CHAPTER

III

MATERIAL AND METHODS

All experiments in the present study were carried out on albino rats (Haffkine strain) of either sex weighing 200 to 250 g. The animals were maintained in our own laboratory for at least one week before any experimentation. Rats were deprived of food but free access to water was allowed for 24 hours prior to administration of any ulcerogenic substances or pyloric ligation for collecting gastric juice. All the drugs were administered in such a way that pyloric ligation could be carried out between 8 to 9 a.m.

PYLORUS LIGATION

On the last day of the treatment only drugs were administered. Rats were deprived of food but allowed free access to water for 24 hours prior to pylorus ligation. One hour prior to the operation even water was witheld. Rats were anaesthetized with pentobarbitone sodium (40 mg/kg i.p.) and the pyloric end of the stomach was ligated according to the standard method of Shay et al (1945), and proper care was taken that no blood vessels were occluded, abdomen was then closed in two layers and the animals were transferred in the cage and care was taken to avoid coprophagy.

After 4 hours of pyloric ligation rats were sacrificed by an overdose of anaesthetic ether. The abdomen was opened and cardiac end of the stomach was ligated. The stomach and the duodenum were dissected out and incised along the greater curvature to examine for lesions developed in the mucosa grossly and by histopathological study to confirm the presence of ulcer and for collection of gastric juice. The gastric juice was collected in centrifuge tubes and the stomach and duodenum were carefully examined to determine the presence of gastroduodenal lesions for calculating ulcer index. Each sample of gastric juice collected was analysed for the estimation of total acidity, pepsin activity, mucin and protein.

GROSS APPEARANCE OF THE STOMACH AFTER PYLORUS LIGATION)

ULCER INDEX

The method of calculation and representation of ulcer index is highly complicated and controversial. Bonnycastle (1964) and Robert et al (1968) suggested a method in which the stomach was given grades (0 to 4) as follows :

0. normal
1. scattered haemorrhagic spots and some ulcers.

2. deeper haemorrhagic spots and some ulcers.
3. haemorrhagic spots and ulcers.
4. perforation.

Lesions were expressed in terms of 'ulcer index' which is derived from (a) percentage incidence of animals with ulcers (b) average severity per group in pluses (from a scale of 0 to 4) and (c) average number of lesions per stomach. The index was computed by dividing values of a, b and c by 10 and then summing up the figures obtained. Severity for a given stomach was that of the most severe lesion for that stomach (Robert et al., 1968).

Presently, the ulcer index is expressed as mm^2 which seems to be more scientific than the previous system of ulcer index calculation. The percentage protection obtained with both the types of ulcer index calculation was compared. No significant difference was observed between these methods.

In the present study the length and width of each lesion was observed carefully and total area of each lesion (mm^2) was measured under the dissecting microscope (10 X). The sum of the areas of lesions in each animal was considered as ulcer index and expressed as ulcer index $mm^2 \pm$ S.E.M. The number of ulcers was also recorded and percentage of protection was calculated for each experiment.

GASTRIC JUICE ANALYSIS

The gastric juice collected after 4 hours of pylorus ligation was centrifuged at. 3000 r.p.m. for 15 min. The volume of the clear supernatent fraction was measured for each experiment separately. The gastric juice was then analysed for total acidity, pepsin activity, and various carbohydrates and protein by standard methods.

PREPARATION OF DRUGS

All the drugs were used in pure (base) form, and prepared freshly for each set of experiments and stored in the refrigerator. All concentrations were made in such a way that the total amount of drug administered did not exceed 0.5 ml.

Tamrabhasma (TABH) : 5% suspension of TABH was prepared in distilled water with trace of Tween 80.

Cimetidine : 1% solution of cimetidine was prepared in distilled water.

Carbenoxolone : 3% solution of carbenoxolone was prepared in distilled water.

After inducing the ulcers, rats were divided in various groups and received the following treatments.

a. TABH 60 mg/kg p.o. for 4 days.
b. TABH 60 mg/kg p.o. for 10 days
c. Carbenoxolone 30 mg/kg p.o. for 4 days.
d. Cimetidine 20 mg/kg p.o. for 4 days.
e. Carbenoxolone 15 mg/kg + TABH 60 mg/kg p.o. for 4 days.
f. Cimetidine 10 mg/kg + TABH 60 mg/kg p.o. for 4 days.

