CHAPTER - II

MATERIAL AND METHODS

I. MATERIAL

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II. METHODS

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I. MATERIAL

Male Albino mice (*Mus musculus*) were used for the present investigation. Breeding pairs of mice were obtained from Hindustan Antibiotics Ltd., Pune. They were breed in the animal house. They were housed in aluminium cages in groups of 4 to 5 and were supplied with Amrut Rat/ mouse feed (Pranav Agro Industry) and water *ad libitum*.

i. Sialoadenectomy :

For sialoadenectomy 10 mice of three months old age, weighing 30 to 35 gms were used. Mice were fasted overnight before operation. Operation tray was cleaned with soapwater, sundried and then scrubbed with 95% alcohol. Scissors, forceps, needles, thread etc. were autoclaved. Sialoadenectomy was carried out under mild ether anaesthesia. Operations were carried out in between 9 A.M. to 10 A.M. Operated mice were maintained in animal house with proper care for two months. Controls were sham operated. Operated mice were sacrificed by cervical dislocation. The stomach and duodenum of mice were dissected out and opened along the greater curvature of stomach and mesentry of duodenum. The general morphological observations were made by fixing the stomach and duodenum in a wax tray containing 0.9% saline chilled at 4^0C. The stomach and duodenum were fixed in 10% neutral buffered formulin (NBF) for 24 hours. After fixation the tissues were washed under running tap water

for 13 to 14 hours and followed for the study of histology and histochemistry.

ii. Cysteamine-HCl Administration :

Ten male mice weighing 30 to 35 gms of 3 months age were selected for cysteamine administration. They were maintained along controlled group in individual cages. Care was taken to avoid coprophagy. The animals were supplied with Amrut mice feed (Pranav Agro Industry) and water *ad libitum*. Body weights of all the animals were recorded. They were initially starved for 24 hrs during which time only water was supplied *ad libitum*. Cysteamine (dissolved in water; 40 mg/ kg body wt.) was administered subcutaneously to the concerned group. Both control and cysteamine administered groups were again starved for further 24 hrs, however water was supplied *ad libitum*. Twenty four hours, after the treatment, both the controlled mice and cysteamine treated mice were weighed and all the animals were sacrificed by cervical dislocation.

iii. Sialoadenectomy and Cysteamine-HCl Administration :

Ten male mice weighing 30 to 35 gms of 3 months age were selected for sialoadenectomy and sialoadenectomized mice were maintained for 40 days. The body weight of all these sialoadenectomized mice were recorded before the administration of cysteamine. For cysteamine administration, they were fasted overnight, during which time only water was supplied *ad libitum*.

Cysteamine (dissolved in water; 40 mg/ kg body weight) was administered subcutaneously to the starved mice. Both the control and sialoadenectomized, cysteamine administered groups were again starved for further 24 hrs, however water was supplied *ad libitum*. Twenty four hrs after the treatment both the controlled and treated mice were weighed and all the animals were sacrificed by cervical dislocation.

II. METHODS :

i. Whole Mount Preparation and Gross Morphology of duodenum :

Duodenum of mice were processed according to the method used by Landboe-Christenson (1944). It involved washing of duodenum (3 cm long from pyloroduodenal junction) which were split opened and pinned in a small wax tray in cold running water.

The duodenum of controlled, sialoadenectomized, cysteamine-HCl administered and sialoadenectomized, cysteamine HCl treated animals were cleared, washed with water and demonstrated with PAS technique and alkaline phosphatase.

Stomach alongwith duodenum were dissected out and the stomach were cut opened along the greater curvature and the duodenum along the mesentry. Stomach and duodenum were examined carefully for lesions with the help of occular magnifier and stereoscopic microscope. Each mice was examined for gastric and

duodenal ulcer so as to critically evaluate the duodenal ulcers accompanied by gastric ulcers.

ii. Ulcer Index :

The duodenal ulcers were critically evaluated with respect to the percentage incidence, number of ulcers per mice and severity. The severity of ulcer was graded according to a scale from 0 to 3 based on the macroscopic appearance and later confirmed by sectioning and staining of the ulcerated region :

0 = no ulcer
1 = superficial mucosal erosion
2 = deep ulcer penetrating to submucosa (involving muscularis propria)
3 = penetrating or perforating ulcer.

The ulcer index was calculated by the following formula :

J Score or Ulcer Index = mean severity + incidence x 2 (i.e.+ve / total)

The method used was described by Szabo (1978).

