MATERIALS AND METHODS
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4.1 EXPERIMENTAL DESIGN

Rats of either sex weighing 150-250 g were selected. They were fed with standard chow diet and were divided into groups of six each. In case of gastric ulceration models, animals were fasted for 36 h before the experiment. Coprophagy was prevented by fasting the animals in cages with grating as the floor. This was not done in duodenal ulceration technique as this method does not require fasted animals, and food and water were made available till the start of the experiment.

Antiulcer activity of calcium channel blockers was evaluated against experimentally induced gastric and duodenal ulcers. The following four models were used for various experimental investigations:

A. Cysteamine induced duodenal ulceration (Szabo, 1978).
B. Aspirin induced gastric lesion method (Hemmati et al., 1973).
C. Pylorus ligated Shay rat model (Shay et al., 1945).
D. Aspirin plus pylorus ligation model (Goel et al., 1985).

4.2 DUODENAL ULCERS

1. Cysteamine (Mercaptamine) Induced Duodenal Ulcers

Experimental duodenal ulcers in rats induced by cysteamine HCl as described by the method of Szabo (1978). Rats were administered p.o. a total dose of 800 mg/kg cysteamine HCl. To avoid acute toxicity, cysteamine HCl was administered in
two doses of 400 mg/kg in 10% aqueous solution at an interval of 4 h. Drugs were administered 30 min before each dose of cysteamine HCl. Animals were sacrificed 24 h after the 1st dose of cysteamine administration. Ulcers were observed in the wall of duodenum close to the pylorus region. The duodenal ulcers were scored for their intensity using a scale of 0 to 3 as follow:

0 = No ulcer
1 = Superficial mucosal erosion
2 = Deep ulcer or transmural necrosis
3 = Perforated or penetrated ulcer

Ulcer Index

Sum of the arithmetic mean of the intensity in a group and the ratio of positive/total multiplied by 2, eg. 2.1 + (9/10 x 2).

4.3 GASTRIC ULCERS

1. Aspirin Induced Gastric Lesions

Aspirin was suspended in 1% carboxymethylcellulose in water and administered orally (p.o.) in the dose of 500 mg/kg in 36 h fasted rats. Four hours later, the animals were sacrificed, stomachs were removed and opened along with its greater curvature for determination of the ulcer index (u.i.). Ulcer index was measured by the method of Ganguli and Bhatnagar (1973) as follow.
Each lesion of stomach was measured along its greatest length. In case of petechie, five of these were considered to be equivalent to 1 mm of ulcer area. The total area of stomach mucosa and that of ulcerated mucosa were measured for determining the ulcer index.

\[
X = \frac{\text{Total area of stomach mucosa}}{\text{Total area of ulcerated mucosa}}
\]

\[
\text{U.I.} = \frac{10}{x}
\]

Area of circular lesion = \(\pi r^2\)

Area of Linear lesion = \(L \times B\)

Area of stomach mucosa = \(\pi r^2/2\)

2. **Pylorus Ligation Technique**

This is the oldest animal model of gastric ulcers developed by Shay et al. (1945). 36 hours fasted rats were anaesthetized with ether and abdomen was opened by a small midline incision below the xiphoid process. Pylorus portion of the stomach was lifted and ligated avoiding traction to the pylorus or damage to its blood supply. The stomach was replaced carefully and the abdominal wall closed by interrupted sutures. The drugs were administered immediately after the pylorus ligation. Six hours later animals were sacrificed, stomachs removed and opened along with the greater curvature. The contents were removed in test tubes and subjected to analysis for free and total acidity. The inner surface of stomach was examined for ulceration. Ulcer index was determined as described above.
3. Aspirin Plus Pylorus Ligation Model

Aspirin suspension in 1% carboxymethylcellulose in water was administered orally in the dose of 200 mg/kg in non-fasted rats once daily for 3 days. Similarly drugs were also administered 30 min. before the aspirin treatment once daily. From 3rd day, food was withdrawn from animals but water was given ad libitum. On fourth day, pylorus was ligated as per the method of Shay et al. (1945). Four hours after the ligation, animals were sacrificed. Ulcer index was determined as described earlier.

4.4 DRUGS AND DOSES

Verapamil, diltiazem and nifedipine were selected for studying their effect against gastric and duodenal ulceration. Verapamil and nifedipine were administered in rats in the dose range of 4-40 mg/kg. Diltiazem was administered in slightly higher dose range i.e. 10-60 mg/kg.

Verapamil and diltiazem solutions were prepared in distilled water. Nifedipine was administered either as suspension in 1% carboxymethylcellulose (CMC) or as its solution in dimethylsulfoxide (DMSO) solvent.