ANTIULCEROGENIC EFFECT OF TABH.

Initially a dose-response study was carried out in dexamethasone induced gastric ulcer in rats. TABH was administered from 1 to 120 mg/kg p.o. for 4 days after dexamethasone. On fifth day rats were sacrificed,stomachs were opened as described earlier, and ulcer index was calculated and expressed as percentage as described by Lynch et al (1962). Maximum protective effect was observed with 60 mg/kg and hence this dose was kept constant throughout the study.

PREVENTIVE EFFECT OF TABH IN DEXAMETHASONE-INDUCED GASTRIC ULCER

TABH (60 mg/kg p.o.) was administered for 4 days before, simultaneously and after dexamethasone-induced ulcer to study the preventive and curative effect of TABH if any. Control animals received vehicle in equal volume.

EFFECT OF TABH PERSE ON GASTRIC JUICE ANALYSIS

Rats were treated with TABH alone (60 mg/kg p.o.) for 4 and 10 days. On the last day gastric juice was collected as described earlier and was analysed for different parameters. Control animals received vehicle in equal volume.

INDUCTION OF ULCERS

DEXAMETHASONE-INDUCED GASTRIC ULCERS Dexamethasone 8 mg/kg s.c. (equivalent to 40 mg/kg ofΔcortisol), was administered daily in single dose for 4 days (Lynch et al., 1962). During this period the animals were kept fasting allowing free access to water ad libitum, and proper care was taken to see that the animals did not eat their faeces during the course of dexamethasone treatment. Control animals received normal saline injection of equal volume.

INDOMETHACIN-INDUCED GASTRIC ULCERS Indomethacin, a non steroidal antiinflammatory drug in a single dose of 20 mg/kg suspended in trace of Tween 80, was given i.p., to fasting rats before any treatment or pylorus ligation (OKABE et al., 1973). Control animals received equal volume of Tween 80 in distilled water.

CYSTEAMINE-INDUCED DUODENAL ULCERS As described by Fujii and Ishii (1975) cysteamine hydrochloride (β-mercaptoethylamine) dissolved in physiological saline immediately prior to use was administered once s.c. for inducing duodenal ulcer 18 hours before any treatment or pylorus ligation. The dose of cysteamine in this study was fixed at 300 mg/kg s.c. as the higher doses were found to be lethal in our experiments. Control animals received equal volume of normal saline subcutaneously.

BIOCHEMICAL ANALYSIS OF THE GASTRIC JUICE

TOTAL ACIDITY determination of the total acidity was carried out as described by Hawk (1965).

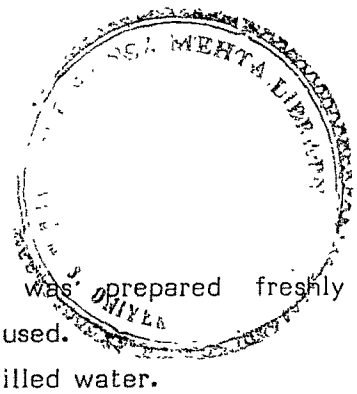
REAGENTS

a. 0.01 N NaOH
b. Topfer's reagent.
c. 1% alcoholic solution of phenolphthalein.

PROCEDURE

0.1 ml of clear gastric juice was taken in a clear vial and mixed with 0.9 ml of distilled water. Then the mixture was titrated with 0.01 N NaOH solution using Topfer's reagent as an indicator till the colour became salmon pink. At this point a drop of phenolphthalein solution was used as indicator and the titration continued till the sample turned pink. The burette reading at this point indicated total acidity. The amount of HCl was calculated and expressed as μEq/4 hrs.of gastric juice ± S.E.M.

PEPTIC ACTIVITY The determination of peptic activity was carried out as described earlier by Anson (1938) and modified by Debnath et al (1974).



REAGENTS

a. 0.1 N and 0.05 N HCl

b. haemoglobin solution : 2% haemoglobin solution was prepared freshly in 0.05 N HCl, filtered and used.

c. Trichloro acetic acid : 10% solution of TCA in distilled water.

d. Phenol reagent : 1 N phenol reagent was prepared by 2 times dilution with distilled water.

e. Alkaline mixture : 2% solution of Na_2CO_3 in 0.1 N NaOH. This solution was always prepared fresh before use.

f. Alkaline reagent : 100 ml of alkaline mixture was mixed with 1 ml of 4% of aqueous solution of potassium tartrate and 1 ml of 2% of aqueous copper sulphate.

g. L. Cystine was used as standard in estimation of digested substrate to indicate pepsin activity.

