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Invivo & Invitro evaluation techniques for Anti-inflammatory and Anti-Ulcer drugs.

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Abstract: Nonsteroidal anti-inflammatory drugs (NSAIDs) produce their therapeutic activities through inhibition of cyclooxygenase (COX), the enzyme that makes prostaglandins (PGs). They share, to a greater or lesser degree, the same side effects, including gastric and renal toxicity. Recent research has shown that there are at least two COX isoenzymes. Effective therapies for peptic ulcers use alternatives that control acidic hypersecretion and its direct effects on the gastric mucosa. The two main classes of drugs used to treat acid-related disorders include proton pump inhibitors (PPI) that inhibit the hydrogen pump in the parietal cell directly, independently of any membrane receptor stimulation, and histamine type 2 receptor antagonists (H2RAs). To evaluate the anti-inflammatory & anti-ulcer activity of drugs there are many in-vivo & in-vitro methods that can be use.

Introduction: Acute inflammatory conditions can be produced in laboratory animals by using various phlogistic agents; however, duration of these inflammatory conditions is quite transitory and these inflammatory conditions can easily be controlled by using currently available antiphlogistic agents. Our main difficulty is dealing with chronic inflammatory conditions which include a large number of diseases and syndromes, caused by one or more unknown factors. Most of the available drugs (steroidal and non-steroidal) to treat these various chronic inflammatory conditions have their limitations due to toxic effects. Hence one should

seek a truely non-toxic, yet potent, broad spectrum anti-inflammatory-antiarthritic drug. Ideally speaking anti-inflammatory drugs should have:

(1) effect on prime causative factors,

(2) inhibitory effect or blocking effect on initial reaction set in a biological model by the prime cause and thereby inhibit the established inflammation,

(3) effect on end results of established inflammation which probably modifies non-specifically the underlying symptoms of inflammation or enhances the repairing process.

