Chapter III

MATERIALS & METHODS
The present chapter includes various techniques/methods for induction of diabetes mellitus in the rabbits by alloxan and streptozotocin using different standardized doses. The route of administration for diabetogenic agents included either intraperitoneal (i.p.) or intravenous (i.v.). When diabetes mellitus was well established which was confirmed by behavioural and biochemical changes, some of the rabbits were sacrificed to observe the deleterious subsequent effects of diabetes mellitus on different organs like pancreas, kidneys, liver, lungs, heart, brain and alimentary canal. The behavioural, biochemical and histopathological changes were noted. Apart from above, this chapter covers treatment of diabetic rabbits by different oral antidiabetic herbal drugs. The treatment was assessed by improvement of behavioural, biochemical and histopathological changes along with blood sugar (F), blood urea and serum creatinine levels.

Animals with Drug Induced Diabetes

The various experimental studies regarding the induction of diabetes mellitus in rabbits by alloxan and streptozotocin was done and the subsequent effects on behaviour, biochemistry and histopathology were studied thoroughly.

Twelve New Zealand white male rabbits of 8-12 months old were used. The rabbits were purchased either from Faculty of Veterinary Sciences and Animal Husbandry (FVSC & AH) Shuhama, Alusteng, Srinagar or Institute of Animal Health and Biological Products (IAH and BP), Zakura, Srinagar or Rabbit Breeding Farm, Wusan Pattan. All the rabbits received human care according to the guidelines outlined in the “Guidelines for the Care and Use of Animals in Scientific Research” prepared by the Indian Science Academy, New Delhi.
Management of Animals

The rabbits were housed in rooms where sufficient space was available so that cages were easily maneuvered and operations such as cage changing, sanitation and investigation by the responsible personnel were easily performed. Cages were large enough to allow the rabbit to freely move about, stretch out when lying down and to facilitate feeding and waste removal. Environmental conditions such as illumination, ventilation and noise free housing was maintained.

The rabbits were fed on green vegetables and commercial pelleted diet so as to maintain their health and weight. Feed was offered to rabbits in a J-feeder attached to the cage floor. Fresh, clean and potable water was provided to the rabbits by means of water bottles attached to cages or bowls placed in the cage. The feeders were regularly cleaned to remove feed dust which might decrease palatability of the food. Further, proper sanitation was maintained. All grossly visible debris was removed by brushing and rinsing with water followed by application of a chemical disinfectant.

Experimental Design

After acclimatizing to standard laboratory conditions for fifteen days, the rabbits were weighed and a mean body weight of 1.33 ± 0.20 kg was recorded. Prior to the alloxan and streptozotocin administration biochemical parameters such as blood sugar (F), blood urea and serum creatinine levels of these rabbits were recorded following twelve hours fasting.

Blood Collection

For biochemical estimation, the following steps were used for percutaneous blood sampling.

1) The hair around the site (ear vein) was shaved and swabbed with an antiseptic such as rubbing alcohol to minimize the chance of introducing skin-associated bacteria into the blood stream.
2) The location of the blood vessel of the ear was identified. The inner side of the ear was grasped between the thumb and index finger.

3) The plunger of the syringe was slightly pulled back before use, thereby breaking the air lock and allowing blood to flow more easily.

4) Using a 25 gauge needle with the beveled edge up at a slight angle into the vessel, the venipuncture was made at a site immediately proximal to the thumb and the plunger of the syringe was gently and slowly pulled back as blood fills the shaft of the syringe. Care was taken not to pull the plunger too aggressively which might collapse the vessel and thereby cease blood flow.

5) Gentle manipulation such as slight changes in the orientation of the needle improved collection of blood. Alternatively, haematoma formation in the tissues around the withdrawal site or the presence of clotted blood in the needle might necessitate changing withdrawal site or replacement of the needle, respectively.

6) Another method was used by making a cut in the marginal vein or the central ear artery near its tip.

7) Once the blood sample was obtained, firm pressure with a gauze pad or little cotton wool was maintained at the sampling site for several minutes until bleeding ceased.

Fig. Location of ear vessels from a dorsal view (top) and from a lateral view, indicating relative orientation of the needle during sampling of blood from the central artery (bottom)
Volume of Sample

4ml of blood was collected for biochemical estimations of blood sugar, blood urea and serum creatinine. Sampling sites were changed each time when blood was withdrawn.

Handling of Sample

Blood samples for harvesting of serum were collected in vials containing no anticoagulants. Clotted blood samples were centrifuged at 3000 rpm for 10-15 minutes for obtaining serum.

Estimation of Blood Glucose

Blood glucose estimations after 12 hours fasting were done using either Ames Glucometer Gx (Bayer Diagnostic India Ltd.) or through enzyme kit method.

Procedure for Blood Sugar Estimations by Glucometer

Glucometer Gx is a battery operated instrument which enables convenient and accurate measure of blood glucose with the help of glucostix reagent strips. The test takes less than one minute to perform. To run blood glucose test following procedure is involved:

Material Required:

- Glucometer Gx : Blood glucose meter
- Glucolet : Automatic lancing device with endcap and lancet
- Glucostix : Reagent strips
- Whatmann's filter paper.

Test Procedure

1) Whatmann's filter paper was folded in quarters (for blotting) and placed on a clean dry surface.
2) The glucolet lancing device was loaded with a lancet and endcap was replaced or a disposable syringe with 25 gauge needle was kept ready for blood collection.

3) Spirit was applied to the marginal ear vein of rabbit with a cotton swab.

Test Proper

It was followed as per the instructions given in the manual which are as follows:

1) The button is pressed to turn the meter on. All the digits and legends appear in the display to show that they are working properly.

