Discussion
Prevention of gastric mucosal lesions and the mechanism of gastric ulcer healing are two multifactorial processes (Sandor et al., 1995), where majority of factors display an overlap. Therefore, the drug designing strategies often flawed the two processes as one while formulating an agent that can act against the ulcerogenic factors. However, the micro evaluation of the two processes provides some vital and astonishing facts that demarcate the mechanism of ulcer prevention and the process of ulcer healing and therefore, should influence both the anti-ulcer therapies as well as ulcer healing approaches.

The key point lies in the process of ulcerogenesis, which occurs due to an imbalance of aggressive and defensive factors of gastric mucosa (Chang and Leung, 2002). Albeit, it is the up regulation of the offensive elements that primarily tilt the balance and eventually craft a lesion in the gastric mucosa. This signifies that the strength of an anti-ulcer agent resides in its capability of arresting the offensive factors, chiefly the acid secretion (Hoogerwerf and Pasricha, 2001). Furthermore, such a drug, even if does not alter the defensive environment of the gastric mucosa, then also it does not impair its efficacy.

On the contrary, 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 (broadly, the 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 (Dharmani et al, 2004). An ulcer-healing drug is therefore required to be a conglomerate of both of these aspects.

However, still, the peptic ulcer disease 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.
Discussion

Present study substantiates this hypothesis of a successful ulcer-healing drug. The comparison of normal and drug mediated ulcer healing has extracted several imperative findings:

(1) Compartmentalization of the participating factors into two broader categories: MAJOR and MINOR contributing factors based on their effect on ulcer healing process.

(2) Cyto-protective or broadly the defensive system provides an inescapable function in the ulcer healing and repairing machinery, where an enzyme COX-2 holds the vital key.

(3) 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 emerges a clearer winner.

In this chapter, we have discussed results of the present study in four sections: (i) Dynamics of normal (or spontaneous) ulcer healing, (ii) major and minor contributing factors (iii) drug mediated ulcer healing with separate emphasize on each of the drug mechanism and their comparison with normal ulcer healing and (iv) finally, the most substantial finding of the study- role of Cyclo-oxygenase-2 (COX-2) in gastric ulcer healing. Both the normal as well as drug mediated ulcer healing process are compared with differential effects of various participating factors, both aggressive and defensive on the healing process and the healing of ulcerated tissue is illustrated in term of various ulcer healing markers like TC: P ratio or total DNA content along with the ulcer area and histopathological examinations.

6.1 Normal (spontaneous) ulcer healing

Ulcer healing corresponds to the filling of the mucosal defect by proliferating and migrating cells (Perini et al., 2003) that involves inflammation, tissue formation (granulation tissue formation, angiogenesis and re-epithelialazation) and tissue remodeling. Results of normal ulcer healing (and that of drug mediated ulcer healing too) compliments significantly to the proposed ulcer healing dynamics discussed in the review section.

According, to the ulcer healing dynamics, the gradual ulcer size reduction is characterized by an organic decay curve (Halter et al., 1995). This reduction occurs in
three phases: early lag phase, when the healing mechanism dominates the ulcerogenic factors. The major effect is seen in the retardation of acid and pepsin volumes followed by the replacement of necrotic tissue. Once the mucosa and submucosa become necrotic, neutrophils and macrophages are attracted to the injured area by a variety of signals such as growth factor released from platelets and fibrin degradation (Tarnawski et al., 1990). This is often termed as cellular restitution. The transition from the early lag phase to the phase of rapid healing is characterized by migration of regenerated epithelial cells to reepithelialize the ulcer crater and by intensive epithelial cell proliferation in the ulcer margin. It is during this phase, when the actual role of cyto-protective and defensive factors comes into play. The transition from the rapid healing phase to late lag phase is characterized by shrinkage of the granulation tissue in the ulcer bed, which gets converted to fibrous tissue. This phase mainly involves tissue remodeling.

The early lag phase lasts for four days (0-4th day), phase of rapid healing works from 5th -12th day while 13th -20th day depicts late lag phase or early remodeling phase (Table 2.13). Findings of the present study correspond significantly to this postulated ulcer healing dynamics. As each of these phases passes, a gradual decrease in the ulcerated crater gets visible. The measurement of ulcer area and the histopathological analysis of the ulcerated tissue on day 0, 5, 10 and 14 portray a highly similar picture. The gastric ulcer showed progressive healing in control rats where ulcer area started reducing in size on day 5, showed a gradual decrease on day 10 and exhibited maximum healing on day 14. Gross appearance of ulcerated bed on four different days (Figure 5.3) and histopathological examination of the ulcerated tissue (Figure 5.14) further complemented the chronology postulated in ulcer healing dynamics. Day 14 analysis depicted maximum ulcer healing in the control (and all other drug treated group as well except CELE treated rats), as by this time the regeneration of the epithelial cells got nearly completed and tissue re-modeling was ongoing at different rates in different treated groups.

On 0 and 5th day, control group was having a grossly well-defined ulcer with damaged mucosal epithelium, glands and inflammatory exudates, proliferated fibroblasts and cellular debris in the ulcerated wall clearly visible on
hostopathiological examination (Figure 5.14 a and b). This is suggestive of the early lag phase, when decrease of acid synthesis is mainly achieved to produce a favorable milieu and no regeneration or reconstruction process really triggers off. Day 10 and 14 results underscore the process of restitution, proliferation, regeneration and slight re-organization (Figure 5.14 c and d). The ulcer bases were considerably reduced in thickness with few necrosis and inflammatory exudates and some extent of glandular organization. This period underline the **phase of rapid healing** as maximum reduction in ulcer size due to filling of the ulcer crater with proliferating cells was observed. The observation of 14th day also signifies that the process of regeneration has almost been completed and **late lag phase** has started, as some glandular re-organization was also visible. However, the observations of 10th and 14th day varies to different extent in different treated groups with comparatively lesser re-organization and re-structuring seen in control group when compared to other drug treated rats. This clearly implies that in the contributing factors that come into play during different healing phases gets affected differently by different drugs.

### 6.2 Contributory MAJOR and MINOR factors

The systematic comparison of the normal and drug mediated ulcer healing has resulted into identification of the major and minor factors that outplays during different phases of ulcer healing processes. Furthermore, it has also provided a direct comparison of the different drugs in terms of their efficacy on ulcer healing and respective causing factors. Overall, 16 different parameters were analyzed in different group of animals in the present study. These 16 parameters can be clubbed into 10 broader categories and the findings identified five **major** and two inter-related **minor** factors while three of the biochemical parameters act like the healing markers although directly involved in the healing process too.