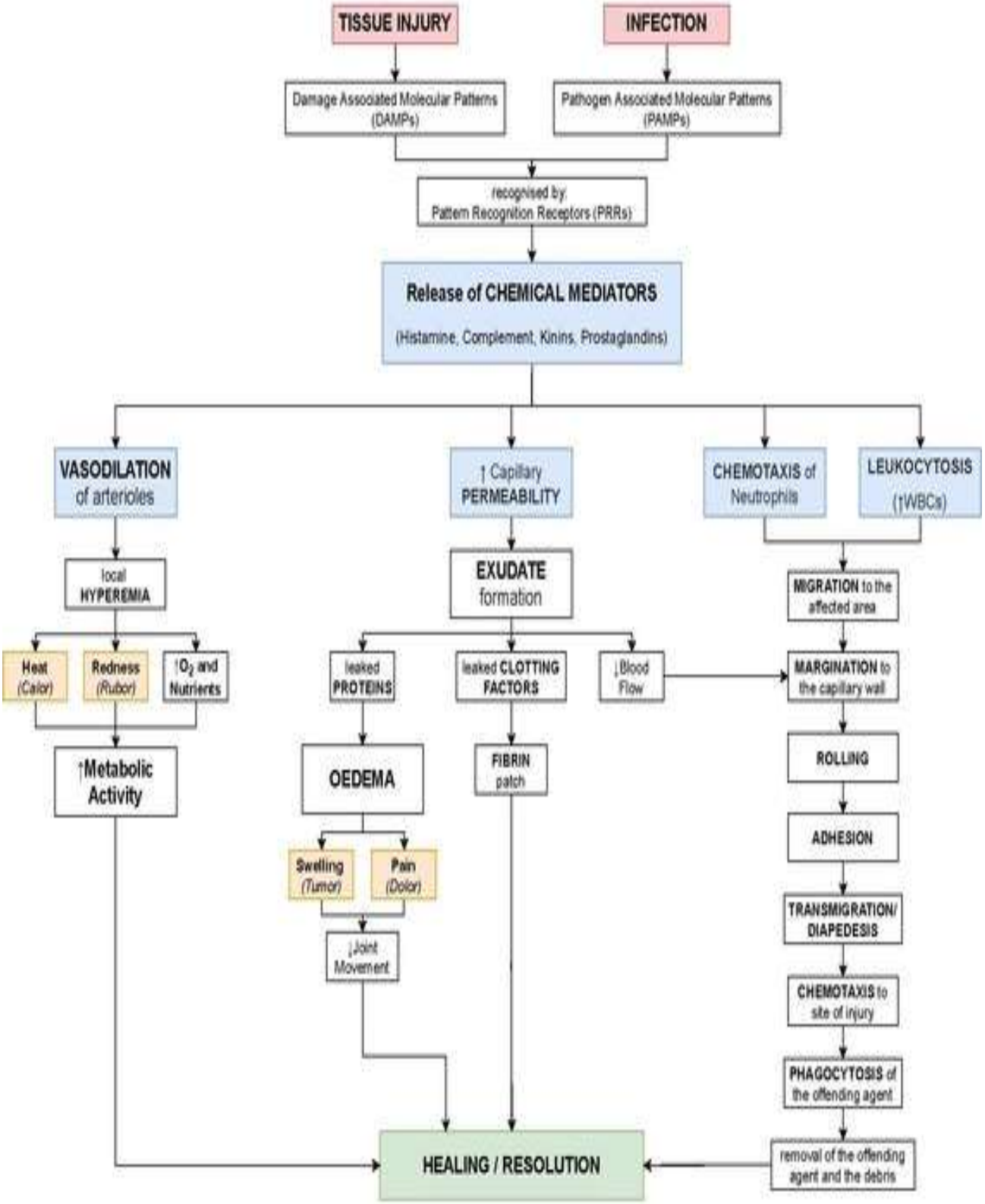

Fig.1 Process of inflammation

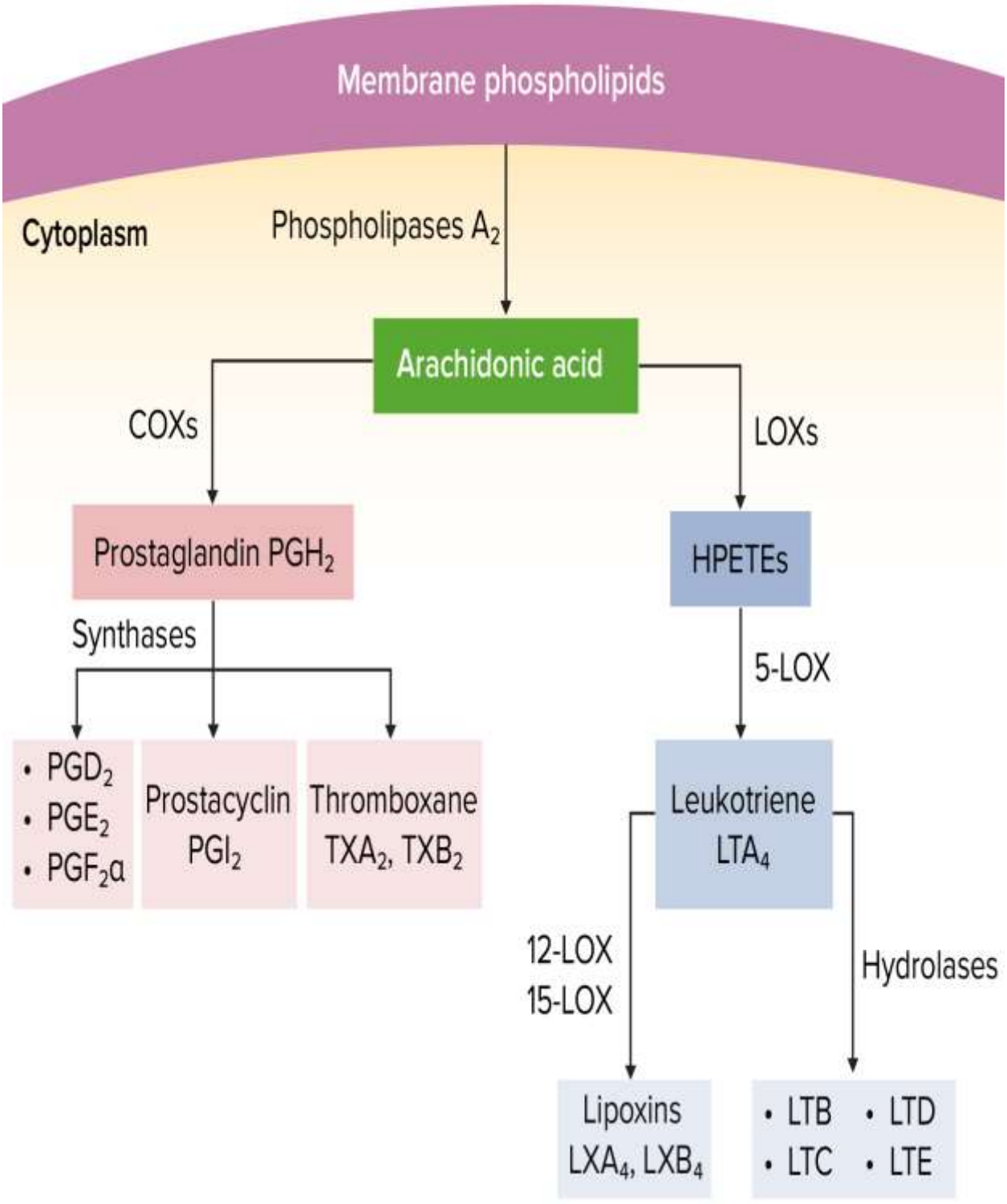

Fig.2 Mechanism of action of Anti-inflammaory drugs

PPI is among the most prescribed drugs in the world; however, it may lead to the development of parietal cell hyperplasia of the gastric glands [1]. Long-term use of H2RAs is associated with

the development of undesirable effects such as gynecomastia and galactorrhea as well as alteration of the bacterial flora of the gastrointestinal tract [2].

