisture.

B. Cortisol Antiserum: (BLUE)

One vial, 11 mL, containing rabbit anti-cortisol serum in a protein-based (BSA) buffer with a non-mercury preservative. Store at 2-8 °C until expiry date.

C. Cortisol Standards:

One vial, 0.5 mL, labeled A, containing 0.1g/dL cortisol and 7 vials, 0.5 mL each, labeled B-H, containing concentrations of approximately 0.5, 1.5, 4.0, 10.0, 20.0, 40.0, and 60.0 g/dL cortisol in protein-based (BSA) buffer with a non-mercury preservative. Store unopened at 2-8 °C until kit expiration date. Store opened vials at 2-8°C for up to 3 weeks. For longer periods, store at -20°C or lower until expiry date.

D. Cortisol Controls:

Two vials, 0.5 mL each, Levels I and II, containing low and high concentrations of cortisol in protein-based (BSA) buffer with a non-mercury preservative. Store unopened at 2-8°C until kit expiry date. Store opened vials at 2-8°C for up to 3 weeks. For longer periods, store at -20°C or lower until expiry date.
E. Cortisol Enzyme Conjugate Concentrate:

One vial, containing 0.3 ml of a solution of cortisol conjugated to horseradish peroxidase in a protein-based buffer (BSA) with a non-mercury preservative. Dilute prior to use in Conjugate Diluent. Store at 2-8°C until expiry date.

F. Conjugate Diluent:

One bottle, 11 ml containing a protein-based buffer (BSA) with a non-mercury preservative. Store at 2-8°C until expiry date.

G. TMB Chromogen Solution:

One bottle, 11 ml, containing a solution of tetramethylbenzidine (TMB) in citrate buffer with hydrogen peroxide. Store at 2-8°C until expiry date.

H. Wash Concentrate:

One bottle, 100 ml, containing buffered saline with a nonionic detergent. Dilute 10 fold with deionized water prior to use. Store at room temperature until expiry date.

I. Stopping Solution:

One vial, 11 ml, containing 0.2M sulfuric acid. Store at 2-8°C until expiry date.

Assay Procedure:

Allow all specimens and reagents to reach room temperature (~25°C) and mix liquid reagents thoroughly by gentle inversion before use. Standards, Controls and unknowns should be assayed in duplicate.

1. Mark the microtitration strips to be used.

2. Prepare the Enzyme Conjugate Solution by diluting the Conjugate in the Conjugate Diluent as described under the Preparation of the Reagents section of this package insert.
3. Pipet 25μl of each Standard, control and unknown into the appropriate wells.

4. Add 100 μl of the Enzyme Conjugate Solution to each well using a semi-automatic dispenser. Gently tap the well holder for 5-10 seconds.

5. Add 100 μl of the Cortisol Antiserum to each well using a semi-automatic dispenser.

6. Incubate the wells at room temperature (~25°C) on a shaker set at 500-700 rpm for 45 minutes.

7. Aspirate and wash each well 5 times with the Wash Solution using an automatic microplate washer. Blot dry by inverting plate on absorbent material.