The **major** factors include:

1. Inhibition of acid and pepsin synthesis (offensive elements)
2. Elevation in mucus secretion (cytoprotective factors)
3. Up-regulation of COX-2 expression at healing ulcer margins
4. Increase in the prostaglandin (PGE₂) content (cytoprotective factors)
5. Stimulation of the growth factors production (cytoprotective factors)
The minor factors include:

(1) Decrease in lipid per-oxidation
(2) Increase in anti-oxidant enzymes activity

Three important ulcer-healing markers along with ulcer area and histopathological examination are:

(1) Decrease in Myeloperoxidase activity (marker of neutrophils infiltration)
(2) Total DNA content of the gastric mucosa
(3) Total carbohydrate: protein ratio (TC: P)

Importantly, there are some crossovers in our classification of these factors and healing markers. However, a proper insight into their assayed role explains these crossovers. On of the major factors, mucus secretion and the healing marker TC: P ratios are completely inter-related, where the former mainly correspond to the nutritious and protective gastric secretion of the lumen while the later indicates its ratio with the leakage of the protein from gastric mucosa to gastric juice. Hence, an increase in the mucin secretion improves the cytoprotective environment that facilitates in the healing process while TC: P ratio describes amount of necrosis still present and hence, the degree of ulcer healing. Secondly, the categorization of MPO, a marker of neutrophil infiltration as the ulcer healing marker because although adherence of neutrophils during the start of healing phase serves various purposes like partial stimulation of COX-2 and growth factors, but as the healing progresses amount of neutrophils and hence the MPO activity starts decreasing. As we have measured the MPO activity only on the 14th day, therefore a decrease in its value on this day indicates towards an increase in ulcer healing.

Based on our results and existing ulcer healing literature, we postulate the chronology of the action of these factors. These major and minor factors get indulged in ulcer healing kinetics at different phases of ulcer healing curve. Inactivation of acid and pepsin synthesis kicks off at first and marks the predominant portion of the early lag phase along with the elevation in mucus synthesis. Neutrophils infiltration comes into act at the cusp of early lag phase and rapid healing phase when cellular restitution occurs. Rest all other factors, COX-2, its product PGs and growth factors participates through different mechanism in rapid healing phase ensuring proper cellular
regeneration, proliferation and re-organization. PGE$_2$ and growth factors also remain involved in late healing phase helping in tissue re-organization (Figure 6.1).

**Figure 6.1:** Chronology of different factors in the ulcer-healing curve

Different phases of ulcer healing curve: (1) Ulcer development, (2) early lag phase, (3) rapid healing phase (4) Late lag phase or early remodeling phase and (5) late remodeling phase

The role played by these major factors in the process of ulcer healing is described in detail in the “Review of literature” section. Largely, inhibition of acid and pepsin synthesis abrupt further damage to the mucosa and produce an environment conducive for defensive factors to work (Goel and Bhattacharya, 1991). Mucus synthesis provides the necessary nourishment and protection (Ito and Lacy, 1985; Wallace and Whittle, 1986). Prostaglandins acts in diverse and dispersed manner affecting number of concerned areas, like they promote blood circulation, rouse mucus synthesis and stimulate growth factors (Scheiman, 1996; Atay et al., 2000). Finally, the growth factors are involved in cellular proliferation, angiogenesis and mucosal reconstruction (Liu et al., 2000; Salcedo et al., 2003). As per the minor factors are concerned, the phenomenon of ROS synthesis is mainly a factor involve in the ulcerogenesis (Oka et al. 1991; Rastogie et al. 1998) and hence is not among the crucial elements involve in healing dynamics as far as the results of present study are
concerned. How different drugs lead to differential effects on these factors is discussed in next section.

**6.3 Drug mediated ulcer healing**

Comparison of normal and drug mediated ulcer healing clearly establishes that the ulcer-healing efficacy of a drug enhances many folds if it carries both the cytoprotective as well as anti-secretory activity. The findings hold good because the ulcer healing effect of drugs is essentially a translation of their action on different contributing factors. OMZ, a proton inhibitor with known cytoprotective properties (Tsuji et al., 2002) showed a 2-4 fold enhancement in ulcer healing and it was closely followed by a PG analogue MISO that is also known to carry the anti-secretory activities (Atay et al., 2000). Rest of the two, RANI or SUC are either anti-secretory or cytoprotective (Hoogerwof and Pasricha, 2001) respectively and were clearly found denounced in their ulcer healing activity compared to the other two drugs (Table 5.1). Effects of these drugs on different major and minor factors that lead to their differential ulcer healing activities are discussed below. The drugs and their effects are described in the order of their healing efficacy i.e. OMZ, MISO, SUC, RANI and lastly CELE.

**1. Omeprazole**

OMZ, a substituted benzimidazole derivative, is a well-known proton pump inhibitor (PPI) that potentially inhibits gastric acid secretion both in humans and animals (Richardson et al., 1998). The PPIs enter the parietal cells from the blood and because of their weak basic nature, accumulate in the acidic secretory canaliculi of the parietal cell, where they are activated by a proton catalyzed process that results in the formation of a thiophilic sulfonamide or sulfenic acid which inactivates the H⁺K⁺ATPase. The parietal cell must then produce new proton pumps or activates resting pumps to resume its acid secretion. OMZ is an irreversible PPI having a profound effect on acid production (Hoogerwof and Pasricha, 2001).

OMZ is unstable at a low pH. The oral dosage forms are supplied as enteric-coated tablets capsules that pass through the stomach intact and are absorbed in the proximal small bowel. Once absorbed, OMZ has a short plasma half-life (about one to two hours). Their duration of action is much longer because of their unique bipartite
mechanism of action (Vanderhoff and Tahboub, 2002). OMZ has been shown to protect the gastric mucosa against necrotizing agents and hemorrhagic shock, these effects are unrelated to the inhibition of acid secretion, but associated with a significant strengthening of gastric mucus barrier (Konturek et al., 1983; Mattson et al., 1983). Furthermore, Ohara et al. (1988) and Blandizzi et al. (1995), have also reported similar findings that OMZ exerts a protective effect possibly through an enhancement of gastric mucus secretion, in addition to inhibition of gastric acid secretion in rats.

Our results, moves one step ahead and provided various clues about the effect of OMZ on various protective factors separately and cumulatively on the process of ulcer healing as whole. As mentioned earlier, OMZ proved to be the most effective drug in the present study and has clearly demarcated discernible effect on various cytoprotective and offensive factors. When effect of OMZ on the 5 major and 2 minor factors was compared and rated with other drugs, it was clearly figured out that OMZ was most effective in all but one of the factors.