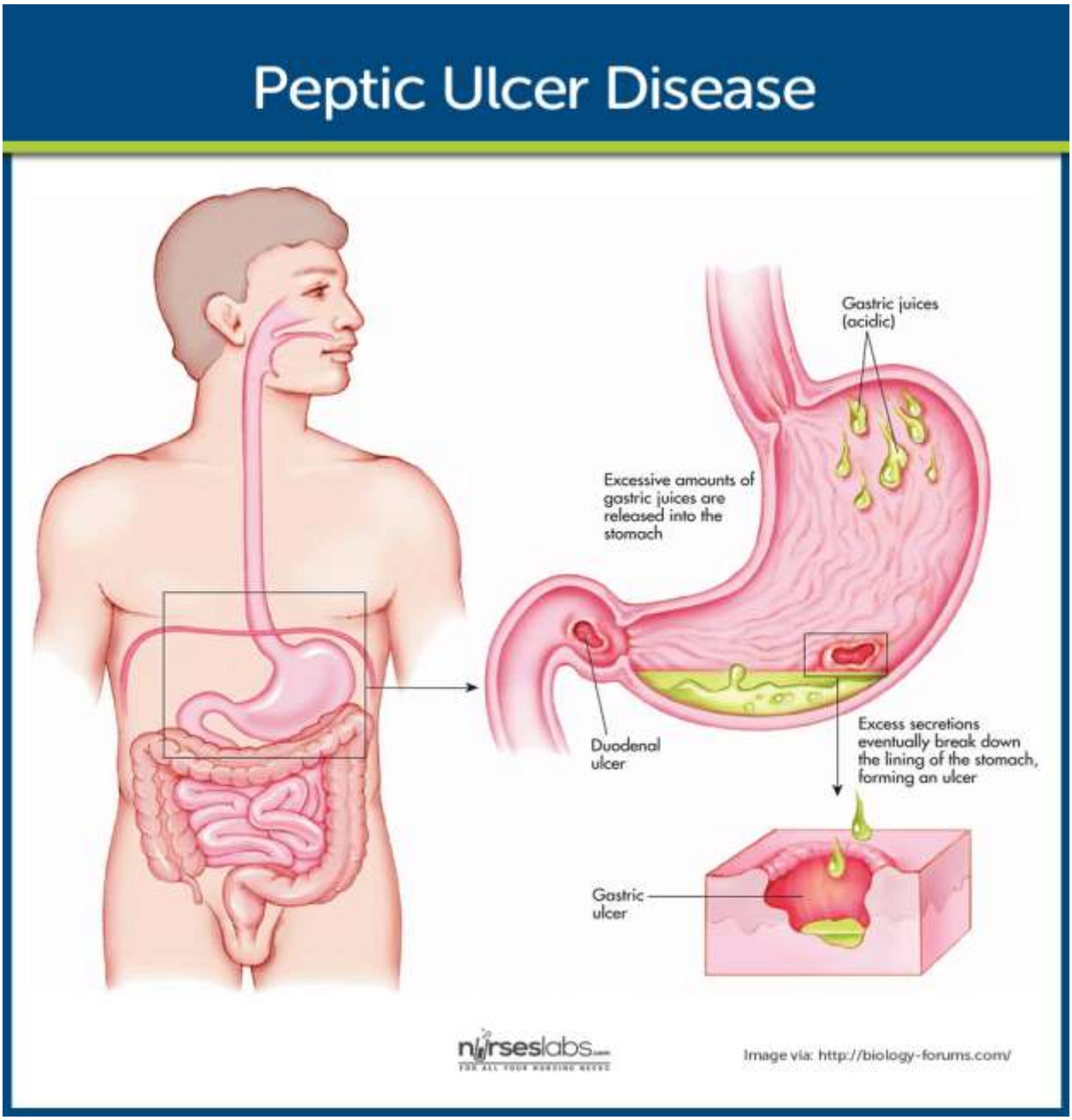

Fig.3 Peptic Ulcer disease

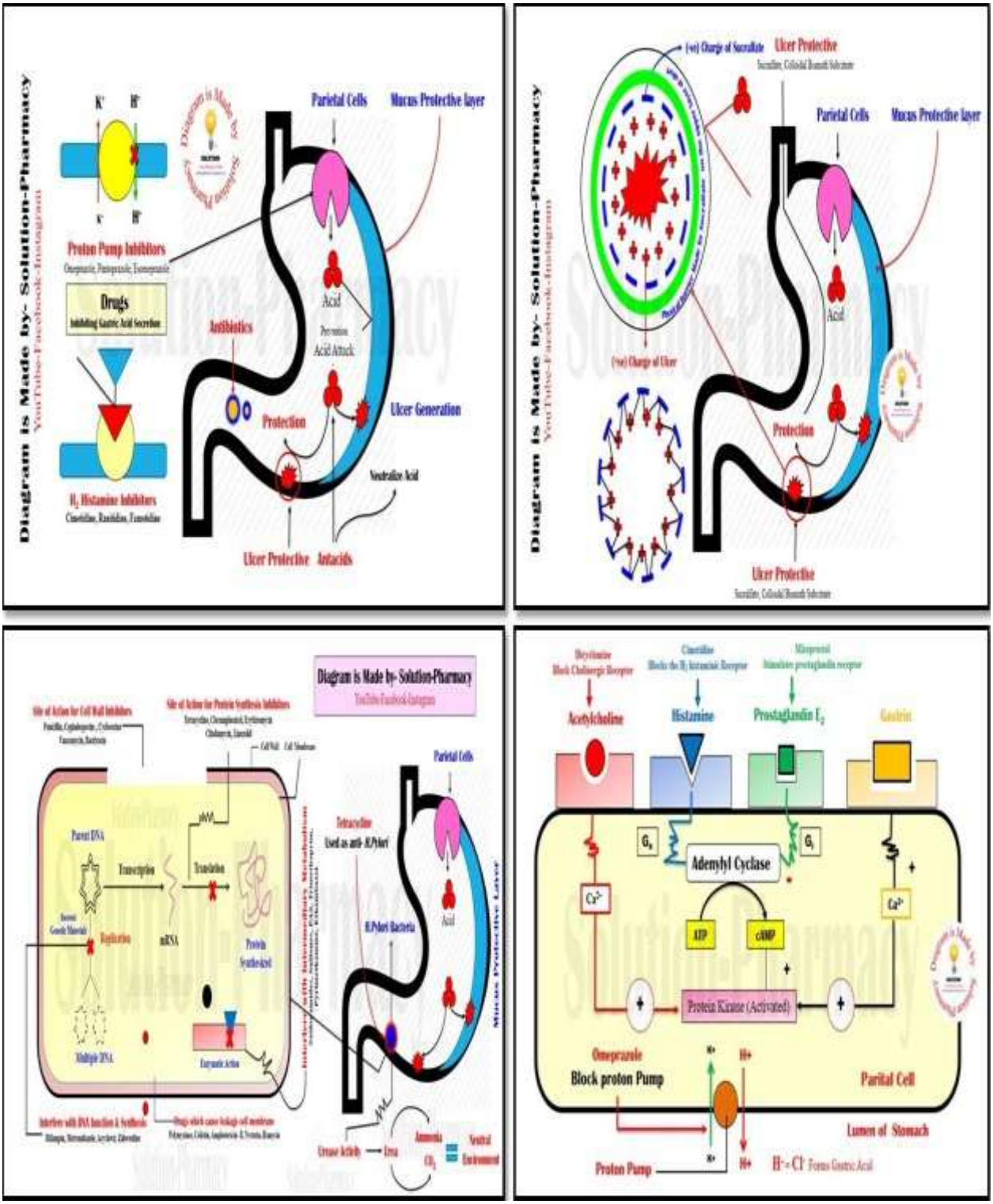

Fig.4 Mechanism of action of Anti-ulcer drugs

In this review we are going to discuss different in-vivo & in-vitro evaluation methods for Anti-Inflammatory & Anti-Ulcer drugs.

