Summary
**Summary**

Peptic ulcer disease (PUD) is one of the most prevalent gastrointestinal disorders that affect millions of people per year. It encompasses both gastric and duodenal ulcer. Clinically, it is defined as disruption of gastroduodenal mucosal integrity of stomach or duodenum leading to a local defect of excavation due to active inflammation. The pathophysiology of peptic ulcer revolves around the imbalance between aggressive and protective factors in the stomach, which disrupts the gastric mucosal integrity. The former include acid, pepsin, *H. pylori*, nonsteroidal anti-inflammatory drugs (NSAIDs), smoking, alcohol, ischemia and corticosteroid use whereas later include mucus, bicarbonate, blood flow, prostaglandins and growth factors. Excess secretion aggressive factors, acid and pepsin and diminished ability of the gastroduodenal mucosal barrier and other defensive factors confers the contributory role in ulcerogenesis.

Approximately 10% of adults suffer annually from peptic ulcer disease and about 5, 00,000 new cases along with 4, 00,000 cases of recurrence are reported each year. An estimated 15,000 deaths per year occur as a consequence of complicated PUD. High global incidence rate, simpler to severe pathophysiology and uniform occurrence across all ages, races and ethnicity, makes PUD, a favorite among clinicians, researchers and pharma companies. Antacids and anti-ulcer drugs alone share 6.2 billion rupees and occupy 4.3% market share in Indian Pharma pie.

The current treatment strategies are mainly focused on two aspects, prevention of gastric mucosal lesions and the mechanism of gastric ulcer healing. However, drug-designing strategies often flawed the two processes as one while formulating an agent that can act against the ulcerogenic factors. Therefore, the PUD is mainly investigated for the role of aggressive factors such as acid, pepsin, *Helicobacter pylori* with little, if any, attention to the healing process. As a result, the treatment of gastro-duodenal ulcers has so far been restricted to the neutralization of intraluminal acid, reduction of its secretion and/or elimination of *H. pylori*. In all these interactions the ulcer is then left to heal by itself in a less hostile environment.

Ulcer healing is a highly complicated process as it comes into action, once the gastric lesion has already occurred. Therefore a two-tier strategy is required, first to down regulate the acid synthesis and other aggressive factors, in order to overcome further
damage and prepare the environment conducive for healing while the second one involves the up regulation of the cytoprotective/defensive elements to infuse cellular repair and mucosal reconstruction. Ulcer healing is an active and complicated array of different mechanisms that involves filling of mucosal defect with proliferating and migrating epithelial cells and connective component so as to reconstruct the mucosal architecture. Ulcer healing is a cumulative effect of several physiological and constitutive processes that occurs in tandem and requires a high level of co-ordination and regulation.

A high degree of coordination and regulation during complex sequence of ulcer healing is carried out by different factors. Among them prostaglandins (PGs) and growth factors have received much attention in recent years. The gastric mucosa contains abundant level of PGs (mainly PGE2 and PGI2) that exhibit a dual character in their functioning because along with being a strong ulcer-healing agent, PGs are also well known mediators in different features of inflammation. This duality of PGs was clarified when two different isoforms of Cyclooxygenase enzyme (COX) were identified. COX is the key enzyme responsible for the synthesis of PGs via arachidonic acid; exist in two isoforms- COX-1 and COX-2. According to the classical COX hypothesis, COX-1 is constitutively expressed and exert different physiological or “housekeeping” effects; while COX-2 is expressed as an inducible enzyme in most cells and is essentially involved in inflammation. Recent advances reveal that the classical COX hypothesis is oversimplistic and COX-2 plays more complex and wider biological role than mere involvement in inflammation and pain. Most important among them are healing process in various gastrointestinal pathologies. Different experiments in this regard have shown a shift in our understanding of ulcer healing mechanisms and COX-2 enzyme and PG synthesis seems to be an important target for the treatment modalities of gastric ulcers.