2) 1 to 3 seconds later a programme number appears in the display.

3) The button is pressed again. The legend programme appears above the programme number. The last programme number entered into memory is shown. Pressing of the button is continued to select the proper programme number. Pressing the button repeatedly scrolls the programme number from 1 to 9 and then back from 9 to 1. The programme number must match the programme number printed on the label on the bottle of glucostix reagent strips being used.

4) Moving it to the left opens the test slide. Three bars (-----) appear in the display, and remain there until the next step is started.

5) Now, a drop of blood collected from the marginal ear vein of rabbit is kept ready and next step is followed quickly.

6) The button is pressed again, and a short beep sound is heard. A 50 appears in the display along with the long beep.

7) Immediately after the beep sound, the drop of blood is applied to the glucose test pads. Both reagent pads are completely covered
with blood. The reagent strip level is kept properly so as to avoid spilling blood off of the test pads.

8) Two short warning beeps which sound at 22 and 21 seconds alert for blotting. At 20 seconds a large beep sounds, the strip is blotted immediately at this time. To ensure a good blot, the reacted strip is placed with pad side up on the folded tissue. The tissue is folded over the test pads and firmly and quickly the tissue is pressed against the pads. It is repeated immediately on a clean area of the tissue. Blotting should be done always on a firm surface.

9) After blotting, the reagent strip is immediately inserted fully into the test slot, making sure the test pads are facing the display.

10) Now, the test sides are closed. The test slide must be closed before the countdown reaches 1 second.

11) After the countdown reaches 1 second, three bars appear briefly, followed by the test result and a long beep. The button is pressed to turn the meter off. The result is automatically stored in the memory.

In order to cross check the results displayed by glucometer Gx, the blood sugar level was also estimated spectrophotometrically using enzymatic kit method. The procedure of the method is given below:

**Enzymatic Kit Method for Blood Sugar Estimation**

For the analysis of blood sugar enzymatic kit method as described by Tietz (1976) was employed. In this single reagent system, glucose oxide converts glucose to gluconic acid and hydrogen peroxide. The peroxide in the presence of horseradish peroxidase forms a coloured complex of hydroxibenzoate and 4-aminophenazene. The intensity of colour formed is proportional to the glucose content present in the sample. 0.1 ml plasma was
used according to the manual instructions. The reagents supplied in the kit include:

- Reagent 1 — Glucose enzyme reagent
- Reagent 2 — Glucose standard 100 mg%
- Reagent 3 — Phenol reagent.

**Preparation of Working Glucose Reagent**

6 x 100 ml pack working glucose reagent was prepared by transferring the contents of one vial of glucose reagent-1 to black plastic bottle, after reconstituting it to 100 ml with distilled water. 5 ml of phenol reagent (reagent 3) was added and these were mixed well and stored at cool dry place at 2 to 8°C. This working solution remains stable for 45 days at 2 to 8°C.

**Protocol For Spectrophotometry**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Blank (B)</th>
<th>Standard (S)</th>
<th>Test (T)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum/Plasma</td>
<td>-</td>
<td></td>
<td>0.2 ml</td>
</tr>
<tr>
<td>Reagent 2: Glucose standard 100 mg%</td>
<td>-</td>
<td>0.2 ml</td>
<td>-</td>
</tr>
<tr>
<td>Working glucose reagent</td>
<td>1.5 ml</td>
<td>1.5 ml</td>
<td>1.5 ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1.5 ml</td>
<td>1.5 ml</td>
<td>1.5 ml</td>
</tr>
</tbody>
</table>

The contents of tubes were mixed well and incubated at 37°C for minutes. The colour intensity was measured in a spectrophotometer at 510 nm against distilled water.

**Calculations**

\[
\text{Serum/plasma glucose mg/100ml} = \frac{\text{Optical Density of Test} - \text{Optical Density of Blank}}{\frac{\text{Optical Density of Standard} - \text{Optical Density of Blank}}{100}} \times 100
\]

After cross checking Glucometer Gx and enzyme kit method for blood sugar estimation, the results were found similar and accurate.
Estimation of Blood Urea

Blood urea estimation was done by Berthelot method (Trinder, 1969). In this method, urea in presence of urease enzyme is converted into ammonia and carbon dioxide. Ammonia reacts with hypochlorite and salicylate in presence of sodium nitroprusside and produces a green colour which is measured at 578nm spectrophotometrically within 60 minutes against reagent blank.

\[
\text{Urea} \xrightarrow{\text{Urease}} \text{Ammonia} + \text{CO}_2 \\
\text{Ammonia} + \text{Hypochlorite} + \text{Salicylate} \xrightarrow{\text{Sodium Nitroprusside}} \text{Green Colour}
\]

Reagents supplied in Kit

For kit 2 x 50ml, contents were as:

- 2 vials of urease and sodium salicylate (R1)
- 1 vial of 50ml alkaline Hypochlorite solution (R2)
- 1 vial of urea standard (40mg/dl)

All reagents were stored at 2-8°C protected from light and contamination.

Reagent Preparation

25ml of distilled water was added to 1 vial of R1 and the contents were dissolved slowly without frothing.

Materials required

- Normal Saline/Distilled water
- Micropipettes upto 1000μl ranges
- Test tubes and test tube racks
- Incubator
- Spectrophotometer.