Evaluation methods for Anti-Inflammatory drugs

***Invivo* Methods**.

1) Adhesion Assay: Adhesion of leukocytes is an important cellular stage during the inflammation process [3]. Adhesion assay involves the estimation of vascular proteins vascular cell adhesion molecule 1, intercellular adhesion molecule 1, and E-selectin in primary cells derived from human umbilical vein endothelial cells and human microvascular endothelial cell line [4]. Human dermal microvascular endothelial cells are the most suitable to predict in vivo situations in the best manner [5]. In the adhesion assay, tumor necrosis factor (TNF)-alpha stimulated cell lines are incubated with test drug [6]. The procedure involves labeling of neutrophil with appropriate fluorescent materials and allowing adhering. Finally, the measurement of fluorescence iscarried out at 485 nm in an ELISA reader [7].

2) COX-1 assay: In this assay, the sample solution was mixed with L-adrenaline-Dhydrogen tartrate and hematin. Then, the incubation of the mixture is carried out with COX-1. Then, the arachidonic acid is added and incubated for 20 min at 37°C. Addition of formic acid to the mixture stops the incubation. Using enzyme immunoassay, measure the concentration of prostaglandin 2 [8].

3) COX-2 assay: The basis of this assay is that N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD) gets oxidized when the reduction of prostaglandin-G2 to prostaglandin-H2 occurs [9]. The oxidation velocity of TMPD is measured spectrophotometrically. The sample is introduced to the mixture of sodium phosphate buffer, gelatin, hematin, and purified COX-2. This mixture is pre-incubated with arachidonic acid before the addition of TMPD. The percentage inhibition is determined by measuring the absorbance at 610 nm [10].

In-vitro methods

1) Protein denaturation has been employee as an in vitro screening method for anti-phlogistic agents by Mizushima and his co-workers. Grant et *al.* (1970) also confirmed their work and reported that anti-inflammatory drug., inhibit protein denaturation. Drug binding to plasma

albumin may inhibit thermal denaturation of albumin which perhaps block $\acute{O}$ -NH_2 groups in case of histidine decarboxylase.[11]

2) Red cell aggregation induced by using various agents like gelatin, carrageenin, nucleotides etc, have proved to be effective screening non-steroidal anti-inflammatory drugs, (Gorog et al., (1970). Actomycin like contractile protein having ATPase activity is situated on the outer surface of erythrocytes. It probably plays an important role in maintaining the form of erythrocyte and distribution of the surface charge on the outer membrane. The non-steroidal anti-infiammatory drugs bind to this contractile protein and inhibit its ATPase activity and its contractile property (Gorog et al., 1970). They have further stated the existence of a relation between the effect in the connective tissues and that exerted on the erythrocyte membrane (Gorog *et al.*, 1970). Famaey and Whitehouse (1975) have suggested that non-steroidal anti-inflammatory drugs may affect various membranes differently.[12]

3) Lysosome membrane stabilization property of drugs can also be determined by using erythrocyte lysis by heat, hypotonic solution or by some other means.[13]

Evaluation methods for Anti-Ulcer drugs

Invivo methods

1) **Reserpine-Induced Peptic Ulcer**

Scientists have also used reserpine to induce ulcers. Reserpine-induced gastric ulceration has been attributed to the degranulation of gastric mast cells consequent to liberation of histamine, believed to be mediated by the cholinergic system [14]. Rats fasted for 36 hours are administered reserpine dissolved in 10% Tween 80 (5–8 mg/kg, i.p.) . Although the model is acid dependent, hypermotility seems to be more important than hypersecretion for the induction of gastric mucosal lesion [15]. Normally, drugs or plant extracts to be evaluated are

administered to the test animals, at least, 30 minutes before the administration of the reserpine. The test animals are then sacrificed 24 hours later.

2) Serotonin-Induced Gastric Ulcer

Serotonin, which has also been used to induce ulcers, is known to cause vasoconstriction, thereby reducing gastric mucosal blood flow (GMBF) resulting in acute mucosal injury [16]. In this model, rats are fasted for 24–36 hours. The fasted animals are denied of water 2 hours just before commencement of the experiments. Glandular lesions are established following the administration of a single dose of serotonin creatinine sulfate (0.5 mL of 50 mg/kg subcutaneous injection). Serotonin is administered by intragastric intubation with the aid of an orogastric cannula. The animals are sacrificed by cervical dislocation 6 hours later.