Based on these observations, present study was drafted to explore the role of COX-2 enzyme in normal and drug-mediated ulcer healing mechanism. An attempt was made to systematically compare the ulcer healing effect of four drugs each of which functions through a different mechanism: omeprazole is a proton pump inhibitor, misoprostol a PG analog whereas sucralfate acts like a cytoprotective agent and ranitidine as a H2 receptor antagonist. The comparisons have been made in light of a broader hypothesis, which assumes that the most efficient and powerful ulcer healing drug is the
one that apart from its known acting mechanism, elevates the expression level of COX-2 enzyme most efficiently, which eventually leads to better and faster repair process. Since the study focused on the expression level of COX-2 enzyme, an additional drug, COX-2 selective NSAIDs- celecoxib was also studied as a negative control. Along with the expression profile of COX-2 at mRNA and protein level, 16 different parameters covering all major contributing factors (offensive and defensive) were also analyzed in different group of animals to offer a more explainable conclusion. The study has been structured into two parts. Initially, a pilot study has been carried out for all the five drugs Omeprazole (OMZ), Ranitidine (RANI), Sucralfate (SUC), Misoprostol (MISO) and Celecoxib (CELE) to deduce the most effective dose and most effective time period. In the second phase different biochemical estimations, expression analysis and other parameters effecting ulcer healing were examined for each category of drug taking the most effective dose and time period respectively.

Total seven experimental groups have been used in all the experiments and 8 rats were allocated for each of the groups. Out of the seven groups, five belongs to that of different drugs while remaining two groups were of normal untreated rats and vehicle treated control rats respectively. Acetic acid induced gastric ulcer model was used to induce chronic gastric ulcers experimentally in Sprague-dawly rats. This model provided the luxury of production of well-characterized ulcers and a benign spontaneous ulcer healing. Ulcer induction was followed by the drug treatment at different doses for different time periods. On last day of drug treatment, gastric juice was collected and gastric mucosa of ulcerated tissue was scrapped. Three logarithmic graded doses for each of the drug and three different time duration (5, 10 and 14 day) of drug treatment were selected in the pilot study. The selection was based on the available literature on study of these drugs in rat models. These studies have suggested that each of the drugs has an optimal dose and treatment time duration, at which it exerts its maximum efficacy. Ulcer area was measured under stereomicroscope with the help of Biovis image analysis software and healing effect of each of the drug was evaluated from evaluation of ulcer area. The extent of ulcer healing was also complemented with the complete histopathological examination of ulcerated tissues.
The pilot study has provided the time kinetics of both normal as well as drug mediated ulcer healing and revealed that chronic gastric ulcers healed in a progressive manner with maximum healing observed on 14 day of treatment. Results of normal and drug mediated ulcer healing in the pilot study compliments significantly to the ulcer healing dynamics, where the early lag phase lasts for four days (0-3rd day), phase of rapid healing works from 4th – 12th day while 13th – 20th day depicts late lag phase or tissue remodeling phase. As each of these phases passes, a gradual decrease in the ulcerated crater was visible in all the groups except that of CELE treated rats. Regeneration of surface epithelial cells and restoration of gastric mucosa was clearly observed on day 14. The effective doses for different drugs omeprazole, ranitidine, sucralfate, celecoxib were found be 10, 30, 500 and 10 mg/kg respectively, whereas the effective dose for misoprostol was 100 μg/kg. When different drugs were compared at different doses on different days, it was clearly established that 10mg/kg dose of OMZ, if given once daily, is most flourishing in healing of preexisting ulcers as it exhibited 84.09% higher healing than that found in control. MISO (100μg/kg) was found to be the second most successful drug with 76.36% healing observed on 14th day followed by 500mg/kg of SUC and 30mg/kg RANI (60.05% and 43.15% of ulcer healing respectively).

Based on the results of the pilot study, all further analysis was carried out for the most effective dose in each category of drugs. Both gastric mucosa and gastric scraping of the ulcerated tissue were used for estimation of various biochemical parameters that either constitutes an offensive or a defensive factor. Expression analysis of COX-2 was carried out from the gastric scraping at mRNA level by RT-PCR and at protein level by western blotting. Effect of the drugs on ulcer damaging factors like free and total acidity and pepsin estimation was carried out from gastric juice along with estimation of proton pump inhibition as a possible reason of decrease in acid synthesis. Other studied factors included protective anti-oxidants enzymes, PGE₂ levels, expression level of different growth factors, estimation of mucus content in term of carbohydrate: protein ratio, total DNA content and myeloperoxidase (MPO) activity as a marker of neutrophils infiltration. All the factors were studied on 14th day of the treatment except the expression profile of
COX-2 mRNA and protein and estimation of PGE₂ levels that was carried out on 5th, 10th and 14th day to have a direct comparison with ulcer healing kinetics.