Sample

- Serum, Plasma
Assay Procedure

<table>
<thead>
<tr>
<th>Dispose</th>
<th>Blank</th>
<th>Standard</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>R₁</td>
<td>1000 µl</td>
<td>1000 µl</td>
<td>1000 µl</td>
</tr>
<tr>
<td>Distilled water</td>
<td>10 µl</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Standard</td>
<td>-</td>
<td>10 µl</td>
<td>-</td>
</tr>
<tr>
<td>Sample</td>
<td>-</td>
<td>-</td>
<td>10 µl</td>
</tr>
</tbody>
</table>

Mix, incubate for 5 minutes at 37°C, then add

| R₂                       | 1000 µl    | 1000 µl  | 1000 µl |

Mix, incubate for 5 minutes at 37°C.

Calculation

Optical density was measured at 578 nm against reagent blank. Concentration of urea was measured in sample in mg/dl.

\[
\text{Concentration of urea} = \frac{\text{Optical Density of sample} \times \text{conc. of standard (i.e., 40)}}{\text{Optical Density of standard}}
\]

Programming Guidelines

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Endpoint (Increasing)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Filter</td>
<td>578 nm</td>
</tr>
<tr>
<td>Temperature</td>
<td>37°C</td>
</tr>
<tr>
<td>Incubation time</td>
<td>5+5 minutes</td>
</tr>
<tr>
<td>Sample volume</td>
<td>10 µl</td>
</tr>
<tr>
<td>Reagent volume</td>
<td>1000 µl + 1000 µl</td>
</tr>
<tr>
<td>Standard</td>
<td>40 mg/dl</td>
</tr>
<tr>
<td>Units</td>
<td>Mg/dl</td>
</tr>
<tr>
<td>Linearity</td>
<td>400 mg/dl</td>
</tr>
<tr>
<td>Blank</td>
<td>Reagent Blank</td>
</tr>
</tbody>
</table>
Estimation of Serum creatinine

The serum creatinine was estimated at weekly intervals throughout the period of experimental study.

Creatinine is an anhydride of creatine and is formed by a spontaneous and irreversible reaction. Free creatine is not reutilized in the process of metabolism. For the measurement of creatinine “Alkaline picrate method” of Baum (1975) was employed. The creatinine reacts with alkaline picrate and produces red coloured complex, which is measured at 520 nm spectrophotometrically.

Working standard solution was prepared by diluting 0.1 ml of stock standard to 10 ml, with diluted water. Firstly, the serum was deproteinized as under:

Serum : 1 ml
Distilled water : 1 ml
Creatinine reagent A : 6.0 ml

These were mixed in a test tube and kept in boiling water for one minute. After cooling the tube was centrifuged till the supernatant became clear.

Test Procedure

The three borosil test tubes were selected and marked as blank, standard and test, respectively.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Blank (B)</th>
<th>Standard (S)</th>
<th>Test (T)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supernatant step I</td>
<td>-</td>
<td>-</td>
<td>4 ml</td>
</tr>
<tr>
<td>Working standard</td>
<td>-</td>
<td>1 ml</td>
<td>-</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1 ml</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Creatinine reagent A</td>
<td>3 ml</td>
<td>3 ml</td>
<td>-</td>
</tr>
<tr>
<td>Alkaline reagent</td>
<td>1 ml</td>
<td>1 ml</td>
<td>1 ml</td>
</tr>
</tbody>
</table>
The tubes were mixed well at room temperature for 20 minutes and the optical density measured against distilled water at 520 nm in a spectro-photometer.

**Calculation**

\[
\text{Serum creatinine in mg/dl} = \frac{\text{Optical Density of Test} - \text{Optical Density of Blank}}{\text{Optical Density of Standard} - \text{Optical Density of Blank}} \times 30
\]

**Induction of Diabetes Mellitus**

Before administration of diabetogenic agents i.e., alloxan and streptozotocin, the rabbits were divided into four groups of four each viz., Group I, Group II, Group III and Group IV. Group II and Group IV received alloxan and streptozotocin respectively while as Group I and Group III received normal saline and served as healthy control.

**Alloxan Administration**

For intraperitoneal administration four doses of alloxan (Loba Chemie) dissolved in 1ml of sterile water was administered @ 80mg/kg b.w. at weekly intervals following the earlier methods (Rastogi et al., 1998; Baqui et al., 2005).

**Streptozotocin Administration**

Diabetes mellitus was induced in New Zealand white rabbits by single iv dose of streptozotocin (Sisco Laboratories Ltd., Mumbai) @ 65mg/kg b.w. dissolved in 1ml of freshly prepared citrate buffer, pH 4.6 as practized earlier (Kedar and Chakrabarti, 1983; Tawfeeg and Sherif, 2001).

**Composition of citrate buffer (pH 4.6)**

**Stock A:** 0.1M disodium citrate (MW 210.0).

2.1gm citric acid dissolved in 20cm³ of normal sodium hydroxide and made upto 100cm³ with distilled water.

**Stock B:** 0.1M Hcl (MW 36.45).

0.8cm³ hydrochloric acid in 100cm³ of distilled water.

Mix 76.6cm³ of stock A with 23.4cm³ of stock B to get citrate buffer with pH 4.6.
Technique for Intraperitoneal Drug Administration

The site for intraperitoneal administration of drug is lower (caudal) right abdominal quadrant and is given as:

1. Normally a long needle (1 inch or greater) was used.

2. The rabbit placed on its back was restrained by securing its head and front quarters with legs and extending the lower portion of the rabbit's body with one hand (Fig.)

Fig. Restraint and intraperitoneal injection in the rabbit.
3) The rabbits hindquarters were held at a 30° to 45° angle to the horizontal.

4) The needle was inserted into the lower right abdominal quadrant just lateral to the midline and directed at an approximately 45° angle to the body wall.