The anti-secretory mechanism of OMZ is due to their inhibitory effects on the H⁺K⁺ATPase (proton pump) in parietal cells (Satoh et al., 1989; Nagaya et al., 1990). The activated form of OMZ reacts by covalent binding with the sulfhydryl group of cysteines from the extracellular domain of the H⁺K⁺ATPase. Binding to 813, in particular, is essential for the inhibition of acid production, which is irreversible for that pump molecule (Hoogerwerf and Pasricha, 2001). Our study also revealed that a sharp decline (43.12 %, Figure 5.17) in total acidity by OMZ is due to potential blockage of the H⁺K⁺ATPase activity (68.75%, Figure 5.20). It was also figured out that blockage of H⁺K⁺ATPase is major mechanism behind inactivation of acid synthesis as two groups that have shown strong anti-secretory properties (RANI and MISO) have also exhibited a significant reduction in both the acid level of stomach as well as proton pump activity. However, these drugs instead of directly affecting the proton pump, mainly blocks one of the participating receptors. The sharp decline in the acid synthesis has also rendered significant decrease in the total pepsin content of the gastric juice (29.92%, Figure 5.17) in OMZ treated group.
OMZ apart from affecting the aggressive factors has tremendously increased PGE$_2$ level via COX-2 expression, which is one of the most underneath finding in our study for cytoprotective nature of OMZ (Okae et al., 1986; Simmons et al., 2004). It also shows the importance of COX-2 enzyme and PGE$_2$ in the process of normal as well as drug mediated ulcer healing. COX-2 is one of the key enzyme, responsible for the conversion of arachidonic acid into prostaglandins (Figure 2.12) and has also been demonstrated to be upregulated in the margins of healing gastric ulcers highlighting that COX-2 represents an important aspect of defense necessary for maintenance of mucosal integrity and healing (Mizuno et al., 1997; Peskar and Maricic, 1998). Increased expression of COX-2 mRNA (COX-2/β-actin mRNA ratio-1.57 on 14$^{th}$ day, Table 5.9) and protein (COX-2/β-actin protein-2.18 on 14$^{th}$ day, Table 5.10) and elevated levels of PGE$_2$ (24.10% in comparison to controls, Figure 5.23) augment strong healing depicted by OMZ. Similar effect was shown by Tsuji et al. (2002), where another PPI, lansoprazole was found promoting mucosal protection by up-regulating COX-2 expression and PGE$_2$ levels in rats during ulcer healing. They have suggested that the gastrin dependent pathway is responsible for up-regulation of COX-2 expression and elevated levels of PGE$_2$, as when lansoprazole was used along with a gastrin receptor antagonist AG-04R, significant reduction in COX-2 expression as well as PGE$_2$ level was observed. Present study also compliment with the findings of Tsuji et al. (2002) and the possible explanation could be that the particular dose of OMZ might be having an acid inhibitory effect as demonstrated in earlier studies (Dharmani et al., 2003, 2004 and 2005a, b), but the parallel increase in the COX-2 expression and PGE$_2$ levels with ulcer healing signifies that OMZ along with its anti-secretory effect also stimulates cytoprotective mechanism by up-regulating the COX-2 expression and prostaglandin synthesis.

Earlier, Shigeta et al. (1998) has also shown that COX-2 contributes to the elevation of PGE$_2$ production during healing of gastric ulcers. The elevation of COX-2 expression both at mRNA and protein level and significant increase in PGE$_2$ levels was found only in the ulcerated mucosa and not in the intact mucosa. The results comply previous reports where higher amounts of PGE$_2$ are detected at the site of ulceration than in non-ulcerated mucosa (Lesch et al., 1998) indicating the role of
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COX-2 and not COX-1 in their production at ulcerated site. Continuous formation of PGs by gastrointestinal mucosa represents a physiological process necessary to maintain cellular integrity (Wallace and Granger, 1996). This PGs play as chief mediator in ulcer healing influencing 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, prevent disruption of mucosal barrier, accelerate cell proliferation, enhances angiogenesis and elevates mucosal blood flow (Robert, 1979; Miller, 1983; Isenberg et al., 1985; Scheiman, 1996; Atay et al., 2000). We support the fact that repeated treatment with PPIs increases the level of PGs in the gastric mucosa mainly by up-regulation of its catalyzing enzyme –COX-2.

Furthermore, PGs have been described as the most potent stimuli for mucus secretion and have been proven to cause almost double the thickness of adherent mucus (Lanas, 2000). Released mucus along with entrapped fibrin, plasma and cellular debris generates a mucoid cap over the ulcerated area, providing a microenvironment favorable to re-epithelization, which is one of the crucial steps in ulcer healing (Ito and Lacy, 1985; Wallace and Whittle, 1986). OMZ in the present study has also enhanced the mucus secretion tremendously as revealed by the high content of total carbohydrates in the gastric juice (an increase of 87.81%, Figure 5.18), probably mediated through high PGE2 synthesis.

Healing of gastric ulcers require a reconstruction of the surface epithelium, glandular epithelial structures, restoration of the lamina propria and most importantly, reconstruction of the micro vascular network essential for delivery of oxygen and nutrients to the healing site. Angiogenesis within granulation tissue is considered to be one of the most important processes in ulcer healing which is reported to be increased with increase in PG content (Wallace and Granger, 1996). The growth of new microvessels through angiogenesis is promoted by angiogenic growth factors such as bFGF, VEGF, PEGF and angiopoietin (Tarnawski, 2002). Increase in COX-2 derived PGs is known to stimulate the expression of growth factors (Bamba et al., 1998; Wallace, 2001), which in turn activate important cellular elements of ulcer healing such as angiogenesis, granulation tissue formation and re-epithelization (Szabo and
Sándor, 2000). Growth factors play fundamental roles in these process, by stimulating chemotaxis and cellular proliferation, by providing signaling among cells of the same and different type, by controlling extracellular matrix formation and angiogenesis, by regulating the process of contraction, and by re-establishing tissue integrity. As soon as blood vessels are disrupted, platelets enter the wound in great numbers and release several growth factors. We found significant increase in levels of bFGF, EGF and VEGF expression in OMZ treated rats and it was seen that protein/β-actin ratio for all the growth factors in OMZ treated rats was much higher to that of the intact mucosa (Table 5.11) suggesting strong cellular proliferation. The protein/β-actin ratio for different growth factors in OMZ treated ulcerated mucosa was second highest after SUC treated rat, which are known stimulater of growth factors (Szabo et al. 1991).

Each of these growth factors are crucial in ulcer healing process. EGF is mainly involved in the reconstitution of the epithelial structures (Milani and Calabro, 2001) and is also known to be potent stimulus for cell proliferation invitro and also reported to stimulate DNA synthesis invivo (Murphy, 1998). The proliferative action of EGF contributes to the normal maintenance of mucosal integrity within the GI tract (Playford and Wright, 1996). EGF promotes all this reparative processes by accelerating epithelial cell migration necessary for re-epithelialization of the ulcer base and also triggers cell proliferation and divisions crucial for filling the mucosal defect, thus enables reconstruction of epithelial structures within the ulcer scar (Tarnawski et al., 1992, 1997; Konturek et al., 1988). The significantly increased level of EGF in OMZ treated gastric mucosa (protein/β-actin ratio 1.22, Table 5.11) indicates that OMZ also mediate the above-mentioned components important for accelerated ulcer healing. The high rate of cellular proliferation and DNA synthesis in OMZ treated rats was also evident from high total DNA content of the gastric mucosa (31.93%, Figure 5.21).

Biological action of bFGF includes the stimulation of migration and proliferation of various mesenchymal cells leading to angiogenesis. It has been already show by Ernst et al. (2001) that neutralizing antibodies against bFGF delay the ulcer healing. VEGF play a major role in the reconstitution of connective tissue and provide the extracellular matrix substrate for cell migration and differentiation. VEGF is the
most potent inducer of angiogenesis, endothelial cell proliferation and capillary permeability (Leung et al., 1989). Activation of VEGF during healing of gastric mucosal injury has also reported previously (Jones et al., 1999). OMZ was also found to increase the level of bFGF and VEGF (Table 5.11), which also comply with the study of Motohiro et al. (2002).

We suggest that increase in the growth factors in OMZ group is also owed to increase COX-2 mediated PGE$_2$ production. PGE$_2$ has been shown to cause vasodilation and stimulate angiogenesis (Forum and Auerbach, 1983). Recent studies have demonstrated that inhibition of COX-2 activity significantly down regulates VEGF expression, inhibits angiogenesis (Liu et al., 2000). Some other studies have shown that PGE$_2$ stimulates VEGF expression in gastric fibroblasts (Takahashi et al., 1998), while other shows that bFGF and VEGF are inducers of COX with subsequent production of PG synthesis in endothelial cells (Kage et al., 1999; Hernandez et al., 2001; Tamura et al., 2002). Blockage of COX activity by non-selective COX inhibitor is also to inhibit 50% action of bFGF- and VEGF-induced angiogenesis (Salcedo et al., 2003). These findings indicated that PGs seems to be on the downstream of bFGF and VEGF in the induction of microvessels formation during gastric ulcer healing. Thus, both VEGF and PGs could co-regulate each other in the induction of angiogenesis and promote ulcer healing in the stomach.