PROCEDURE

0.4 ml of the diluted gastric juice (1:250 dilution) and 1 ml of 2% haemoglobin solution in 0.05 N HCl were taken in separate test tubes. Both the test tubes were kept in the incubator at 37°C for 10 min. The haemoglobin solution was then added to the diluted gastric juice and this mixture was incubated at 37°C for 20 min. Digestion was stopped by adding equal volume i.e. 1.4 ml of ice cold trichloroacetic acid and kept in ice bath for 15 min. The mixture was then filtered out to separate the precipitated undigested protein.

0.4 ml of filtrate was taken to determine the concentration of liberated amino acid tyrosin by first adding 4 ml of alkaline reagent followed after 10 min by adding 0.4 ml of diluted phenol reagent as per the method of Lowry et al (1951).

The optical density was determined with Spectronic 20 spectrophotometer set at 610 mμ against the blank prepared similarly using 0.01 N HCl instead of diluted gastric juice after 10 min of adding phenol reagent. The peptic activity was calculated in terms of mmoles of tyrosin ± S.E.M. liberated per 4 hours of gastric juice.

MUCIN Mucin being a glycoprotein, the study included the carbohydrates namely hexoses, hexosamine, fucose, sialic acid and protein. Sanyal and coworkers (1983) compared the qualitative information in terms of carbohydrate : protein ratio in lyophilised nondialysable fraction of gastric juice with those of alcoholic precipitate

of the same gastric juice. The results were qualitatively similar. Hence in the present study also various carbohydrates and proteins were estimated from the alcoholic precipitate of the gastric juice.

ALCOHOLIC PRECIPITATION

1 ml of gastric juice and 9 ml of 95% alcohol was mixed, shaken and then the mixture was centrifuged at 3000 r.p.m. for 15 min to obtain the precipitation. This precipitation was dissolved in 1 ml of 0.1 N NaOH. From this 1 ml NaOH reconstituted solution 0.1 ml was taken to estimate the protein. To the rest 0.9 ml was added 4 times of its volume i.e. 3.6 ml of 6 N HCl. The mxiture was hydrolysed in boiling water for 2 hours. The hydrolysate was neutralised by 4 N NaOH using phenolphthalein as indicator and volume was restored to 9 ml with distilled water. It is from this 9 ml of hydrolysate that the total hexoses, hexosamine and fucose were estimated.

In the second phase 0.5 ml of gastric juice was mixed with 4.5 ml of 95% alcohol, shaken and centrifuged at 3000 r.p.m. for 15 min to obtain precipitate. The precipitate was dissolved in 0.5 ml of 0.1 N H_2SO_4. This was for estimating sialic acid.

TOTAL HEXOSES The estimation of total hexoses was carried out as described by Winzler (1958).

REAGENTS

a. Orcinol: 1.6g of orcinol dissolved in 100 ml of distilled water.

b. H_2SO_4 : H_2O mixture (3:2): 150 ml of concentrated sulphuric acid mixed with 100 ml of distilled water.

c. Orcinol-sulphuric acid reagent: 1 volume of reagent 'a' mixed with 7.5 volume of reagent 'b' just before use.

d. Galactose-mannose : equal amount of each was taken as standard.

PROCEDURE

To 0.4 ml of the hydrolysate 3.4 ml of orcinol reagent was added. The mixture was heated in the boiling water bath for 15 min. This was then taken out, cooled under running tap water and the intensity of the colour was read in a spectronic 20 spectrophotometer (Baush and Lomb) set at 540 mμ against the blank prepared of water instead of hydrolysate. The total hexose content was read from the standard curve of galactose-mannose and expressed as μg/ml of gastric juice ± S.E.M.

HEXOSAMINE Estimation of hexosamine was carried out as described by Dische & Boronfreund (1950).

REAGENT:

a. Acetyl-acetone reagent : 0.3 ml of acetylacetone mixed with 9.7 ml of 1.5 N sodium carbonate just before use.

b. Ehrich's reagent : 1.6 g of p-dimethyl amino benzyldehyde in 30 ml of con. HCl and kept in refrigerator.

c. (D+) glucosamine HCl was taken as standard for hexosamine estimation.