8. Add 100 μl of the TMB Chromogen Solution to each well using a semi-automatic dispenser.

9. Incubate the wells at room temperature (~25°C) for 10-15 minutes on a shaker set at 500-700 rpm.

10. Add 100 μl of the Stopping Solution to each well using a semi-automatic dispenser.

11. Shake the plate by hand for 5-10 seconds.

12. Read the absorbance of the solution in the wells within 30 minutes, using a microplate reader set to 450 nm.

HEMATOLOGICAL PROFILE

Coulter Principle

The Coulter principle of counting and sizing particles/cells is based on measurable changes in electrical resistance produced/created by nonconductive particles/cells suspended in conductive isotonic electrolyte. EDTA anticoagulated blood sample was aspirated in the instruments, blood is highly diluted with the isotonic solution (the isotonic solution is called the diluent reagent), then this dilution is divided into 2 portions/parts.
First portion will be enforced through the tubing toward the RBC chamber, where it is further diluted with the isotonic solution, and then cells/particles are passed through the aperture, in which red blood cells and platelets are counted and sized according to the total number and heights of created pulses. Cells/particles which have a volume between 2-20 fl are considered and counted as platelets, while cells between 36 to 360 fl are considered and counted as red blood cells. The second portion of diluted blood sample will be moved through tubing towards the WBC chamber, where it is further diluted with a lyse reagent; not the diluent; this lyse reagent has several functions: (1) will lyse the red blood cells, so that red blood cells will not be counted or interfere with white blood cells; whereas in RBC chamber WBC’s are not lysed, but because of their low count (WBC’s, are in thousands/cumm) in comparison to the very high RBC count which are in millions per cumm, they will not affect significantly the total red blood cell count, only in cases of very high WBC count as seen in leukaemia’s, they may affect the RBC total count, (2) lyse reagent contains Drabkin’s solution which is provided for haemoglobin determination, when the red cells are lysed they release haemoglobin. (3) lyse reagent will puncture the WBC membranes so that they will collapse around the nucleus with their granules. So, when the WBC’s pass through the aperture they are counted and sized in the same manner as red blood cells and platelets are counted and sized, but the difference here is the counter in counting and sizing the WBC nuclei because their membranes are punctured by the lyse reagent. By this the counter has counted the total WBC count. After the white blood cells have been counted and sized, the remainder of the lysed dilution is transferred to the haemoglobin Flow Cell to measure Haemoglobin concentration. The packed cell volume is also counted in this.

5.1.7 WET WEIGHT

- The wet weight of Testis, Seminal vesicle, Epididymis, Prostate, Kidney and liver of all 5 group (Group I, II, III, IV, V) has been assessed. (Phase 1), at end of 30th day.

- And for Phase 2 animals (Group VI, VII, VIII & IX) on 45th day.
5.1.8 HISTOPATHOLOGICAL ASSESSMENT

- Prostate
- Seminal vesicle
- Epididymis
- Liver
- Kidney of phase 1 and phase 2 animals are collected in Buffered formalin for Histopathology assessment, stained with H & E.
- Where as testis is collected on Bouins fluid for Histopathology and *vivo* antioxidant study
- Special Stain- toluidine Blue is done for Testis and PAS for Liver
- Immuno Histochemistry – Ki67 is done for Testis

The procedures to prepare staining solutions for histopathological study.

5.1.8.1 Bouin’s Fixative (Bouin’s Fluid) - for Testicular tissue

REAGENTS:

- Saturated picric acid - 3000.0 ml
- Formaldehyde - 1000.0 ml
- Glacial acetic acid - 200.0 ml

Purpose

Bouin’s is generally used for testicular fixation because it preserves nuclei and chromosomes especially observes well during meiosis. Formalin is a more general fixative, used widely for other tissues when mitotic or meiotic cycles are not necessary to observe. Also used as a mordant for staining procedures.
**FIXATION TIME**

For small biopsies fix in 2-4 hours, for large specimens may remain in fixative for a maximum of 72 hours

**PROCEDURE**

1. Fixed tissue may be retained in 10% formalin or 70% alcohol

2. Prior to staining, remove the picric acid from the tissue by
   a) washing in tap water
   b) grades of alcohol (50%)
   c) or 70% alcohol saturated with lithium carbonate.

5.1.8.2 **10% NEUTRAL BUFFERED FORMALIN – for other tissues**

**PURPOSE:** The fixative is a good routine fixative. This solution is hypotonic in buffer ions and has a pH of 6.8

**REAGENTS:**

**10% NEUTRAL BUFFERED FORMALIN** (Purchase through CMS)

Sodium phosphate, monobasic 4.0 gm

Sodium phosphate, dibasic 6.5 gm

Formaldehyde, 37% 100.0 ml

Distilled water 900.0 ml
5.1.8.3 HAEMATOXYLIN AND EOSIN STAIN:

Harris’s haematoxylin:

Preparation of solution:

Haematoxylin 2.5g
Absolute alcohol 25 ml
Potassium alum 50g
Distilled water 500ml
Mercuric oxide 1.25g
Glacial acetic acid 20ml

The haematoxylin is dissolved in the absolute alcohol, and is then added to the alum which has previously been dissolved in the warm distilled water in a 2 litre flask. The mixture is rapidly brought to the boil and the mercuric oxide is then slowly and carefully added. The stain is rapidly cooled by plunging the flask into cold water or into a sink containing chipped ice. When the solution is cold, the acetic acid is added, and the stain is ready for immediate use. The glacial acetic acid gives more precise and selective staining of nuclei.