5) The syringe plunger was gently pulled prior to injection to ensure that neither viscera nor blood vessels have been penetrated. For example, aspiration of yellow fluid implies that the needle has penetrated urinary bladder, while green fluid suggests that the intestinal tract has been penetrated. Unexpected contamination by such materials necessitates discarding the compound to be given and obtaining a fresh sample.

**Intravascular Drug Administration**

Intravascular administration was done slowly to avoid consequences of an unexpected adverse reaction. The common sites for intravascular administration included marginal ear vein or jugular vein. Before injection of the drug, the syringe plunger was pulled back slightly to confirm the presence of needle within the vessel which was checked by the presence of blood in the hub of the needle and the tip of the syringe shaft. Care was taken not to form a bleb or blister within the skin around the vessel which indicates that the needle is not within the blood vessel.

**Behavioural Study**

All the groups of rabbits were observed closely throughout the study period for their behavioural patterns, food intake, water intake and urination.

**Body Weight Profile**

Body weight profiles of all the groups of rabbits were measured prior to the start of experiment and extended to the entire experimental study. The changes in the body weight were recorded at weekly intervals.
Histological Procedure

In order to observe the deleterious effects of diabetes mellitus, induced by alloxan and streptozotocin, on different organs of the rabbits, 50% of all groups of rabbits were sacrificed for histological/histopathological study. The different organs viz., pancreas, kidneys, liver, lungs, heart, brain and gut of both diabetic and normal rabbits were processed for histological/histopathological study.

The rabbits were killed by injecting a blow of air, using a 5ml syringe, into the central ear vein which resulted in the sudden death of animals. The skins of the animals were incised along the ventral midline with the scalped blade beginning at the lower jaw and continuing along the midline caudally to the pubis. Using the scalpel, the skin was then gently reflected laterally and the subcutaneous tissues and underlying musculature was examined. The abdominal wall was then incised and the abdominal cavity exposed using the dissecting scissors.

![Incised abdomen with exposed viscera.](image)

The organs and peritoneal surfaces were examined for abnormal colouration, presence of masses, traumatic damage or any other abnormal appearance.
The abdomen was then thoroughly explored so as to find the retroperitoneal location of pancreas. The pancreas is relatively inaccessible as its retroperitoneal location in the upper abdomen means that it is almost completely hidden by the stomach, transverse colon and mesocolon deriving its blood supply from numerous branches arising from major branches of the coeliac and superior mesenteric arteries and further the anatomical relationship of pancreas is summarized as, "the pancreas cuddles the left kidney, tickles the spleen, hugs the duodenum, cradles the aorta, opposes the inferior vena cava, dallies with the right renal pedicle, hides behind the posterior parietal peritoneum of the lesser sac and wraps itself around the superior mesenteric vessels" (Moossa, 1982). However, the pancreas was mobilized out of the retroperitoneum. It was then extracted and placed in normal saline.

Fig. Photograph showing location of pancreas in situ

The other organs viz., kidneys liver and different parts of gut were removed and placed in normal saline so as to remove the adherent blood. The thoracic cavity was then exposed by cutting the diaphragm and then clipping
the ribs using the bone cutting forceps. The clipped portion of the rib cage was then lifted off and removed or reflected laterally. The lungs, heart and pleural surfaces were examined for abnormalities as for the abdominal cavity. The organs were removed by cutting the trachea and all attachments of trachea, lungs, and heart caudally to the diaphragm.

For removal of brain, the skin over the skull was reflected forward and was held firmly in position with one hand. With the tips of heavy jawed bone shares or strong scissors, a nip or cut was made just through the bone to the cranial cavity on both sides of the head, beginning at the occipital foramen and proceeding forward laterally to the mid point at the anterior edge of the cranial cavity. The cut portion of the brain was lift off and the entire brain was exposed. The nerves and the attachments were cut carefully while the head was tipped upside down to collect the loosen brain. After collection, the brain was divided longitudinally into two halves with sharp scalpel.

After removing the organs from the animals, these were cut into desirable sizes with the help of sharp scalpel and were processed for histological/histopathological study using routine histological methods (Weesner, 1968; Luna, 1968). The different processing steps include:

**Fixation**

The tissues were immediately placed in Bouin's fluid or 10% neutral buffered formalin keeping the volume of fixatives 30 to 50 times the volume of the tissue. The procedure for preparation of these fixatives is as follows:

**Bouin's Fluid**

It is composed of:

- Picric acid, saturated aqueous solution $750.0 \text{ ml}$
- 37-40% formalin $250.0 \text{ ml}$
- Glacial acetic acid $50.0 \text{ ml}$
The fixation was done for 4-12 hours or several days. After fixing the blocks/tissues in Bouin’s solution, these were washed in several changes of 50% alcohol for 4-6 hours, agitating constantly, to ensure proper removal of the picric acid, and then stored in 70% alcohol.

**10% Neutral Buffered Formalin**

It is composed of:

- Sodium dihydrogen phosphate, monohydrate (Na$_2$H$_2$PO$_4$-H$_2$O) 4.0 g
- Sodium monohydrogen phosphate anhydrous (Na$_2$HPO$_4$) 6.5 g

After fixing the material in 10% neutral buffered formalin, it is washed in water or alcohol. A convenient stopping place is to bring the tissues, after washing in water, through 50% alcohol and then to 70% or 80% alcohol. At these alcohol concentrations tissues can be stored for several weeks or months without harming the tissue.

**Post Fixation Treatment**

After fixation, the excess fixative was washed out as recommended for the particular fixative used so as to prevent interference with subsequent processing of the tissue. As paraffin and water are not miscible, the tissues were first dehydrated and cleared, then infiltrated and embedded in paraffin.