Among the minor factors, the LPO content was decreased in OMZ treated rats (31.32%, Figure 5.22). Enhance LPO in control could be mediated by activated neutrophils infiltrated into the ulcerated region and administered OMZ could exert an attenuating action on the enhanced gastric mucosal LPO by inhibiting neutrophil infiltration in the ulcerated region. Oxidative damage is considered to be a common factor in ulcerogenesis. Apart from acid and pepsin free radical generation is also involved in the delay of healing. Increased LPO is seen in acetic acid induced ulcer model. This is due to increase in the generation of reactive oxygen species (ROS) leading to oxidative damage. The increase in ROS leads to decreased activity of COX and loss of gastric mucosal cytoprotection (Goel et al., 2001). OMZ is a well-known powerful anti-oxidant (Lapenna et al., 1996). Increased gastric mucin level is another reason of anti-oxidant nature of OMZ as it is reported to interact with oxygen free
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radicals invitro (Grisham et al., 1987). It has also shown that OMZ attenuates the production of oxygen free radicals in activated neutrophils by increasing intralysosomal pH in the cells in-vitro (Suzuki et al., 1996). Our results also showed an elevation in SOD (23.59%, Figure 5.22) and CAT (31.38%, Figure 5.22) activity in OMZ treated group. SOD scavenges the superoxide radical \( (\text{O}_2^-) \), one of the ROS responsible for LPO (Fridovich, 1986). This reaction leads to increase in generation of peroxyl radicals \( \text{H}_2\text{O}_2^+ \), which is further reduced by CAT (Das et al., 1997).

Finally, gastric mucosal MPO activity, an index of neutrophil infiltration was also found significantly low in OMZ group (4.5 U/mg protein in comparison to 24.90U/mg protein found in controls, Table 5.6). These neutrophils phagocytize necrotic tissue and releases pro-inflammatory cytokines, which in turns activates COX-2 (Tarnawski et al., 2001) probably during the start of rapid ulcer healing phase. Initial infiltration of the ulcer with neutrophils is reduced after few days and is followed by accumulation of macrophage and therefore induction of COX-2 also starts decreasing, which was clearly visible in COX-2/B-actin ratio on day 10 and 14 (discussed in the next section). Therefore, the up-regulation of both COX-2 and growth factors is also induced by neutrophil adherence partially. As we have measured the MPO activity only on the 14th day, therefore the lower value is suggestive of strong ulcer healing which was also reported elsewhere (Wandall, 1992). Significantly, level of MPO activity can also be correlated with the ulcer area. As the acid attacks the ulcerated portion, because of lack of tight junctions, neutrophil might accumulate at this site. OMZ, a strong acid inhibitory agent doesn't allow further breaking of tight junctions in the regenerating mucosa so inhibits the activity of neutrophils and reduces the MPO activity.

The strong effect of OMZ on various major and minor factors was clearly evident in its strong ulcer healing activity. Apart from the decrease in ulcer size (84.09% as compared to controls, Table 5.1), and MPO activity, OMZ treated rats exhibited most significant score on other ulcer healing markers too. Histologically, OMZ-treated rats exhibited a flat ulcer margin and this may have been due to the protection of the newly formed epithelial cells in the ulcer margin and granulation tissue (including microvessels in the ulcer bed) from direct gastric acid-pepsin damage.
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(Shigeta et al., 1998). The results are consistent with other studies indicating that PPIs promote ulcer re-epithelization (Folkman et al., 1991). Moreover, OMZ treated rats showed a thinner granulation tissue in the ulcer base (including microvessels in the ulcer bed). This observation is consistent with the high expression of growth factors indicating that the stimulation of angiogenesis in the ulcer base may be one of the main mechanisms of the acceleration of ulcer healing (Schmassmann et al., 1996).

Among the other two markers, total DNA content of gastric mucosa, which represents cellular proliferation and DNA synthesis, was found maximum in OMZ group (31.93% increase from control group, Figure 5.21), nearly similar to that of normal intact mucosa. Similarly, the high TC: P ratio where high value TC corresponds to high mucin content, while low protein value represents lesser cell death. OMZ treated group depicts maximum TC: P ratio (2.41, Table 5.5) among all other groups.

Overall, our results clearly establish that OMZ being a strong anti-secretory, cytoprotective and anti-oxidant moiety represented the most effective class of ulcer healing drug.

2. Misoprostol

PGE₂ and PGI₂ are the major PGs synthesized in gastric mucosa. Chemically, MISO (15-deoxy-16-hydroxy-16-methyl-PGE₁) is a synthetic analog of PGE₁ with an additional ester group at C1 (resulting in an increase in potency and in the duration of the antisecretory effect) and a switch of the hydroxyl group from C-15 to C-16 along with an additional methyl group (resulting in improved activity when given orally, increased duration of action, and improved safety profile). MISO is rapidly absorbed and undergoes an extensive and rapid first-pass metabolism (deesterification) to form MISO acid (free acid), the principal and active metabolite of the drug. Some of this conversion may infact occur in parietal cells. The elimination half-life of the free acid is about 20-40 minutes (Hoogerwerf and Pasricha, 2001). Synthetic PGs are more potent than naturally occurring prostaglandins, have fewer side effects, are longer acting and resist rapid metabolism, making them effective with oral administration (Collins et al., 1985, Wilson et al., 1987).

The exact mechanism of MISO cytoprotection is uncertain; several concepts have been developed to explain its varied effects. The major contribution in the
healing mediated through MISO is by an increase in PG level. The role of endogenous PGs in the gastric mucosal integrity is well established but there are several mechanisms through which exogenously supplied PGs promote healing of the ulcers. These mainly include inhibition of acid secretion, replacement of deficient PG levels in the gastric mucosa, and stimulation or modulation of the factors involved in the healing process such as angiogenesis, epithelial cell proliferation and regeneration, ulcer contraction and blood flow (Helpap et al., 1981; Helander, 1983; Penney et al., 1994).

Our results correspond to these facts as MISO has shown best results among all the other drugs than OMZ for the ulcer healing mechanism (76.36% increase on 14th day in comparison to control, Table 5.1). We found MISO not only enhances the defensive factors but also credibly reduced the aggressive factors. When effect of MISO on the 5 major and 2 minor factors was compared and rated with other drugs, the possible mechanism of action of MISO was nearly figured out.