3) Indomethacin, Plus Histamine-Induced Duodenal Ulcer

Another method for inducing duodenal ulcers described by Takeuchi et al. involves administering indomethacin and histamine to rats. In this model, indomethacin (5 mg/kg) is first given subcutaneously to rats fasted for 24 hours followed 30 minutes later by histamine dihydrochloride (40 mg/kg also subcutaneously) three times at 2.5-hour intervals. After 3 hours, duodena are excised carefully, cut opened along the antimesentric side, and the ulcer area(s) is measured. This combined treatment has been reported to induce one or two round lesions in the proximal duodenum at an incidence of 100%, and a few lesions in the corpus and antrum of the stomach as well. The development of duodenal lesions induced by indomethacin and histamine in rats is due to both an increase in gastric acid secretion and an impairment of acid-induced duodenal HCO^{3-} secretion. This model for duodenal ulcers is useful for studying the pathogenesis of duodenal ulcers and for screening antiduodenal ulcer drugs or agents [17].

4) Ferrous Iron-Plus Ascorbic Acid-Induced Gastric Ulcer Model

This type of gastric ulcer model is induced by the local injection of ferrous iron with ascorbic acid (Fe/AS A) solution into the gastric wall. The ulcers produced resemble human gastric

ulcers that penetrate the muscularis mucosa. Lipid peroxidation mediated by oxygen radicals plays a crucial role in the pathogenesis of the gastric ulceration induced by the Fe/AS A solution [18].

In-vitro methods

1) Acid Neutralizing Capacity: The aqueous extract of acid-neutralizing capacity value are 100mg, 500mg, 1000mg, 1500mg. The aluminium hydroxide and magnesium hydroxide (500mg) have compared for the standard. The total volume was 70ml with the addition of 5ml of a quantity of the mixture and remaining with water to make up the total volume; mix this for one minute. To the standard and test preparation, the 30ml of 1.0 N HCl was added and stirred for 15 minutes after that phenolphthalein was added and mixed. With 0.5N Sodium hydroxide, the excess HCl was immediately titrated until the pink colour is attained 15 . The moles of acid neutralized is calculated by, Moles of acid neutralized = (vol. of HCl × Normality of HCl) - (vol. Of NaOH × Normality of NaOH) Acid neutralizing capacity (ANC) per gram of antacid = moles of HCl neutralized divided by Grams of Antacid/Extract.[19]

2) H+/K+ - ATPase Inhibition Activity: Preparation of H+/K+ - ATPase Enzyme: To prepare H+/K+ - ATPase enzyme sample the fresh goat stomach has purchased from the local slaughterhouse, the gastric mucosa of the fundus was cut-off and opened, the inner layer of the stomach has scrapped out for the parietal cell. The parietal cell obtained from the stomach has homogenized in 16mM Tris buffer with PH of 7.4, which has 10% Triton X-100 and centrifuged at 6000 rpm for 10mins after centrifuged the supernatant solution has used for the H+/K+- ATPase inhibition Protein content are used to find out according to Bradford's method were BSA are used for standard. Assessment of H+/K+ ATPase inhibition: Per-incubated for 60 min at 37 ºC for the reaction mixture of the sample containing 0.1ml of enzyme extract (300µg) and plant extract with different concentration (20µg, 40µg, 60µg, 80µg, 100µg). The reaction was initiated by adding substrate 2 mM ATP (200µL), in addition to this 2mM MgCl2 (200µL) and 10mM KCl (200µL) has added. After 30 min of incubation at 37 ºC the reaction was stopped by 4.5% ammonium molybdate, and 60% perchloric acid was added and centrifuged at 2000rpm for 10 min, and in spectrophotometrically inorganic phosphate was released and

measured at 660nm by following the Fiske-Subbarow method. Briefly, at 10 min at room temperature, 1ml of supernatant 4ml of Millipore water, 1ml of 2.5% of ammonium molybdate, 0.4ml of ANSA was added. At 660nm inorganic phosphate, absorbance has been measured at various doses of the extract; the enzyme activity has been calculated as micromoles of Pi released per hour. Results were compared with the known anti-ulcer PPA inhibitor Omeprazole and expressed as Mean ± SEM 16 % enzyme inhibition has calculated using the formula: Percentage of inhibition = [Activity (control) - Activity (test)/Activity (control)] × 100.[20]

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