The study has extracted several imperative findings like various participating factors of the ulcerogenesis and mucosal regeneration contributes differentially to the process of ulcer healing and hence can be classified as major or minor factors, cytoprotective or broadly the defensive system provides an inescapable function in the ulcer healing and repairing machinery, where an enzyme COX-2 hold the vital key and finally, the differential effect of drugs on different factors leads to their differential efficacy in ulcer healing, where a drug that affects both the offensive and the defensive factors is most successful.

Among the offensive factors, acid and pepsin synthesis were found to be an important contributing factor. OMZ (43.12% and 29.92% respectively) and RANI (33.56% and 16.22% respectively) have produced maximum decrease in both of these factors; MISO has also produced significant reduction (32.92% and 19.63% respectively), which accounts for its strong anti-secretory activity, while SUC has marginal effect (16.35% and 6.69% respectively). The negative control CELE has not shown any significant effect despite of the fact that it has clearly delayed the process of ulcer healing. It was also figured out that blockage of H⁺K⁺ATPase is major mechanism behind inactivation of acid synthesis as all the three groups that have shown strong anti-secretory properties (68.75% decrease in OMZ, 43.75% RANI and 29.2% MISO) have also exhibited a significant reduction in both the acid level of stomach as well as proton pump activity. However, OMZ was more effective being a proton pump inhibitor while the other two instead of directly affecting the proton pump, mainly blocks one of the participating receptors. The results indicates that that attenuation of acid synthesis is albeit an important feature, but cannot alone trigger the ulcer healing process as was seen in case of RANI treated rats.

Among the defensive factors, the study has conveyed various important aspects of ulcer healing; most crucial among them is the role of COX-2 and PGs in the process of mucosal regeneration and repair. First of all, it was clearly established that an elevation in PGE₂ level, whose synthesis is catalyzed by COX-2 enzyme is pivotal in ulcer healing process, because its estimation have shown an almost two-fold increase in PGE₂ levels on
Summary

10\textsuperscript{th} and 14\textsuperscript{th} day in the ulcerated region as compared to the intact mucosa in all the treated groups. Secondly, it was clearly evident that COX-2/β-actin mRNA and protein ratio and PGE\textsubscript{2} levels showed a parallel increase in control and different treated groups suggesting that up-regulation of COX-2 expression was the main reason behind the elevation of PGE\textsubscript{2} production at ulcerated margins in all the groups as no expression of COX-2 enzyme was found in the intact mucosa. Finally, an analogous and completely corresponding rapport was figured out between COX-2 expression, PGE\textsubscript{2} levels and ulcer healing kinetics.

A sequential increase of the enzyme and its product from 5\textsuperscript{th} to 10\textsuperscript{th} day and then a slight decrease on 14\textsuperscript{th} day indicates parallel run with the curve of ulcer healing dynamics. Both of these factors gets stimulated with the mucosal damage, but showed a low expression on 5\textsuperscript{th} day, suggesting the diminution of the offensive factors in early phase. Both the COX-2 expression as well as PGE2 level reached its crest on 10\textsuperscript{th} day i.e during the phase of rapid healing suggesting strong role in epithelial cell regeneration and proliferation in the ulcer margin. Finally, COX-2 and PGE\textsubscript{2} depicted a trivial down regulation on 14\textsuperscript{th} day in all the groups except CELE treated rats as the normal mucosal architectures re-establish and tissue remodeling gets on its way during late lag phase. The differential expression of COX-2 and PGE\textsubscript{2} levels was not only found complementing the ulcer healing kinetics but also showed an expression profile highly matched to the healing efficacy of different drugs, with maximum expression found in OMZ (1.57 and 2.18 COX-2/β-actin mRNA and protein ratio respectively on 14\textsuperscript{th} day), followed by MISO (1.34 and 1.78 respectively), SUC (1.15 and 1.48 respectively) and RANI (1.06 and 1.45 respectively). The CELE treated rats although showed COX-2 expression comparable to that of controls but lacked severely in PGE\textsubscript{2} levels, indicating the inhibition of COX-2 activity.