Our study provides the direct evidence that PG analogues inhibit the acid secretion (Ishibashi et al., 1979; Wilson, 1987; Atay et al., 2000). The mechanism through which MISO shows its acid inhibitory property (32.92%, Figure 5.17) is probably mediated through the specific receptor present on the parietal cell (EP3 receptor). The binding of MISO with EP3 receptor results in activation of inhibitory subunit of adenylate cyclase and lead to decreased levels of intracellular cyclic AMP, which don’t allow H⁺K⁺ATPase to get activated. It was because of this reason; MISO has also depicted a reduction in the proton pump activity (29.2%, Figure 5.20). However, this acid inhibition by MISO is not clinically superior to histamine receptor antagonists or PPIs (Atay et al., 2000) that might account for its lesser efficacy in bringing down the acid level as compared to OMZ and RANI. Additionally, MISO is also reported to inhibit basal, nocturnal and stimulated gastric acid secretion (Wilson, 1987). Some of the reports have even suggested that MISO is as effective as cimetidine (a H₂ receptor antagonist) in their acid inhibitory response. Decrease in acid secretion also accounts for reduced proteolytic action of pepsinogen and hence also decrease the peptic activity (19.63 % decrease in comparison to control, Figure 5.17).
In addition to the antisecretory mechanism, MISO has also exhibited numerous gastro-duodenal mucosal protective effects. The rapid increase in healing by MISO is mainly through enhancement of endogenous PGE$_2$ (16.80% increase in comparison to control on 14$^{th}$ day, Figure 5.23) due to elevation in COX-2 expression assessed at both mRNA (COX-2/β-actin mRNA ratio-1.34 on 14$^{th}$ day, Table 5.9) and protein COX-2/β-actin protein-1.78 on 14$^{th}$ day, Table 5.10) level. This is probably the most unexpected finding of the present study, as there is contradiction regarding the role of an exogenous PGE$_2$ analogue in promoting the synthesis of endogenous PGE$_2$. Some authors suggest that supply of exogenous PGE$_2$ retards the production of endogenous PGE$_2$. However, there are several reports available in recent times that categorically states MISO or any other exogenous PGE$_2$ analogue (dimethylprostaglandin E$_2$) up-regulates the expression of COX-2 m-RNA enzyme and elevates the levels of endogenous PGE$_2$ both invivo (Wang et al., 1989; Tjandrawinata et al., 1997; Buluc et al., 2002) and invitro (Minghetti et al., 1997; Bonazzi et al., 2000; Hinz et al., 2000). Recently, MISO has been shown to increase the level of COX-2 protein in the leukocytes in a model of carrageenan air pouch inflammation (Buluc et al., 2002), albeit, none of the study has reported such effect of MISO in ulcer healing. We therefore provide the substantial proof that MISO elevates the level of endogenous PGs via up-regulation of COX-2.

Along with increasing endogenous PGE$_2$, exogenous supplied PGE$_2$ is also found to increase other major contributing factor, growth factors leading to stimulation of angiogenesis, cellular reconstruction and proliferation. The angiogenic effects of MISO have also been shown earlier (Tranawsky et al., 1990). Most probable reason behind this could be the rise in COX-2 expression. Earlier, Sawaoka et al. (1998) have also shown that when gastric endothelial cells are cultured on metrigel they form tubular capillary like structure reflecting angiogenesis. They concluded that COX-2 is an important mediator of angiogenesis. The significant rise in the level of COX-2 mRNA as well as protein expression in MISO treated rats is therefore suggested to be an important supporting parameter in the gastric ulcer healing process as it promotes both synthesis of endogenous PGs and angiogenesis.
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Increases in mucosal blood flow have been postulated as another mechanism of MISO-induced gastric protection as it supplies increased oxygen as well as nutrients to the site of regenerating mucosa. However, several experimental studies have produced contradictory results regarding the role of mucosal blood flow in MISO-induced gastric protection (Wolfe et al., 1999). In an experimental model of isolated versus perfused canine gastric mucosa, pre-treatment with 16, 16-dimethyl prostaglandin was shown to protect against injury caused by topical application of aspirin in vivo, but not in vitro, suggesting that blood flow and neural factors may be required to mediate the effect of PGs (Henagan et al., 1989).

In addition to these protective effects, MISO has also increased the mucin secretion tremendously (46.12% increase in comparison to controls, Figure 5.18) but the exact mechanism is uncertain. Enhancement of local mucus production may explain some of the effect. MISO has been shown to increase gastric mucus production in humans in a dose-dependent manner (Wilson et al., 1986). In rats, the adherent layer of mucus can be increased up to three-fold in thickness, with 70% of the response noted within 5 minutes (Sellers et al., 1986). This suggests that MISO stimulates the release of pre-formed mucus (Allen and Carroll, 1985). This increased mucus production has been postulated to prevent the back-diffusion of hydrogen ions, resulting in mucosal protection. Support of gastric mucosal turnover and regeneration is also postulated to be a factor in MISO cytoprotection.

Among the minor factors, MISO has also exhibited some of its anti-oxidant properties. Pawlowska et al. (2001), has reported earlier that MISO increase the SOD level in rabbit blood, we also found similar results in rat model as MISO was found increasing the SOD level in the treated rat mucosa (19.45%, Figure 5.22). An increase, albeit non-significant was also observed in activity of CAT (10.79%, Figure 5.22). However, the most important clue regarding the anti-oxidant activity of MISO was evident from the highly significant decrease in LPO activity (20.42%, Figure 5.22).

We therefore conclude that the strong mucosal protective and moderate anti secretory properties of MISO are responsible for its strong ulcer-healing efficacy. Apart from the decrease in ulcer size (76.36% as compared to controls, Table 5.1),
MISO treated rats exhibited significant score on other ulcer healing markers too. High TC: P ratio (1.55, Table 5.5), reduced MPO activity (64.25%) and increased total DNA content (18.52, Figure 5.21) rates MISO to be the second most potent ulcer healing drug after OMZ. Goodlad et al. (1990 and 1992) has also shown that increased DNA and cell proliferation, or decreased cell loss is responsible for significantly increase canine gastric mucosal mass. The authors have demonstrated that the increase in gastric mass associated with administration of MISO is related to increased cell proliferation, rather than decreased cell turnover.

Conclusively, MISO was found to be second most powerful ulcer healing drug in present study due to the superlative combination of antisecretory and mucosal protective properties.

3. Sucralfate

SUC, a complex of sucrose octasulfate and aluminum, is a known to protect gastric mucosa against the damaging action of strong irritants and to accelerate healing of chronic ulcers. Its pharmacological activity may reside in the entire parent molecule or its components sulfate, aluminum and sucrose octasulfate. In the acid environment (pH<4), it undergoes extensive cross-linking and polymerization to produce a viscous, sticky gel that adheres strongly to epithelial cells and even more strongly to ulcer craters for as long as 6 hrs after a single dose. In addition to inhibition of hydrolysis of mucosal proteins by pepsin, SUC have cytoprotective effects such as stimulation of local production of PGs and EGF (Szabo et al, 1991; Hoogerwerf and Pasricha, 2001).

Present study revealed that SUC mainly targets the contributing factors of mucosal defensive mechanism and is rather futile in its anti-secretory properties. Comparison of 5 major and 2 minor factors for their possible role in acting mechanism of SUC imply that SUC is rated down in the order mainly because of its inefficiency in regulating the acid synthesis.

Biochemical analysis of different major and minor contributing factors showed that SUC does not have any significant effect on acid synthesis or contents and hence it is ineffective in controlling the peptic activity too (Figure 5.17). The drug has small buffering activity and during its reaction with acid, it gets converted to a sticky gel
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like substance (SUC polyanion) that binds to proteins in vitro and at the site of ulcer. This complex in turn inhibits the rate of diffusion of acid and pepsin into ulcer crater and protects the regenerating mucosa. As reported earlier, SUC does not inhibit the activity of pepsin; the formed complex only restricts pepsin to digest the gastric mucosa (Samloff and O'Dell, 1985). Furthermore, an earlier study of Sandor et al. (1995) has also shown that SUC enhances chronic gastric ulcer healing without decreasing gastric acidity and pepsin secretion.