PROCEDURE

0.5 ml of the hydrolysated fraction was taken and 0.5 ml of acetylacetone reagent was added. The mixture was heated in boiling water bath for 20 min, taken out, and after cooling 1.5ml of 95% of alcohol was added, followed by the addition of 0.5 ml of Ehrich's reagent. The reaction was allowed a time of 30min to get completed. The colour intensity was measured on the Baush and Lomb spectrophotometer set at 530 mμ against the blank prepared from distilled water, instead of the hydrolysate. The hexosamine content of the sample was found out with the help of the standard curve prepared by (D+) glucosamine HCl. The content was expressed as μg/ml of gastric juice ± S.E.M.

FUCOSE Estimation of fucose was carried out as described by Dishe and Schettles (1948).

REAGENT

a. H_2SO_4 - H_2O mixture (6:1) : Six volumes of concentrated pure sulphuric acid was added to one volume of distilled water slowly with constant stirring and stored in refrigerator.

b. Cysteine reagent : 3% solution of cysteine HCl was prepared weekly in distilled water and kept in refrigerator.

c. (D+) Fucose was used as standard for estimation of fucose.

PROCEDURE

Three test tubes were taken. In one of them 1 ml of distilled water as blank and in rest two 1 ml of hydrolysate in each was taken. To all these 4.5 ml of 6:1 H_2SO_4

was added by keeping all test tubes in ice cold water bath to prevent breakage by strong exothermic reaction. After this, the mixture was heated in boiling water bath for 10 min the tubes were taken out and cooled. To the blank and one of the hydrolysate containing tubes (unknown) 0.1 ml of cysteine reagent was added while nothing was added to the last tube containing hydrolysate (unknown blank). After having allowed 90 min for completion of the reaction the reading was taken in Baush and Lomb spectrophotometer at 395 mμ setting zero with distilled water. True optical density for fucose in hydrolysate was calculated from the difference in the reading obtained at 395 and 430 mμ and substracting the values without cysteine. This was again read with the standard curve prepared with (D+) fucose and expressed in μg/ml of gastric juice ± S.E.M.

$$\text{The true optical density} = \frac{(\text{Unknown O.D. 395} - \text{O.D.430}) - (\text{Unknown blank O.D.395} - \text{O.D.430})}{(\text{O.D.395} - \text{O.D.430})}$$

SIALIC ACID Estimation of sialic acid was carried out as described by Warren (1959).

REAGENTS

a. 0.1 N sulphuric acid

b. Sodium meta periodate : 0.2M sodium meta periodate in 9 M orthophosphuric acid was prepared every 15 days and stored in glass stoppered bottle.

c. Sodium arsenite : 10% sodium arsenite in 0.5 M sodium sulphate was prepared every 15 days in 0.1 N H_2SO_4 and stored in glass bottle.

d. Thiobarbituric acid : 0.6% thiobarbituric acid in 0.5 M sodium sulphate was prepared every 15 days, filtered and stored in glass stoppered bottle.

e. Cyclohexanone.

f. Sialic acid was used as a standard for estimation of sialic acid.

PROCEDURE

The precipitate dissolved in 0.1 N H_2SO_4 was hydrolysed in boiling water bath for 1 hour in corked hydrolysing test tube to prevent evaporation. After hydrolysis, the volume of 0.5 ml was restored with 0.1 N of H_2SO_4, out of this 0.2 ml was taken to estimate the sialic acid. To the 0.2 ml of hydrolysate 0.1 ml of sodium meta periodate was added, mixed by shaking, 20 minutes were allowed to elapse

before addition of 1 ml of sodium arsenite solution to this mixture. The brown colour was made to disappear by shaking. Then 3 ml of the thiobarbituric reagent was added and the mixture was heated in boiling water bath for 15 minutes. After taking out and cooling, 4.3 ml of cyclohexanone was added and shaken for 15 seconds till all the colour was taken up by cyclohexanone. The mixture was centrifuged to get a clear pink layer of cyclohexanone. This supernatent was pippetted out and the colour intensity was measured in Baush and Lomb spectrophotometer at 550 mμ. The sialic acid content of the sample was found by use of the standard curve of sialic acid and the sialic acid content was expressed as μg/ml of gastric juice ± S.E.M.