Eosin:

Eosin Y (eosin yellow, eosin water soluble) C.I.NO45380 (C.I.Acid Red 87)
Water and alcohol soluble Eosin 0.5%
Distilled water 500ml
Thymol crystal 0.5g
Acetic acid 0.5ml
5.1.8.4 Toluidine blue staining (According to Mayer; Metzner)

Subsequent to immunohistological reaction, counterstaining of tissue sections with the thiazin dye toluidine blue (Toluidine blue O) is an alternative to other dyes (e.g. haematoxylin, carmine, methyl green) for nuclear staining.

Chemicals

- Toludine Blue O
- Iron ammonium sulphate (Ammonium ferric sulphate)
- Ethanol
- Distilled Water

Chemical Solution

- Toluidine blue dye solution: 0.1 g toluidine blue dissolved in 100.0 mL distilled water

  - Iron ammonium sulfate solution 3.0-5.0 g dissolved in distilled water

Staining procedure

Immuno-stained sections are passed through distilled water and stained:

- Iron ammonium sulfate solution 45 min
- distilled water rinse
- toluidine blue dye solution 10-20 min
- differentiate in 50% ethanol until dye veils are no longer seen
- 96% ethanol 1 min
Slides are dehydrated in absolute ethanol, cleared in xylene or xylene substitute and mounted in resinous medium under coverglass.

5.1.8.5 Ki67 Staining Protocol for Paraffin Sections

Solutions and Reagents

A. 10X Phosphate Buffered Saline (PBS):
To prepare 1 liter,
Na2HPO4 --------------- 10.9 g
NaH2PO4 --------------- 3.2 g
NaCl ------------------ 90 g
Distilled water --------- 1000 ml
Mix to dissolve and adjust pH to 7.4

B. Antigen Retrieval Solution:
10mM Sodium Citrate Buffer:
To prepare 1000 ml,
Sodium citrate ----------- 2.94 g
Distilled water ----------- 1000 ml
Adjust pH to 6.0

C. 3% Hydrogen Peroxide:
To prepare 100 ml,
30% H2O2 -------------- 10 ml
PBS or methanol ------- 90 ml

D. Blocking Solution:
2% Normal Goat Serum in PBS:
To prepare 100 ml
Normal goat serum ------ 2 ml
PBS ------------------------ 98 ml
Mix to dissolve.
E. Primary Antibody:
Rabbit anti-human Ki67 (Novocastra Cat# 301103). Optimal dilution 1:3000 in PBS.

F. Secondary Antibody:
Goat anti-rabbit IgG (H+L), biotinylated (Vector Laboratories, Cat# BA-1000). Optimal
dilution 1:400.

G. ABC Reagent:
HRP-streptavidin (Vector Laboratories, Cat# HA-5004). Optimal dilution 1:400.

H. DAB Reagent:
0.02% DAB and 0.003% H2O2 in PBS

To prepare 100 ml
DAB ----------------------- 20 mg
PBS ----------------------- 100 ml

Stir to dissolve. Add 10 ul of 30% H2O2 and filter. Use the solution immediately.