Despite of its ineptness in controlling the offensive factors, SUC still turned up to be an effective and competent ulcer healing drug in the present study (ulcer healing being 60.45% on 14th day in comparison to control, Table 5.1), largely due to its beneficial effects on different mucosal defense mechanisms such as the superficial mucus bicarbonate barrier, mucosal hydrophobicity, cellular function (including regeneration and restitution), mucosal blood flow, endogenous mediators of tissue injury and repair as well as local production of PGs. The drug is reported to be truly cytoprotective as it protects isolated epithelial cells from damage by noxious agents (Romano et al., 1990). Present study revealed that SUC has increased the total carbohydrates, an important part of mucin significantly (61.89%, Figure 5.18), which was appreciably higher than that of MISO (46.2%, Figure 5.18), despite of the fact that MISO (76.36%) has shown much higher healing than that of SUC (60.45%). This increase in mucus secretion is primarily because of activation of phosphatidylinositol kinase and phospholipase C enzyme in epithelial cells (Slomiany et al., 1991). Later on Hill et al., 1991 drew the attention towards the highly hydrophobic nature of canine gastric mucosa and suggested that this was attributable to a layer of surface-active phospholipids adsorbed directly onto the apical membrane of surface epithelial cells. Their view was supported by the existence of 8-10 bilayers of phospholipid overlying the rat gastric mucosa treated with SUC (Hill et al., 1991). Absence of this lining led to hydrophilic mucosa and development of ulceration. This was an added process of securing the gastric mucosal from acid attack. These layers protect the regenerating and proliferating cells from the further damage and facilitate prompt restoration of the damaged epithelium.
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Based on our results, we inferred that like OMZ and MISO, SUC also mediate its protective action mainly by increasing the local PGE$_2$ level through increased COX-2 expression (Table 5.8-5.10). However, the elevation of COX2 and PGE$_2$ in SUC treated group was not as higher as that in OMZ or MISO treated groups that might have been another reason for low efficacy of SUC in healing of gastric lesions. This illustration is supported from the study of Wallace et al. (1988) that has shown that if COX inhibitors are given along with SUC than its protective mechanism is abolished showing that COX is involved in the synthesis of protective PGs.

The ability of SUC to prevent the deeper gastric tissues depends upon the preservation of mucosal blood flow. The microvasculature plays an essential role in transporting oxygen and nutrients to various layers of the mucosa and its integrity is a prerequisite for energy-dependent re-epithelialization following ulceration. Chen et al. (1989) have studied gastric blood flow using ex vivo chamber preparation and laser Doppler flowmetry and observed that SUC reduces the magnitude of mucosal damage and blood flow induced by topical ethanol. They also reported that SUC increases the blood flow in a dose dependent manner but they could not unveil the mechanism of action. Based on our results, we postulate that increase in the blood flow is mainly due to increased PGE$_2$, which is reported potent vasodilator. This increased blood flow stimulates angiogenesis, formation of new blood vessels and granulation tissue.

SUC, being a potent cytoprotective involves the protection of the regeneration zone and the maintenance of blood flow to the mucosa. Among the trophic agents like PGs, growth factors, gastrin etc that exhibits mucosal growth-promoting action, growth factors, especially EGF might contribute exceedingly to the healing effects of SUC. We found highest increase in EGF level in growing ulcerated tissue of SUC treated rats (1.45 protein/β-actin ratio on 14th day, Table 5.11). SUC at acidic pH is a strong binder of EGF and therefore concentrates this agent at the site of ulcer craters. In this way EGF may promote growth of epithelial cells and fibroblasts in the vicinity of ulcer, thus accelerates healing. These effects of SUC are related to their stimulation of cell proliferation, that was also evident from its high total DNA content obtain from gastric mucosa (13.82%, Figure 5.21). Among the minor contributory factors, SUC
has not shown any anti-oxidant property but has reduced the lipid peroxidation significantly (17.3%, Figure 5.22). This reduction may further be related to decrease in the MPO activity in the regenerating gastric mucosa.

Conclusively, SUC is rated relatively high for its role in healing of gastric ulcers, apart from its known importance in the treatment of peptic ulcer disease. Healing provided by SUC is not as efficacious as with OMZ and MISO as this drug doesn’t carry a property of being anti-secretory in nature. But SUC has shown better results than RANI indicating that defensive factors are imperative in the ulcer repair process.

4. Ranitidine

RANI is a member of the H₂ (histamine blocker) family of drugs, which prevents the release of acid into the stomach. Chemically, it has an imidazole ring containing an additional furan ring. RANI is popularly used to treat stomach and duodenal ulcers. The most prominent effects of H₂ receptor antagonist are on basal acid secretion; less profound but still significant in suppression of stimulated acid production. This agent thus is particularly effective in suppressing nocturnal acid secretion. Histamine receptor antagonists are generally known to have a weak effect on the healing of chronic gastric ulcers (Okabe et al., 1994). The reason why the antagonists do not exert an appreciable effect on ulcer healing seems to be primarily the short action of duration of the anti-secretory activity.

Results of the present study also falls in the same line as when 5 major and 2 minor contributing factors in ulcer healing were compared for RANI treated rats, it was clearly evident that RANI is exceedingly successful in retaining the acid secretion but is relatively ineffective on other factors. This clearly describes the reason of lowest rank of RANI among the four studied drug for their ulcer healing capabilities.

We found that RANI has potentially decreased the acid secretion (33.56%, Figure 5.17), due to binding of RANI to H₂ receptors on the basolateral membranes of parietal cells (Hoogerwerf and Pasricha, 2001). The histamine receptors on the basolateral membrane of acid-secreting parietal cells are of the H₂ type. The occupation of H₂ receptors by histamine, which is released by mast cells and possibly by other cells, activates adenylate cyclase, increasing intracellular concentration of cAMP. This increased cAMP activates the proton pump of the parietal cells to secret
hydrogen ion. RANI competitively and selectively inhibit the binding of histamine to H₂ receptors, thereby reducing both intracellular concentration of cAMP and the secretion by the parietal cells, thus decrease the acid secretion. As previously reported by Kubo et al. (1995), H₂ receptor antagonists act on the initial step, blocking the H₂ receptor site on the plasma membrane of the parietal cells. As a result of inhibiting the secretion of gastric acid and raising gastric pH, peptic activity gets reduced (Feldman and Burton, 1990). Our results provide ample proof for this mode of action of RANI as the drug was found both reducing the total acid content of the gastric juice (33.56%, Figure 5.17) as well as the activity of H⁺K⁺ATPase activity (43.75%, Figure 5.20) significantly.

Ulcer healing analysis has shown that although RANI has exhibited slowest healing among all the other used anti-ulcer drugs but still carries a significant edge over control group. The results sustain the findings of previous studies and establish that RANI is not cytoprotective in nature. It is mainly an anti-secretory drug whose action also lies for a shorter duration. We found that all other drugs OMZ, MISOP and SUC studied in the present study carry strong cytoprotective properties, where former two are also efficient anti-secretory drugs. On the contrary RANI carries only anti-secretory properties. This probably explains the lowest rank of RANI among all the studied drugs, cementing the concept that during the process of regeneration and reconstruction of mucosal architecture, cytoprotective and other defensive contributing factors are most vital and mere reduction in offensive machinery only restricts further damage leaving the regeneration process to occur seldom.