PROTEIN Estimation of protein was carried out as described by Lowry et al. (1951).

REAGENTS

a. Alkaline reagent : Same as used in peptic activity.

b. Phenol reagent..

c. Bovine albumin used as a standard for estimation of protein.

PROCEDURE

To the 0.1 ml of solution of alcoholic precipitation of gastric juice in 0.1 N NaOH was added 0.9 ml of distilled water. Out of this constituted 1 ml solution, 0.4 ml was taken in another test tube. 4 ml of alkaline reagent was added to this test tube and kept for 10 min. Then 0.4 ml of phenol reagent was added and again 10 min were allowed for colour development. Readings were taken against the blank prepared with distilled water at 610 mμ in the Baush and Lomb spectrophotometer. The protein content was obtained by calculating with the use of standard curve prepared with bovine albumin and was expressed in terms of μg/ml of gastric juice ± S.E.M.

EFFECT OF TABH ON EPITHELIAL CELL TURNOVER

A sophisticated method of measuring cell production in gastric mucosa has been developed by Hart Hansen et al (1975). In this method gastric biopsies obtained by fiber optic endoscope are placed in a radium containing tritiated thymidine. This radio active marker can be demonstrated by autoradiographs and the rate of mitosis measured. An alternative method of estimating cell turnover in man is to perfuse the human stomach with normal saline and measure the rate of loss of DNA from the mucosa (Croft et al., 1966).

Effect of TABH on epithelial cell turnover was carried out only in dexamethasone and indomethacin induced gastric ulcers. After inducing the ulcers rats were treated with TABH 60 mg/kg for 10 days; control animals received vehicle. As it is difficult to perfuse the rat stomach, the following procedure was adopted for collecting stomach wash. Gastric juice was collected as described earlier. The stomach was opened and the mucosa was washed smoothly and gently with about 5 ml of normal saline. This saline wash alongwith gastric juice secreted in 4 hours of pylorus ligation was used for estimating DNA from the epithelial cells.

DNA ESTIMATION

Extraction from cell was carried out as described by Sekiguchi and Takagi (1960) and estimated by using the diphenylamine reaction of Burton (1956).

REAGENTS

a. 0.5 N Perchloric acid.

b. Diphenylamine reagent : Dissolved 1 g. of pure diphenylamine in 100 ml of glacial acetic acid and added 2.5 ml of con.sulphuric acid. Stored in glass bottle in refrigerator.

PROCEDURE

To 9.5 ml of gastric wash along with gastric juice, 0.5 ml of perchloric acid was added and kept for 30 minutes in ice, then centrifuged at 7000 r.p.m. and the residue was collected and mixed with 2 ml of 0.5 N perchloric acid, heated for 15 min at 90°C. Again the mixture was centrifuged after cooling and supernatent of 1 ml was taken and 2 ml of DPA reagent was added and again heated for 10 min at 100°C. After cooling the DNA content was read at 595 mμ on Spectronic 21 (Digital) spectrophotometer against the blank prepared from 0.5 N perchloric acid and calculated from the standard curve of DNA and expressed as total DNA μg ± S.E.M

HISTOPATHOLOGICAL STUDY

Severity and the extent of the lesions induced by dexamethasone, indomethacin and cysteamine were studied histopathologically. After inducing ulcers, rats were treated with either TABH 60 mg/kg or other drugs as per protocol. Sections of duodenum and stomach of treated rats were also studied histopathologically for healing and regeneration of the mucosa.

It is well known that there is a close relationship between the mucus in the mucosa and the surface epithelium. This can be demonstrated by histological stains of mucus with Periodic Acid Schiff Reagent (PAS)., most of the mucus in the gastric mucosa is in the surface of epithelium.

PROCEDURE

The tissue with lesion was selected immediately after removal from the body to avoid autolytic changes. Then dehydration was done by passing the tissue through ascending grades of alcohol (70 to 100%). The tissue was cleared by different clearing agents like xylol, chloroform for infiltration with paraffin wax and paraffin tissue blocks were prepared. From this block a thin section of 2-3 microns was cut in microtome.