Procedure
1. Deparaffinize sections in xylene for 3x5min.
2. Hydrate with 100% ethanol for 2x5min.
3. Hydrate with 95% ethanol for 2x5min.
4. Rinse in distilled water.
5. Antigen Retrieval: Use steamer-citrate buffer antigen retrieval method.
6. Rinse sections in PBS for 1x5min.
7. Hydrogen Peroxide: incubate sections in 3% H2O2 in PBS (or methanol) for 10-15
   minutes to block endogenous peroxidase activity.
8. Rinse in PBS for 1x2min.
9. Blocking: incubate sections with 2% normal goat serum in PBS for 20 minutes to
   block non-specific binding of secondary immunoglobulin.
10. Rinse in PBS for 1x2min.
11. Primary antibody: incubate sections with rabbit anti-human Ki67 diluted 1:3000 in
    PBS for 1 hour at room temperature.
12. Rinse in PBS for 3x5 min.
13. Secondary antibody: incubate sections with biotinylated goat anti-rabbit IgG
diluted 1:400 in PBS for 30 minutes at room temperature.
14. Rinse in PBS for 3x5min.
15. ABC: incubate sections with HRP-streptavidin reagent diluted 1:400 in PBS for 30 minutes at room temperature.
16. Rinse in PBS for 3x5min.
17. DAB: incubate sections with DAB solution for 2-10 minutes.
18. Rinse in distilled water 2x5min.
19. Counterstain with hematoxylin if desire.
20. Rinse in distilled water 2x5min.
21. Dehydrate through 95% ethanol for 5min, 100% ethanol for 2x5min.
22. Clear in xylene for 2x5min.
23. Coverslip with mounting medium.

**Results**

1. Ki67 positive staining -------------- brown
2. Nuclei ---------------------------------- blue

**5.1.8.6 PAS TECHNIQUE: for Liver**

Sections- Paraffin sections

Preparation of solutions:

a. Periodic acid solution

   Periodic acid 1g
   Distilled water 200ml

b. Schiff’s reagent

   Dissolved 1g basic fuchsin in 200ml of boiling distilled water, removed the flask of water from the Bunsen just before adding the basic fuchsin. Allow the solution to cool to 50oC, and added 2g potassium metabisulphite with mixing. Allowed to cool in room temperature then added 2ml concentrated hydrochloric acid, mixed, then added 2g activated charcoal and left overnight in the dark at room temperature. Filter through a NO.1 whatman paper when the solution should be either clear or a pale yellow colour. Store in a dark container at 4°C.
5.1.9 SPERM ANALYSIS [152, 153]

- Epididymis of one side (after recording wet weight) is exposed by dissection and punctured in an ice-cold phosphate buffer to collect the semen.
- Using haemocytometer, the semen samples are observed for:
  - Total sperm Count
  - Motility (active, sluggish, non-motile)
  - Morphology

**Dilution of semen**

The caudal epididymal duct on one side was exposed and incised. The connective tissue capsule around the caudal epididymis was teased out and the epididymal duct was uncoiled. The semen that oozed into the cavity block was quickly sucked into a capillary tube up to 0.05 μl and transferred, diluted 200 times (0.05 μl of sperm with 199.95 μl of PBS) in physiological saline. After thorough mixing, the sperm suspension was used for analysis of motility, morphology and counts.

**Assessment of sperm motility**

A drop of dilute semen transferred, using a Pasteur pipette, on to a cover glass. The cover glass was inverted over a cavity slide to obtain a hanging drop. The edges of the cover glass were sealed with vaseline and the hanging drop was observed under research microscope (Carl Zeiss Axio 2Plus, Germany) at magnifications 200x and 400x. The preparation was observed at regular intervals in such a way as to find the duration, in min, of the progressive motility of the last motile sperm. For each animal two separate hanging drop preparations were made, and two independent observers assessed the motility. The data from each animal were used to obtain the average.

**Sperm counts**

The semen diluted was thoroughly mixed and a drop of the dilute semen was transferred to an improved Neubauer counting chamber with a cover glass overlaid. The counting chamber was observed under a research microscope at 400x magnification and sperm in the central square were counted. The central square has 25 large squares. The volume of each of the 25 squares is 0.1 mL. The sperm counts were calculated using the
following formula: \( \frac{\text{Number of sperm in 25 squares}}{25} \times 10 \times \text{dilution factor} \times 2000 \) which gives the number of sperms in one mL. Data on each group were used to calculate the mean and the standard deviation (Mean ±SD).