**Dehydration of the Tissues**

Here the tissues were passed through different types of graded alcohol series (80%, 90%, 100% alcohol) keeping the volume of the alcohol 10 times the size of the tissue. The time/duration in each alcoholic concentration is given below in the flow chart:
Flow Chart for Dehydration

<table>
<thead>
<tr>
<th>Step</th>
<th>Timing</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Holding point</td>
<td>80% alcohol</td>
</tr>
<tr>
<td>2.</td>
<td>Two hours (1(^{st}) change)</td>
<td>95% alcohol</td>
</tr>
<tr>
<td>3.</td>
<td>One hour (2(^{nd}) change)</td>
<td>95% alcohol</td>
</tr>
<tr>
<td>4.</td>
<td>One hour (1(^{st}) change)</td>
<td>100% alcohol</td>
</tr>
<tr>
<td>5.</td>
<td>One hour (2(^{nd}) change)</td>
<td>100% alcohol</td>
</tr>
<tr>
<td>6.</td>
<td>One hour (3(^{rd}) change)</td>
<td>100% alcohol</td>
</tr>
</tbody>
</table>

**Clearing/Dealcoholization**

The tissues after dehydration were cleared in xylene keeping the volume of the clearing agents 10 times the size of the tissues. The process of clearing with duration is as follows:

<table>
<thead>
<tr>
<th>Step</th>
<th>Timing</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.</td>
<td>One hour (1(^{st}) change)</td>
<td>Xylene</td>
</tr>
<tr>
<td>8.</td>
<td>One hour (2(^{nd}) change)</td>
<td>Xylene</td>
</tr>
</tbody>
</table>

**Infiltration or Impregnation**

The tissues were then removed from the clearing agent, drained and placed in the first paraffin beaker in the paraffin for a period of 2 hours. The temperature of the paraffin oven was maintained at 60°C and the volume of the paraffin wax was kept 15 to 20 times the volume of the tissue. The tissue were then transferred to the second and third paraffin beakers for two hours and 1½ hours, respectively.
<table>
<thead>
<tr>
<th>Step</th>
<th>Timing</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>9.</td>
<td>2 hours (1st change)</td>
<td>Paraffin</td>
</tr>
<tr>
<td>10.</td>
<td>2 hours (2nd change)</td>
<td>Paraffin</td>
</tr>
<tr>
<td>11.</td>
<td>1½ hours (3rd change)</td>
<td>Paraffin</td>
</tr>
</tbody>
</table>

**Embedding or Casting or Blocking**

Upon completion of infiltration, the tissues were removed from the tissue capsule and were transferred to a small container of freshly melted paraffin with warm forceps. Care was taken that the tips of forceps, while heating, might not get overheated which might result in the cooking of the tissue. The small container of paraffin was placed on the same paraffin oven with maintained temperature. Embedding paper boats or block holders were filled with molten paraffin. With the help of warm forceps, the tissues were picked up gently and placed into mold keeping the tissue towards the bottom of the mold and centered, leaving a margin of several millimeters around the tissue. Manipulation of the tissue in the mold with proper orientation was quick, so that paraffin did not begin to harden. The paraffin block was allowed to harden and then immersed into shallow, cool (10°C) water bath for 10 to 15 minutes to hasten solidification of the paraffin. When the paraffin was completely hardened, it was then removed from mold and labelled with a code number.

**Trimming of the Block**

The paraffin block was carefully trimmed before attaching to the microtome peg with the help of a scalpel with a clean even edge. The excess paraffin was removed so as to maintain proper size of the block. The block now appeared to be transparent.

**Mounting the Tissue Block on the Block Holder or Microtome Peg**

The paraffin tissue block was attached to a base or peg for clamping into the microtome. The mounting was done by holding the block holder on the left
hand and a little wax was placed on the upper surface of block holder to which tissue was to be mounted. Spatula or backside of scalpel was heated and the wax placed on the block holder was evenly distributed. It was repeated till the surface coating of the block holder was about 1 mm thick. With the help of heated spatula, a central cavity was melted into the surface of the block holder and the trimmed tissue block was immediately placed into the cavity so that the front of the block (the face along which sections were to be to cut) is upper most, the tissue block was to be gently but firmly pressed down against the peg with the help of forefinger placed on top of the block. The tissue block was kept exactly perpendicular to the surface of the block holder. While setting the base, the block holder containing the tissue block was immersed in cold water in a beaker for complete setting.

A label with code number was attached to the block holder/peg by encircling it with a piece of cellophane tape.

**Microtomy or Sectioning**

The solid paraffin block containing the tissue was sliced into thin desired sections of 5 micron on a rotary microtome (WESWOX).

Before sectioning the paraffin block containing the tissue block was mounted on the microtome. Proper orientation of the block in the microtome was done. The thickness of the sections was achieved by setting the thickness adjustment scale at desired microns (5 microns). After checking all the parts of microtome, the drive wheel of the microtome was rotated and the subsequent sections of tissue in the form of smooth straight ribbons were picked up with the help of two clean ‘camels hair’ brushes.

**Affixing or Attaching Sections to Slides**

Before affixation, the slides were cleaned scrupulously. The sections of tissues were attached to these slides using Mayer’s affixative whose composition is:
Mayer’s Affixative

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Egg albumin</td>
<td>50.0 ml</td>
</tr>
<tr>
<td>Glycerin</td>
<td>50.0 ml</td>
</tr>
<tr>
<td>Sodium salicylate</td>
<td>1 gm</td>
</tr>
</tbody>
</table>

A small drop of Mayer’s egg albumin was smeared over the surface of the slide with the finger and the excess rubbed off. The tissue sections in the form of ribbons were placed in tissue floatation bath whose temperature was maintained according to the melting point of the paraffin wax and were picked on the center of the slides. The slides were dried on a slide-warming place. Using a diamond point pencil each slide was clearly labelled. After the desired number of sections were taken all blocks were sealed with paraffin wax so as to prevent drying and other damages of the exposed tissue/material.