Among other defensive factors, total mucin content and growth factors appears to be an important contributing factors where former mainly correspond to the nutritious and protective gastric secretion of the lumen while later activate important cellular elements of ulcer healing such as angiogenesis, granulation tissue formation and re-epithelization. Estimation of mucin in the form of total carbohydrates in the gastric juice revealed that OMZ, MISO and SUC were highly effective in their elevations (87.81%
Summary

46.12% and 61.89% respectively), while RANI have showed no effect. The results substantiates the cytoprotective properties of SUC and offers an explanation for its high ulcer healing efficacy than that of RANI despite of poor anti-secretory properties. This was also evident in various other healing markers like high TC: P ratio suggesting lesser cell death and high mucin content for OMZ, MISO and SUC treated groups (2.41, 1.55 and 1.83 respectively) while in CELE treated rats TC: P ratio showed a value (0.67) lower than even that of control rats (0.73) indicating a delayed ulcer healing. Similarly, the expression analysis of three growth factors were found up-regulated in OMZ, SUC and MISO treated groups indicating high level of cellular proliferation and tissue remodeling. High Total DNA content in the gastric mucosa of OMZ, MISO and SUC treated rats (31.93%, 18.52% and 13.82% increase in comparison to control respectively) also signifies rapid cellular proliferation. Decrease in LPO levels and increase in anti-oxidant levels were found to have the least effect on ulcer healing, suggesting they are mainly involved in the ulcerogenesis and not in the healing mechanism as such. Still, OMZ had exhibited a strong anti-oxidant activity and found increasing the activity of both CAT and SOD enzymes (31.38% and 23.59% respectively). The other drugs were not found so effective in tendering the anti-oxidant activity.

Based on the systematic comparison of the different factors involved in the normal and drug mediated ulcer healing, we have categorised 16 parameters (belonging to 10 broader group) into five major and two inter-related minor factors while three of the biochemical parameters act like the healing markers. The major factors include inhibition of acid and pepsin synthesis, elevation in mucus secretion, stimulation of the growth factors production and most importantly an up-regulation of COX-2 expression at healing ulcer margins and therefore an elevation in the prostaglandin (PGE_2) content. The minor factors include decrease in lipid per-oxidation and increase in anti-oxidant enzymes activity, while the three important ulcer-healing markers along with ulcer area and histopathological sections are decrease in Myeloperoxidase activity (marker of neutrophils infiltration), total DNA content of the gastric mucosa and total carbohydrate: protein ratio (TC: P). Overall, the results indicates that a rise in the PGE_2 levels mediated through an up-regulated COX-2 expression is probably the most focal reason behind the strong effect of OMZ and MISO and to some extent of SUC on various defensive factors.
Summary

As the reported literature suggested that PGs play as chief mediator in virtually every component of mucosal defense: they inhibit acid secretion, stimulates mucus and bicarbonate secretion, inhibits mast cell activation, decrease leukocyte adherence to the vascular endothelium, inhibits apoptosis, elevate mucosal blood flow, prevent disruption of mucosal barrier, accelerate cell proliferation, enhances angiogenesis and elevates mucosal blood flow.

Conclusively, the foremost finding obtained from the comparisons and interpretations of COX-2 expression profile and PGE$_2$ levels in different treated groups revealed that the most efficient and powerful ulcer healing drug is the one that apart from its known acting mechanism, elevates the expression level of COX-2 enzyme most efficiently, which eventually leads to better and faster repair process. It was also figured out that the cyto-protective or broadly the defensive system endow with an inexorable role in the ulcer healing and repairing mechanism. The study along with establishing the role of COX-2 enzyme in the process of both natural and drug mediated ulcer healing, will also be helpful in providing necessary information for the development of better ulcer therapeutic modalities where an ulcer-healing drug should be a conglomerate of a two-tier strategy, a fall in the levels of offensive factors to prepare an environment conducive for healing, and strengthening of the defensive mucosal system to infuse cellular repair and mucosal reconstruction.