4.5 PARAMETERS UNDER INVESTIGATION

(a) Physical Parameters
- Ulcer index and total lesion area.
- Volume of gastric secretion (Hawk, 1965).
(b) Biochemical Parameters

- Free acidity (Hawk, 1963).
- Total acidity (Hawk, 1965).
- Pepsin activity (Debnath et al., 1974).
- Mucus content (Corne et al., 1974).
- Total carbohydrates (TC).
  - Total hexose (Winzler, 1958).
  - Hexosamine (Dische and Borenfreund, 1950)
  - Fucose (Dische and Schettles, 1948).
  - Sialic acid (Warren, 1959).
- Protein content (PR) (Lowry et al., 1951)

4.5.1 FREE ACIDITY AND TOTAL ACIDITY

Gastric contents were assayed for free and total acidity by titration against 0.01 N NaOH to pH 3.5 using Topfer's reagent and to pH 8.0 using phenolphthalein as indicators. Topfer's reagent will change its red color to yellow color (indication of free acidity) and further yellow color changes to pink color in presence of phenolphthalein indicator (indication of total acidity). The amount of HCl was calculated and expressed as mEq/L. The results may also be expressed in terms of ml of 0.01 N NaOH (which is equal to the same amount of 0.01 N HCl) required for 100 ml of gastric juice.
4.5.2 PEPSIN ACTIVITY

The determination of pepsin 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 Na₂CO₃ 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.Tyrosine 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 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 Tyrosine 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 nm 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 μg/ml of Tyrosine ± S.E.M. liberated per 4 hours of gastric juice.
4.5.3 MUCUS CONTENT

A modified procedure of Corne et al. (1974) was used. The glandular segments, from stomachs which had been opened along their greater curvatures were removed and weighed. Each segment was transferred immediately to 10 ml of 0.1% w/v Alcian Blue solution, the later was made by freshly dissolving Alcian Blue 8GX (Sigma) in 0.16 mol/L sucrose solution buffered with 0.05 mol/L sodium acetate, and finally adjusting the pH to 5.8 with 1 mol/L HCl. Tissues were stained for 2 h in Alcian Blue solution, excess dye was removed by two successive rinses, soaking the tissue each time in 10 ml of 0.25 mol/L sucrose, first for 15 and then for 45 min. Dye complexed with the gastric wall mucus was extracted from the glandular tissue with 10 ml 0.5 mol/L magnesium chloride which was intermittently shaken for 1 min at 30 min intervals for 2 h, 4 ml of blue extract solution was then shaken vigorously with an equal volume of diethylether. The resulting emulsion was centrifuged at 3,600 rpm for 10 min and the aqueous layer used to determine the concentration of Alcian Blue. Colour absorbance was recorded by a spectrophotometer at an optimum wave length of 598 nm. The quantity of Alcian Blue extracted per gram of wet glandular tissue was then calculated, from standard curves which obeyed the Beer-Lambert law at the dye concentration used.
4.5.4 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 mixture was centrifuged at 3000 rpm 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 mixture 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 rpm
for 15 min to obtain precipitate. The precipitate was dissolved in 0.5 ml of 0.1 N H₂SO₄. This was used for estimating sialic acid.

4.5.5 TOTAL HEXOSES

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

Reagents:

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

b. H₂SO₄ : H₂O 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 nm 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.
4.5.6 HEXOSAMINE

Estimation of hexosamine was carried out as described by Dische and Borenfreund (1950).

Reagents:

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 benzaldehyde 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 1.5 ml 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 30 min to get completed. The colour intensity was measured on the Baush and Lomb spectrophotometer set at 530 nm 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.
4.5.7 FUCOSE

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

Reagents:

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 nm 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 nm and subtracting 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.

The true optical density = \frac{(O.D. 395 - O.D. 430) - (O.D.395 - 0.D.430)}{(O.D.395 - 0.D. 430)}

4.5.8 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 orthophosphoric 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 H₂SO₄ 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 pippeted out and the colour intensity was measured in Baush and Lomb spectrophotometer at 550 nm. 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 $\mu$g/ml of gastric juice $\pm$ S.E.M.
4.5.9 PROTEIN

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

Reagent:

a. Alkaline reagent: Same as used in pepsin 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 nm 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.

4.6 EXPRESSION OF RESULTS AND STATISTICS

The significance of the differences between mean values for various treatments was tested using the Student's 't' test as described by Ghosh (1984). However, in case of non-parametric data, significance between these values was tested using the Wilcoxon's rank sum test (Ghosh, 1984).