Staining Procedure

Routine Haematoxylin and Eosin stain was used for histological/histopathological study.

Specimen Preparation: 5 micron thick sections.

Reagents Required

1) **Harris Haematoxylin**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haematoxylin crystals</td>
<td>- 5.0gm</td>
</tr>
<tr>
<td>Ethanol</td>
<td>- 50.0ml</td>
</tr>
<tr>
<td>Ammonium or Potassium Alum</td>
<td>- 100.0gm</td>
</tr>
<tr>
<td>Distilled water</td>
<td>- 1000.0ml</td>
</tr>
<tr>
<td>Mercuric oxide (red)</td>
<td>- 2.5gm</td>
</tr>
</tbody>
</table>

The haematoxylin in the alcohol was dissolved and the alum in the water by the aid of heat. The two solutions were mixed and brought to a boil as rapidly as possible. The heat was limited to less than one minute and stirred often. The solution was removed from heat and the mercuric oxide was added
slowly. It was reheated to a simmer until dark purple. The vessel was plunged into a basin of cold water until cool. 2-4ml of glacial acetic acid per 100 ml of solution was added to increase the precision of the nuclear stain and was filtered before use.

2)  **Eosin Solution**
   1% stock alcoholic eosin
   Eosin y. water soluble  - 1.0gm
   Distilled water  - 20.0ml
   Dissolve and add:
   Alcohol, 95%  - 80.0ml
   **Working Eosin Solution**
   Eosin stock Solution  - 1 part
   Alcohol, 80%  - 3 parts
   Just before use, 0.5ml of glacial acetic acid was added to each 100 ml of stain and stirred.

3)  **1% Acid Alcohol**
   Acid Alcohol  - 1 ml
   90% alcohol  - 100ml

4)  **0.1% Ammonium Water**
   Ammonia  - 0.1ml
   Distilled water  - 100ml

**Routine Harris’ Haematoxylin and Eosin Staining Method**

1. **Deparaffinization**
   Xylene I  - 15min.
   Xylene II  - 15min.
   Xylene III  - 15min.

2. **Hydration**
   Absolute Alcohol I  - 5min.
   Absolute Alcohol II  - 5min.
   90% Alcohol  - 5min.
3. **Staining**

- **Harris’ Haematoxylin**: 7min.
- **Distilled water**: Rinse
- **Acid Alcohol (1%)**: one dip
- **Distilled water**: Rinse
- **Ammonia water (0.1%)**: Till optimum blue
- **Tap water**: Wash
- **Distilled water**: Rinse
- **Eosin stain (Alcoholic)**: 4 minutes

4. **Dehydration**

- **95% alcohol**: 30 sec.
- **Absolute Alcohol I**: 30 sec.
- **Absolute Alcohol II**: 30 sec.
- **Absolute Alcohol III**: 30 sec.

5. **Clearing**

- **Xylene I**: 30min.
- **Xylene II**: 30min.
- **Xylene III**: 60min.

6. **Mounting**

Mounted in DPX mountant

In order to demonstrate the different types of cells in the islets especially the beta cells, certain special stains were employed *viz.* “Rapid staining of Beta cell Granules in Pancreatic Islets” (Scott, 1952) and Gomorri Modified Stain (Halmi, 1952). The staining methods of these stains are given as:
Rapid staining of Beta cell Granules in Pancreatic Islets (Scott, 1952)

A modification of Gomori’s staining technique (Gomori, 1950) was developed by Scott (1952) to demonstrate the pancreatic islets of the mouse. The method is more rapid and precise than Gomori’s staining method.

Fixative: - Bouin’s fluid

Reagents required

1) 0.5% Potassium Permanganate

Potassium permanganate - 0.5gm
Distilled water - 100.0ml

0.5% Sulphuric Acid

Sulphuric acid - 0.5ml
Distilled water - 100.0ml

Both the solutions were mixed in equal proportions.

2) 2% Sodium Bisulphite

Sodium bisulphite - 1.0gm
Distilled water - 100ml

3) Gomoris Aldehyde Fuchsin

Basic Fuchsin - 1.0gm
Paraldehyde - 2.0ml
Conc. Hcl - 1.0ml
Ethanol - 60ml
Distilled water - 40ml

The basic fuchsin was dissolved in the alcoholic distilled water. The hydrochloric acid and the paraldehyde were added and the solution was allowed to ripen for 2-7 days at room temperature, then filtered and stored at 4°C.

4) 0.5% Phloxine

Phloxine - 0.5 gm
Distilled water - 100 ml
5) **5% Phosphotungstic Acid**

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphotungstic Acid</td>
<td>5 gm</td>
</tr>
<tr>
<td>Distilled water</td>
<td>100 ml</td>
</tr>
</tbody>
</table>

6) **0.2% Fast Green FCF**

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fast Green FCF</td>
<td>0.2 gm</td>
</tr>
<tr>
<td>Distilled water</td>
<td>100 ml</td>
</tr>
</tbody>
</table>

**Method**

1) Bring deparaffinized sections to water.
2) Oxidize in a mixture of equal parts of 0.5% potassium permanganate and 0.5% sulphuric acid for 2 minutes.
3) Rinse in distilled water.
4) Decolourize in 2% sodium bisulphite
5) Wash under tap for 2 minutes
6) Stain in Gomori’s aldehyde-fuchsin for 2 minutes.
7) Rinse in 3 changes of 95% alcohol.
8) Run sections down to water.
9) Stain in 0.5% phloxine for 2 minutes
10) Rinse in distilled water.
11) Place in 5% Phosphotungstic acid for 1 minute.
12) Wash under tap for 2 to 5 minutes.
13) Stain in 0.2% fast green FCF for 30 seconds.
14) Rinse in 95% alcohol for 15 seconds.
15) Place in absolute alcohol for 30 seconds.
16) Xylene (2 changes) and mount.