**SPERM MORPHOLOGY:**

Sperm morphology was stained with Papanicolaou stain (PAP) The Criteria of Wyrobek abd Bruce [154] and Zaneveld and Polakoski [155] were employed for evaluation of sperm abnormalities.

**Papanicolaou stain**

**Procedure**

Wet fixed smear of the sperm samples on microscope slides

**Conventional staining, manual:**

1. Wash with 96% alcohol
2. Wash with 80% alcohol
3. Wash with 70% alcohol
4. Wash with 50% alcohol
5. Wash with distilled water
6. Stain in Harris' haematoxylin solution 3 min
7. Rinse under weak stream of tap water 3-5 min
8. Wash with 50% alcohol
9. Wash with 70% alcohol 10. Wash with 80% alcohol
11. Wash with 96% alcohol
12. Stain in Papanicolaou's stain OG6 solution 3 min
13. Wash with 96% alcohol
14. Stain in Papanicolaou's solution EA50 3 min
15. Dehydrate with 96% alcohol
16. Dehydrate with absolute alcohol 5 min
17. Dehydrate with equal parts of absolute alcohol and xylene or xylene substitute.
18. Clear with xylene or xylene substitute 2 min
19. Mount with DePeX® or DPX mountant

Specimens for use in histology and cytology must be completely anhydrous prior to being mounted. Xylene should be added as a final stage in order to prevent turbidity brought about by solvents containing water. To carry out the mounting process, drop approximately 0.5 ml mounting agent onto a horizontal slide using a glass rod. This fills the space between slide and cover glass. As soon as the specimen has been covered with a homogeneous solution, cover with a cover glass, taking care to avoid air bubbles. Allow to harden over a period of 20-30 minutes in a horizontal position.

Result

Cytoplasm Cyanophilic (basophilic) - blue to green

Cytoplasm Eosinophilic (acidophilic) - Pink

Nuclei – Blue, Dark violet

5.1.10 INVIVO ANTIOXIDANT STUDY OF TESTICULAR TISSUE

- Superoxide dismutase (SOD) Kakkar et al., 1984 [156]
- Lipid peroxidase (TBARS) Ohkawa et al., 1979 [157]
- Catalase (CAT) Sinha, 1972 et.al; [158]
Assay of superoxide dismutase (EC.1.15.1.1, SOD)

The activity of superoxide dismutase was estimated according to the method of Kakkar et al., 1984.

Reagents:

1) Sodium pyrophosphate buffer (0.025M): 1.115g in 100ml of distilled water.

2) Phenazonium Metho Sulphate (PMS) (186µM): 3mg in 10 ml of distilled water (930µM). Then 1:5 dilutions were carried out to obtain 186µM.

3) Nitro Blue Tetrazolium (chloride) (NBT) (300µM): 3mg in 10 ml of phosphate buffer.

4) NADH (780µM): 6mg in 10 ml of phosphate buffer.

Procedure:

Superoxide dismutase was assayed by taking 0.05ml of spleen homogenate followed by addition of 0.3ml of sodium pyrophosphate buffer (0.025M, PH 8.3), 0.025ml of PMS (186µM) and 0.075ml of NBT (300µM in buffer of PH 8.3) The reaction was started by addition of 0.075 ml of NADH (780µM in buffer of PH 8.3). After incubation at 30°C for 90 seconds, the reaction was stopped by addition of 0.25ml glacial acetic acid. Then the reaction mixture was stirred vigorously and shaken with 2.0ml of n-Butanol. The mixture was allowed to stand for 10 minutes and centrifuged. 1.5ml of n-butanol alone was served as blank. The colour intensity of the chromogen was read at 560nm.

Lipid peroxidation (TBARS)

The procedure of Ohkawa et al., 1979 was followed for the estimation of lipid peroxidation.

Reagents:

1) ThioBarbituric Acid (TBA) (0.8%): 0.8gms in 0.5N HCL
2) Butylated Hydroxyl Toluene (0.05%) : 10.05gms in methanol.

3) Saline (0.9%): 0.9g in 100ml distilled water.