Results were interpreted with student's "t" test statistical methods.

iii. Histology :

To study the histological structure of duodenal mucosa, the duodenums were fixed in 10% neutral buffered formaldehyde (NBF) for 24 hrs. at 4^0C. The tissues were washed under running tap water

for 14 hrs. and dehydrated through alcoholic grades, cleared in xylene and embedded in paraffin. The sections were cut at 6μ on a rotary microtome. The sections were mounted on albuminized glass slides and routinely stained with haematoxylin -eosin (H-E).

iv. Histochemistry :

Polysaccharides are classified as glycogen and muco-polysaccharides. The mucopolysaccharides are further classified into neutral mucopolysaccharides and acidic mucopolysaccharides (Spicer *et al.*, 1965). Neutral glycoproteins, immunologically reactive glycoproteins, fucomucins and mannose rich mucosubstances are neutral mucopolysaccharides and all are per iodate reactive. Sulfated mucosubstances and carboxyl mucosubstances are called as acid mucosubstances. Some of them are per iodate reactive and some are per iodate non-reactive (Roberts,1977). But Alcian blue, 1% (pH 2.5 – 2.7) stains both the types of acid mucopolysaccharides. Alcian blue (0.5%) at pH 1.0 stains only sulfated mucosubstances (Spicer *et al.*, 1967; Pearse, 1968).

a) Per iodic Acid Schiff Reaction :

The periodic acid-Schiff reaction includes two steps of reaction

Oxidation by periodic acid :

Periodic acid is an oxidizing agent that is known for its ability to oxidize 1, 2 glycols to form dialdehydes. Many other oxidizing

agents could be theoretically used as well, and some have been actually substituted for periodic acid. However, other oxidizing agents are not convenient to use, because the aldehydes are further oxidized to carboxyl groups.

Reduction by Na-metabisulfide :

Aldehydes are demonstrated with Schiff's reagent and tissue sections are given several rinses in 0.5% aqueous solution of sodium metabisulfide as reducing agent. The PAS procedure demonstrates glycogen and this can be used to demonstrate glycogen by using diastase digestion control. To demonstrate mucosubstances in tissues, periodic acid-Schiff's technique was first introduced by McManus (1946), followed by Hotchkiss (1948).

Method :

i. After dewaxation and hydration, the sections were brought to distilled water through alcohol grades.

ii. Sections were oxidized with 0.5% periodic acid for 10 minutes.

iii. Sections were washed with distilled water and treated with Schiff's reagent for 10 minutes followed by rinsing, 3 times in 0.5% sodium metabisulfide.

iv. Sections were washed thoroughly in distilled water, dehydrated through alcohol grades, cleared in xylene and mounted in Distrene diphenyl Pthalate Xylene (DPX).

b. Alcian Blue Staining Method :

Alcian blue 8GS, the textile dye, was adopted as histochemical stain specific for 'mucins' by Steedman (1950). The specificity was improved by Mowry (1956), using higher concentration of dye at lower pH. Alcian blue is unique in three ways :

i. It does not have affinity for nucleic acids.

ii. It stains carboxyl groups at pH substantially below their pK value.

iii. It has high solubility even in the presence of high concentration of salts.

Spicer (1960) in his comparison of affinity to alcian blue and azure A observed that alcian blue exhibited greater resistance to acid and alkali decoloration than that of azure A. This property of alcian blue led Spicer (1960) to suggest that alcian blue is not held by a salt type linkage like that of which is apparently involved in azure A binding. Alcian blue stains both sulfated and non sulfated acid glycosaminoglycans, when used in a 3 percent acetic and solution (pH 2.5 to 2.7) but at a pH^1, only sulfated ones stain (Pearse, 1968), Sialoglycoproteins and also stained at pH 2.5 (Spicer *et al.,* 1967). Spicer (1960) studied the effect of pH on alcian blue affinity and observed that dilute solutions of the dye show a strong affinity for mucins from pH 1.0 to 3.0, but almost none at pH 3.0 to 4.0.

Method :

i. Sections were deparaffinized in xylene, hydrated through alcoholic grades and brought to distilled water.

ii. The sections were treated with 3% acetic acid for 5 minutes and then stained with alcian blue for 30 minutes (1% alcian blue in 3% acetic acid, pH 2.5).

iii. Sections were washed in running tap water for 5 minutes, dehydrated through grades of alcohol, cleared in xylene and mounted in DPX.

III. CHEMICALS :

Following chemicals used for the present investigation, were obtained from different sources :

Sr. No.	Name of the chemical	Batch No.	Source
1.	Cysteamine –HCl	130347/1 54099	Fluka Biochemica USA
2.	Fast Red RR	7109	Chroma Cesell Schaft Schino and Com.
3.	Naphthol-AS-MX Phosphate	70476	Fluka Biochemica, USA

Other chemicals were used of analytical grade.