RANI has neither shown a significant increase in the level any of the components of total carbohydrate, nor in the level of other cytoprotective elements like PGE₂, growth factors etc. However, due to its property of pepsin inhibition (16.22%, Figure 5.17), it has reduced the protein leakage in comparison to control (42.2%, Figure 5.19). But overall, ineffectiveness of RANI in promoting carbohydrate levels results into no effect on total mucin content. Earlier study by Ichikawa et al. (1997) has shown that histamine administration caused a significant increase in mucin content. RANI showed suppression of mucin biosynthesis probably because of being histamine receptor antagonist.
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Apart from mucin, no increase was observed in the PGE₂ level in the RANI treated rat mucosa (6.25% on 14th day, Figure 5.23). Konturek et al. (1981) has also showed very less mucosal generation of PGs in animals treated with RANI. The lowered level of PGE₂ in RANI treated rats is probably because COX-2 expression, both at mRNA (COX-2 / β-actin ratio, 1.06 on 14th day, Table 5.9) as well as protein (COX-2 / β-actin ratio, 1.45 on 14th day, Table 5.10) was not significantly different from control group. As seen in other treated groups that an elevation in COX-2 expression has resulted in significant rise in the levels of PGE₂, which in turn has affected the synthesis of various other cytoprotective factors.

Among the minor contributing factors, LPO was significantly reduced in RANI treated rats (15.17%, Figure 5.22) but the other antioxidant enzyme level was not changed. RANI is reported to possess anti-oxidant property in stress-induced model (Lapenna et al., 1994) but in healing mucosa, no change was observed in the two analyzed mechanism-SOD and CAT (Figure 5.22). Ineffectiveness of RANI in triggering the synthesis of mucin and growth factors was well marked in other ulcer healing markers like very low TC: P ratio despite of significant reduction in the protein leakage in the gastric juice (1.0, Table 5.5). Even MPO activity was found significantly reduced (Table 5.6), which could be due to its acid inhibitory action. Similarly, histopathological examination of RANI treated gastric mucosa showed very poor signs of mucosal re-organization. Giaccio et al. (1996) in their study showed that RANI did not stimulate cell migration but it enhances cell proliferation. They also showed that RANI treatment didn’t increase the mRNA expression of TGF and EGF.

Conclusively, RANI, an acid inhibitory drug is very useful in treating stress ulcers but it has less potential for ulcer healing as this drug lacks cytoprotective property.

5. Celecoxib

CELE (a COX-2 selective NSAID), used as negative control in the present study has shown to retard the ulcer healing in comparison to vehicle treated control. CELE was developed after cloning of COX-2 exclusively for its inhibitory activity against COX-2, when it became clear that inhibition of COX-1 resulted in removal of protective PGs and causes injury to the gastric mucosa. The sulfonamide group on
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CELE binds into the side pocket within the channel of the active site of COX-2 and confers COX-2 selectivity for CELE. CELE inhibits COX-2 competitively, but this becomes converted into an irreversible, slow, time-dependent inhibition; however, CELE weakly inhibits COX-1 (Copeland et al., 1994; Gierse et al., 1995). The advancement of COX-2 inhibitors represents a major step forward in the pharmacological approach to the treatment of arthritis. These specific inhibitors have been shown in clinical trials to relieve pain and inflammation associated with arthritis as effectively as conventional NSAIDs with significantly less adverse side effects as gastric ulceration (Schnitzer and Hochberg, 2002).

Analysis of ulcer healing and its different contributory factors have revealed that the basic property of CELE, i.e. inhibition of COX-2 activity is major hurdle in the healing of pre-existing ulcers observed in CELE treated rats. When different contributing factors were compared between CELE and other groups, it was clearly evident that CELE exhibited a delayed ulcer healing by diminishing the COX-2 activity manifested by severe retardation in the PGE$_2$ levels (Table 5.8) COX-2 protein expression (Table 5.10) despite of the marginal fall in the expression of COX-2 mRNA (Table 5.9) in comparison to the control group. Therefore, stumbling consequence of CELE treatment in healing process is mainly because of the underperformance of COX-2 activity.

A delay in ulcer healing by COX-2 inhibitor CELE provides a clear line of support for COX-2 being a major candidate for gastric ulcer healing. Lesch et al. (1998) have proposed that the chemical structure with a sulphonamide moiety may account for the healing delay, although the exact mechanism for this impairment is not known. Retardation in ulcer healing in CELE treated rats was visible as even on 14th day CELE treated group has shown ulcer area greater than that of the control group (-25.7% on 14$^{th}$ day, Table 5.1). Interestingly, none of the aggressive factors were found affected by treatment of CELE as both acid and pepsin synthesis in CELE and control groups was nearly similar (Figure 5.17). Among other major factors, PGE$_2$ was found significantly reduced in CELE group on 10$^{th}$ as well as on 14$^{th}$ day of treatment, whereas no significant change was found on 5$^{th}$ day (Table 5.8). CELE was found suppressing the PGE$_2$ production significantly by inhibiting COX-2 activity and
to some extent by down regulating COX-2 expression. Some of earlier reports have also shown that selective COX-2 inhibitors along with suppressing the COX activity also results in retardation of its expression, however no possible mechanism has been suggested for this effect (Tanaka et al., 2002). We postulate that the failure of COX-2 enzyme in producing PGE₂ due to coxib activity might be having a plummeting effect on its expression.

Decrease in level of PGE₂ further retards other ulcer healing mechanisms such as mucus and bicarbonate secretion (Figure 5.17) etc. Delay in ulcer healing can be further correlated with decrease in angiogenesis, which is also very crucial for acceleration of mucosal regeneration. As previously reported, COX-2 selective inhibitors are known to delay healing by impairing angiogenesis (Guo et al., 2002). In the present study, we found low expression level of bFGF and VEGF protein whereas EGF was not influenced by the treatment (Table 5.11). As previously reported blockage of COX-2 activity inhibits 50% of bFGF and VEGF- induced angiogenesis (Luo et al., 2004). The anti-angiogenic action of COX-2 inhibitors through inhibition of angiogenic growth factor bFGF may contribute significantly to its inhibitory effect on gastric ulcer healing as bFGF is reported to be a potent angiogenic endothelial mitogen. Reduction in the angiogenic protein exerts synergistic effect in delaying healing by both reducing angiogenesis and decreasing granulation tissue. These findings suggest that decrease in PGE₂ seems to downstream bFGF and VEGF expression in the induction of microvessel formation during the ulcer healing process.

According to Fujito et al. (1998), suggested another potent reason for the delay in ulcer healing as decrease in antichemotactic activity i.e. increase in chemotactic activity in the ulcerated mucosa of CELE treated group, leading to persistent neutrophil infiltration. The selective inhibition of COX-2 by CELE resulted in pronounced reduction in ulcer healing was accompanied by lesser decrease in granulocyte infiltration as measured by MPO activity (Table 5.6), which was insignificantly more than the control group. This increased MPO lead to significant increase in LPO, whereas no significant change in anti-oxidant enzyme was found. Recently, Burak et al. (2003), has also shown the ineptness of CELE in affecting the anti-oxidant activities.
In summary, our study demonstrates that CELE as a highly selective inhibitor of COX-2 delayed healing of acetic acid induced gastric ulcer healing in rats by inhibiting COX-2 induced increased PGE$_2$, which in turn down regulates angiogenic growth factor bFGF. CELE doesn’t play any role in the ulcerogenesis but it has major role in delaying healing of pre-existing ulcers mainly by blocking cytoprotection offered by COX-2.