Procedure

The method involved heating of homogenized spleen sample with 0.8ml saline, 0.5ml of BHT and 3.5ml TBA reagent for 1 1/2 min in a boiling water bath. After cooling, the solution was centrifuged at 2,000 rpm for 10 min and the precipitate obtained was removed. The absorbance of the supernatant was determined at 532 nm using spectrophotometer against a blank that contained all the reagents minus the biological sample. The values were expressed in mg/g tissue.

Assay of catalase (EC.1.11.1.6, CAT)

The activity of CAT was estimated by the method of Sinha, 1972.

Reagents

1) 0.01M phosphate buffer

2) 0.2M H$_2$O$_2$

3) Stock dichromate/acetic acid solution: This reagent was prepared by mixing 5% solution of potassium dichromate with glacial acetic acid.

4) Working dichromate/acetic acid solution: stock solution was diluted to 1:5 with water to make the working solution.

Procedure

CAT causes rapid decomposition of H$_2$O$_2$ to H$_2$O. The method is based on the fact that dichromate in acetic acid is reduced to chromic acetate when heated in the presence of H$_2$O$_2$ with the formation of perchloric acid as an unstable intermediate. The chromic acetate thus formed was measured at 610 nm. The reaction mixture consisted of 0.5 ml of 0.2 mM H$_2$O$_2$, 1 ml of 10 mM sodium phosphate buffer (pH 7.0) and 0.4 ml water. 0.2 ml of the diluted enzyme was added to initiate the reaction. To this, 2 ml of the
dichromate/acetic acid reagent (5% solution of potassium dichromate in water with glacial acetic acid in the ratio of 1:3, diluted 1:5 with water) was added after 30 and 60 sec of incubation. To the control tube, the enzyme was added after the addition of the acid reagent. The tubes were then heated for 10 min and the color developed was read at 610 nm. The activity of CAT was expressed as mM of H₂O₂ consumed/min/mg protein.

5.1.11 MORPHOMETRIC ANALYSIS [159]

- Diameter of seminiferous tubules
- Number of Spermatogonia
- Number of Primary Spermatocytes
- Number of Secondary Spermatocytes
- Number of Spermatids

Diameter measurement of seminiferous tubule

The diameter of a seminiferous tubule was defined as the shortest distance between two parallel tangent lines of the outer edge of the tubule. Dozens of tubules were needed to estimate the average diameter of the whole structure. Rectangular measurement frames were placed on each field of view to select the interested tubules. The tubules within the measurement frame or intersectant with the right or upper edges were selected while those intersectant with the left or bottom edges, or with the extended lines of the right and left edges were not. The mean value of the diameters of the tubules selected from a group of fields was calculated and considered to be the estimated average diameter of seminiferous tubules of the testicular tissue. The Stereological image processing program was used to measure the diameter of the seminiferous tubules.

The Stereological image processing program

The program in the present study was developed with Borland Delphi 2007. The parameters such as the number, the size and the place of measurement frames or crossed measurement points could be adjusted by the observer according to the region of interest.
The program also provided the function of distance measurement, including transforming the pixels pitch to the real distance automatically by input of the coefficient. After the measurement, the program could save the altered images and output the measurement results to “.csv” files which could be opened by spread sheet software such as Microsoft Excel for further analysis.

Number of Spermatogonia, Primary spermatocytes, secondary spermatocytes and spermatid were counted in AP view software using digital microscope.

5.1.12 STATISTICAL ANALYSIS

Analysis 1:

Repeated measures ANOVA was carried out to find the significant difference within each group for each parameter. Sphericity assumption was verified for a $p$ value of >0.05 using Maulchy’s test. If the $p$ value is less than 0.05, then greenhouse geisser correction epsilon has to be taken into account.

Analysis 2:

Oneway Analysis of Variance was carried to find the significant difference between the groups 1, 2, 3, 4 & 5 which is followed by the Tukey’s Honestly Significant Difference(HSD) Test for the Post hoc analysis. Paired $t$-test was performed to find the significant difference between 2-6&7, 3-8&9.