**Result**

After counterstaining by this method, the cytoplasmic background of the beta cells is stained light green in strong contrast to the deep purple granulation. The cytoplasm of the other cells in the islet and of any duct cells present is also stained light green, while nuclei and erythrocytes exhibit the light red colouration of the phloxine.
Modified Aldehyde Fuchsin Stain (Halmi, 1952)

Halmi in 1952 modified Gomori's aldehyde fuchsin stain and is summarized as:

Specimen Preparation

5 μ thick paraffin sections were cut from tissues fixed in Bouins fluid or 10% neutral buffered formalin.

Reagents Required

1) Lugol's Iodine
   Iodine Crystals - 1.0g
   Potassium Iodide - 2.0g
   Made up to 100 ml with distilled water

2) Sodium Thiosulphate
   Sodium thiosulphate - 5.0gm
   Distilled water - 100.0ml

3) Aldehyde Fuchsin
   Pararosaniline (Cl 42500) - 0.5gm
   70% ethanol - 100.0ml
   Paraldehyde - 1ml
   Conc. Hcl - 1ml
   Pararosaniline in ethanol was dissolved. The paraldehyde and HCl were added. The stain was allowed to ripen at room temperature for 3 to 5 days and then stored at 4°C.

4) Light Green/Orange G
   Light Green SF yellowish (Cl 42095) - 0.2gm
   Orange G (Cl 16230) - 1.0gm
   Phosphotungestic Acid - 0.5gm
   Distilled water - 100.0ml
   Glacial Acetic Acid - 1.0ml
5) **Celestine Blue**

Celestine blue B - 2.5gm
Ferric ammonium sulphate - 25.0gm
Glycerin - 70cm$^3$
Distilled water - 500cm$^3$

The ferric ammonium sulphate in cold distilled water were dissolved and stir well. The celestine blue to this solution was added, then the mixture was boiled for a few minutes. After cooling, the stain was filtered and the glycerin was added.

6) **0.2% Acetic Acid**

Acetic Acid - 2.0ml
Distilled water - 100.0ml

**Method**

1) Dewax and rehydrate sections.
2) Place sections in Lugol’s Iodine for 10 minutes.
3) Wash in water.
4) Decolourize with sodium thiosulphate for 2 minutes
5) Wash in water.
6) Rinse in 70% alcohol
7) Immerse sections in aldehyde Fuchsin staining solution for 15 – 30 minutes. Check staining microscopically.
8) Rinse in 95% ethanol.
9) Wash in water.
10) Stain nuclei with celestine blue or alum haematoxylin. differentiate and blue.
11) Rinse in distilled water.
12) Counterstain with light Green/Orange G for 45 seconds.
13) Rinse briefly in 0.2% acetic acid, then in 95% ethanol.
14) Dehydrate, clear and mount.

**Result**

<table>
<thead>
<tr>
<th>Nuclei</th>
<th>Blue/Black</th>
</tr>
</thead>
<tbody>
<tr>
<td>B-cell granules</td>
<td>Purple</td>
</tr>
</tbody>
</table>
A-cell granules - Yellow
D-cell granules - Green
Collagen - Green

The results obtained by this method were not satisfactory, as the stain did not differentiate different types of cells in islets of Langerhan's. So, a modification of the stain was done by substituting the lugol's iodine with equal parts of 0.5% KMNO₄ and 0.5 sulphuric acid, and sodium thiosulphate with 2% sodium bisulphite respectively. Further, the duration of treatment of sections was reduced from 45 seconds to 5 seconds in the counter stain (Light Green / Orange G). The protocol for staining is as:

1) Xylene I - 15min.
2) Xylene II - 15 min.
3) Xylene III - 15 min.
4) 100% alcohol I - 5min.
5) 100% alcohol II - 5min.
6) 90% alcohol - 5min.
7) 80% alcohol - 5min.
8) 70% alcohol - 5min.
9) 50% alcohol - 5min.
10) Distilled water - 15 min.
11) 0.5% KMNO₄ + 0.5%H₂SO₄ - 2min.
12) Distilled water - Rinse
13) 2% Sodium Bisulphite - Decolorize
14) Tape water - 2min.
15) 70% alcohol - Rinse
17) 95% alcohol - Rinse
18) Tap water - wash
19) Alum Haematoxylin - 10min.
20) Acetic Acid - Rinse
21) 1% ammonium water - Till optimum blue
22) Tap water - wash
23) Light Green / Orange G - 5 seconds
24) 0.2% Acetic acid - Rinse
25) 95% ethanol - Rinse
26) 100% ethanol - 5 minutes
27) Xylene I - 15 min
28) Xylene II - 30 min.
29) Xylene III - 30 min.
30) Mount in paramount/DPX mountant.

This method gave satisfactory results and the alpha and beta cells were qualitatively and quantitatively checked.

Photomicrography

The microscopic study of the stained tissue sections for histopathological study was done with the help trinocular microscope using different lens combinations. Photomicrographs were taken using PM-6.

Animals with Therapeutic Study on Experimentally Induced Diabetic Rabbits

During the course of present investigation the diabetic rabbits were given water extracts of Abroma augusta and Syzygium jambolanum orally so as to study the improvement with regard to biochemical, behavioural and histological changes. An Allopathic drug, glimepiride was also tested in this experimental study.