The paraffin of the section was dissolved with xylol and treated with absolute alcohol and then with descending grades of alcohol. Then the section was washed in running tap water for 3 minutes and treated with haematoxyline for 10 to 20 min. After that the section was washed with tap water and with 1% acid alcohol for 1 min to remove excess haematoxyline and again put in water till the section became blue and treated with counter stain, 0.5 eosin solution for 1 to 2 min and washed with absolute alcohol, cleared with xylol and mounted. Sections were carefully observed under the photomicroscope (CARZEISS, Model. I.) with 4 and 10 X magnification.

CHRONIC TOXICITY STUDY

Rats were treated with TABH 120 mg/kg p.o. daily for 30 days. The control animals received vehicle. On the last day rats were sacrificed, blood samples were collected and analysed for haemoglobin, total RBC count, total WBC count and differential count, S.G.O.T., S.G.P.T. by routine methods used in clinical laboratory. The adrenal glands were removed and their wet weight was compared with control. Other organs like heart liver, kidney and spleen were also examined.

TRACE CHEMICAL ANALYSIS

TABH is a traditional preparation and is generally prepared as described earlier. The major chemical ingredients of TABH are copper, iron and ascorbic acid. The reported composition of TABH is $CuO \geqslant 44.45\% \leqslant 66.13\%$ $Fe_2O_3 < 6.03\%$ and sulphur $< 2.75\%$ (Raghunathan., 1976). Trace chemical analysis of the TABH (Gujarat

Ayurveda Vikas Mandal Pharmacy of Junagadh) used in the present study was carried out by well established spectrophotometic method of chemical analysis. The 100 mg of the TABH was digested with 25 ml mixture of concentrated HCl and nitric acid (10:1) and then centrifuged. The contents were diluted to 100 ml with double distilled water. An aliquot of this solution was used for the estimation of metals spectrophotometrically (Agrawal and Patel, 1980).

A VSV-2P CZ-Spectrophotometer with 10 mm Silica cell was used for measurement and a systronic digital pH meter was used for pH adjustment.

STATISTICAL ANALYSIS

Unpaired 't' test was applied to determine the level of significance. $p < 0.05$ was considered as statistically significant (Mahajan, 1984).

DRUGS USED

Absolute alcohol (Alembic Chemicals Ltd , Baroda), Acetyl acetone (S.D. Chemicals, Bombay), Bovine albumin (Sigma, U.S.A.), Carbenoxolone sodium (Bio-chem, Bombay), Cimetidine (Keptab Pharmaceutical, Baroda), Copper sulphate (Samir Tech Chem, Baroda), Cyclohexanone (Samir Tech chem, Baroda), Cysteamine HCl (Riedel - De haenay Scelze-Hannover, Germany), DNA (Sigma, U.S.A.), Dexamethasone (Cadila Lab , Ahmedabad), (D+) Fucose (S.D.Chem , Bombay), Diphenylamine (S.M. Chem, Baroda), (D+) Mannose (S.D.Chem, Bombay), D -glucosamine HCl (Loba Chemicals, Bombay), Galactose (Loba Chem , Bombay), Haemoglobin powder (Loba Chem , Bombay), Hydrochloric acid (S.M. Chemicals, Baroda), Indomethacin (Cipla, Bombay), L-Cystein HCl (Loba, Bombay), L-tyrosin monohydrochloride (Loba Chem , Bombay), N-acetylneuraminic acid (Koch-light, Lab. Germany), NaOH (S.M. Chem , Baroda), Orcinol (B.D.H., England), p-dimethyl amino benzaldehyde (Loba Chem., Bombay), Pentobarbitone sodium (Apetho & Research Chem Lab , Bombay), Perchloric acid (S.M. Chem , Baroda), Phenol reagent (Loba Chem , Bombay), Sodium potassium tartarate (Samir Tech Chem, Baroda), Sodium Carbonate anhydrus (S.M. Chem , Baroda), Sulphuric acid (S.M. Chem , Baroda), Sodium meta periodate (Loba Chem , Bombay), Sodium arsenite (E.Merk. Darmstadt), Sodium sulphate (S.M. Chem , Baroda), Tamrabhasma (Guj. Ayurveda Vikas Mandal Pharmacy, Junagadh.), Thiobarbituric acid (Loba Chem , Bombay), Topher's reagent (B.D.H. England) Trichloroacetic acid (Reidel-de hean, Germany).