**6.4 Role of COX-2 in gastric ulcer healing**

Present study was drafted with the major aim of deducing the role of COX-2 enzyme in normal and drug-mediated ulcer healing mechanism. Hence, the estimation of COX-2 expression was carried out both at mRNA transcript and the protein level on three different days: 5$^{th}$, 10$^{th}$ and 14$^{th}$, to have a direct comparison with the PGE$_2$ synthesis in general and ulcer healing kinetics in particular. 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 lead to better and faster repair process.

Overall, the study has conveyed various important aspects about the role of COX-2 and PGE$_2$ in ulcer healing or substantiated the earlier reports with better evidences. First of all, it was clearly established that an elevation in PGE$_2$ level, whose synthesis is catalyzed by COX-2 is pivotal in ulcer healing process, because its estimation have shown an almost two-fold increase in PGE$_2$ levels on 10$^{th}$ and 14$^{th}$ day in the ulcerated region as compared to the intact mucosa in all the treated groups (Table 5.8). These findings have authenticated the earlier report of Mizuno et al. (1997) that also demonstrated a significant increase in the PG generation in ulcerated but mucosa of mice. Peskar and Maricic, 1998 have also highlighted that COX-2 represents an important aspect of defense necessary for maintenance of mucosal integrity.

Secondly the most crucial outcome of the present study was manifested when COX2/β-actin mRNA and protein ratio and PGE$_2$ levels were considered together, it was clearly evident that all the three showed a parallel increase in control and different
treated groups. Combined analysis of the three parameters reveals that up-regulation of COX-2 expression was the main reason behind the elevation of PGE\(_2\) production at ulcerated margins in all the groups as complete lack of COX-2 enzyme was revealed by RT-PCR and western blot analysis in the intact tissues along with extremely low levels of PGE\(_2\) (Figure 5.23). Similarly, as the expression profile of COX-2 mRNA and protein starts increasing from day 5 to 10 and then showed a slight fall on day 14\(^{th}\), similar pattern was also observable for PGE\(_2\) production (Figure 5.23). The only exception to this pattern of elevation was seen in CELE treated group that furnished another proof in favor of COX-2 being an important architecture behind ulcer healing process. As in CELE treated group, although COX-2 mRNA and protein expression was well defined but a noteworthy decline in their expression was observed in comparison to the controls. However, the PGE\(_2\) levels remains severely suppressed mainly due to the fact that CELE suppresses the COX-2 activity with marginal effect on its expression (Tanaka et al., 2002). The finding clearly establishes the role of COX-2 and PGE\(_2\) in repair, regeneration and healing process. These results are endorsed from some of the earlier reports suggesting that up-regulating COX-2 expression also increases PGE\(_2\) levels that promotes mucosal protection during ulcer healing (Tsuji et al., 2002; Shigeta et al., 1998).

Finally, an analogous and completely corresponding rapport was figured out between COX-2 expression, PGE\(_2\) levels and ulcer healing kinetics as shown in Figure 6.2. A sequential increase of the enzyme and its product from 5\(^{th}\) to 10\(^{th}\) day and then a slight decrease on 14\(^{th}\) day indicates that expression of COX-2 enzyme and PGE\(_2\) production runs in parallel with the curve of ulcer healing dynamics. Both of these factors gets stimulated with the mucosal damage, although a significantly low expression was found on 5\(^{th}\) day, suggesting that during early lag phase, when main focus remains on diminution of the offensive factors (Tarnawski et al., 1991), COX-2 and PGE\(_2\) levels also remains poorly categorized, however, if a drug persuade the COX-2 expression as early as in the early lag phase, decreases the time required for the lesion getting completely healed as evident in OMZ treated group which has exhibited maximum healing on 14\(^{th}\) day (84.09%, Table 5.1) and COX-2 mRNA (Table 5.9) and protein (Table 5.10) expression was even evident on 5\(^{th}\) day.
Figure 6.2: Comparative effect of increase in COX-2 mRNA and protein expression and PGE₂ levels on ulcer healing
Discussion

Both the COX-2 expression as well as PGE\textsubscript{2} level reached its crest on 10\textsuperscript{th} day i.e during the *phase of rapid healing* when regenerated epithelial cells begins to re-epithelialize the ulcer crater and intensive epithelial cell proliferation was visible in the ulcer margin. COX-2 via PGE\textsubscript{2} triggers the regeration process as PGE\textsubscript{2} has been reported to accelerate cell proliferation and angiogenesis and elevates mucosal blood flow (Isenberg et al., 1985; Scheiman, 1996; Atay et al., 2000). Finally, as the normal mucosal architectures re-establish and tissue remodeling gets on its way during *late log phase* (Bennet and Schultz, 1993), both COX-2 and PGE\textsubscript{2} flaunt a trivial down regulation on 14\textsuperscript{th} day in comparison to the 10\textsuperscript{th} day expression profile in all the groups except CELE treated rats. This indicates that COX-2 and therefore PGE\textsubscript{2} are active participants in the ulcer healing process mainly during *phase of rapid healing*, where they performs number of function including stimulation of growth factors (Liu et al., 2000). These growth factors actually take over from PGE\textsubscript{2} in *late log phase* where the focus shifts from regeneration to re-modeling. This is supported by both a slightly decreased COX-2 expression and a significant elevation in all the growth factors (VEGF, bFGF and TGF) on 14\textsuperscript{th} day.

Overall, the results of COX-2 expression analysis both for mRNA and protein and estimation of PGE\textsubscript{2} level on three different days corroborate the robust role assayed by COX-2 and its product PGE\textsubscript{2} in the ulcer healing process that apart from stimulating other defensive factors like mucus secretion or growth factor production, gets directly involved in the process by assisting in various mechanism like inhibition of acid secretion or elevation of mucosal blood flow etc. The study also proposes the possible chronology of action of COX-2 and PGE\textsubscript{2} during different stages of ulcer healing kinetics. Furthermore, comparison of the COX-2 expression profile in normal and drug mediated ulcer-healing processes clearly figured out that COX-2 hold the key for better defensive mechanism and a drug that effect its expression profile maximum, also manifest maximum ulcer healing.

Conclusively, the leading finding emerged from the assessment and interpretation of COX-2 expression profile and PGE\textsubscript{2} levels in normal and different treated groups revealed that the most competent and potent ulcer healing drug is the one that up regulate the expression profile of COX-2 enzyme most efficiently, apart
from its known acting mechanism, which eventually lead to enhanced and rapid repair process. It was also elucidate that factors of the defensive system bequeath a pivotal role in the ulcer healing and repairing mechanism The study along with ascertaining the role of COX-2 enzyme in the process of both natural and drug mediated ulcer healing, will also be helpful in providing crucial information for the development of superior ulcer therapeutic modalities where an ulcer-healing drug should be a capable of diminishing 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.