New Zealand white male healthy rabbits of 8-12 months old were selected for the study. The rabbits were purchased from Institute of Animal Health and Biological Products, Zakura Srinagar. Before the start of the experiment the animals were acclimatized to standard laboratory conditions for a period of 15 days. Fiber rich vegetables and commercially available pelleted diet was provided to rabbits in three divided doses daily.

Induction of Diabetes Mellitus

After acclimatization to standard laboratory conditions, the animals were made diabetic by intravenous administration of alloxan (Wasan et al., 1998).
For intravenous administration a single dose of alloxan @ 100mg/kg b.w. dissolved in 1 ml of sterile water was given after twelve hours of fasting. The fasting glucose levels above 250mg/dl of rabbits were considered for therapeutic studies. For this set of therapeutic experiments, the methods/techniques (biochemical and histopathological) were same as elaborated in previous pages of this chapter.

**Drug Treatment**

1) *Abroma augusta*: The water extract of dried powder of leaves of *Abroma augusta* was purchased commercially from Dr. Wellman’s Homeopathic Laboratory Pvt. Ltd. Wazirpur India. It was given orally to the diabetic animals @ 2 ml daily for a period of fifteen days.

2) *Syzygium jambolanum*: The water extract of *syzygium jambolanum* was purchased commercially from Dr. Wellman’s Homeopathic Laboratory Pvt. Ltd. Wazirpur India. The rabbits received an oral dosage of *Syzygium jambolanum* @ 2 ml daily for a period of 15 days.

3) *Glimepiride*: One group of diabetic rabbits received allopathic drug, glimepiride @ 2mg/kg b.w. daily for a period of 15 days. The drug was dissolved in 5ml of sterilized water and the homogenous fluid was given.

**Method of Oral Administration of Drugs**

The oral administration of antidiabetic drugs included either oral gavage or administration by syringe.

**Technique for Oral Gavage**

1) The total length of the tube to be inserted was estimated as the length from the mouth to the last rib and was marked on the tube before insertion begun.
2) A speculum was placed in the rabbits mouth to prevent chewing of the tube. A small block of wood with a hole drilled in the middle allow passage of the tube was sufficient.

3) The tube (usually an infant feeding tube) was lightly lubricated with petroleum jelly.

4) The tube was passed through the speculum and back to the pharynx. When the rabbit demonstrated the gag reflex, the tube was advanced into the esophagus and on into the stomach.

5) The location of the tube in the stomach was confirmed by examining for a lack of air passage through the tube as the rabbit breath so as to avoid accidental administration of the drug to the respiratory tract.

6) Drugs were administered slowly by a syringe attached to the stomach tube. A small volume of water was subsequently administered to rinse any residual drug into the stomach.

7) After administration, the tube was kinked, to prevent flow of residual material in the tube into the respiratory tree as it passes through the pharynx, and the tube was slowly withdrawn.

Fig. Orientation of the stomach tube for oral gavage in the rabbit.
Administration by Syringe

By this method the tip of the syringe was placed at the corner of the rabbits mouth and the material was slowly injected.

Grouping of Animals

Five groups of rabbits were selected for part II of the experiments. Group I comprised four normal healthy rabbits. Group II, III, IV and V comprised alloxanized diabetic rabbits of four animals each

- Group I served as normal control and received normal saline orally.
- Group II were untreated alloxanized diabetic rabbits and received normal saline orally.
- Group III alloxanized diabetic rabbits received water extract of *Abroma augusta* @ 2 ml daily.
- Group IV alloxanized diabetic rabbits received aqueous extract of *Syzygium jambolanum* @ 2 ml daily.
- Group V alloxanized diabetic rabbits received glimepiride @ 2mg/kg b.w.

Assessment of Treatment

The efficacy of drugs was based on the improvement of behavioural, biochemical and histopathological changes. Biochemical estimation was restricted to blood sugar (F), blood urea and serum creatinine. Biochemical values were estimated on day 7th, day 15th and day 21st. After 21 days of treatment 50% of all groups of rabbits were sacrificed. Histological study was carried to the pancreas, kidneys, liver, heart, lungs, gut and brain so as to check the efficiency of drugs up to the tissue level.

Quantitative Assessment of Beta Cells

In all the groups of rabbits viz. control (saline treated healthy rabbits), diabetic-untreated (alloxan-induced and streptozotocin-induced diabetic rabbits) and diabetic-treated rabbits, the percentage of beta cells were counted. Cells of
approximately four islets on each tissue and forty islets of each group were counted under a light microscope at a magnification of ×100.

**Statistical Analysis**

**Student's 't' test** was used for statistical analysis of the data and value \( P \) was calculated for evaluating statistical significance. The value of 't' was calculated (Prasad, 2000) according to the following equation:

\[
t = \frac{\bar{X}_1 - \bar{X}_2}{SE_D}
\]

Where, \( \bar{X}_1 = \text{Mean of one variable (Diabetic)} \)
\( \bar{X}_2 = \text{Mean of second variable (Saline Control)} \)
\( SE_D = \text{Standard error of difference between two means} \)

The standard error was obtained by using following formula:

\[
SE_D = \sqrt{SE \bar{X}_1^2 + SE \bar{X}_2^2}
\]

where, \( SE \bar{X}_1 = \text{Standard error of the first mean} \)
\( SE \bar{X}_2 = \text{Standard error of the second mean} \)

\( SE_{M} \) was obtained with the help of following formula:

\[
SE_{M} = \frac{\sigma}{\sqrt{N-1}}
\]

'\( P \)' value was obtained from the distribution of 't' probability chart.