SUMMARY

GERD is a common and chronic disorder which requires long-term, even lifelong, therapy. GERD occurs when there is an abnormally prolonged contact time between the esophageal mucosa and refluxate, which is believed to be primarily gastric acid. The resultant reflux of acid and other materials from the stomach may induce pain or damage the esophageal mucosa. This damage to the esophageal mucosa may lead to esophagitis which is characterized by inflammation of the esophageal mucosa, bleeding, cytological changes, peptic esophageal stricture, esophageal ulcer and Barrett's metaplasia, depending on the severity of the disease.

The present mainstay therapy for GERD is based on gastric acid-suppression, and healing of injured oesophageal mucosa to prevent further complications. Although these therapies are effective in producing acute symptom relief and mucosal healing by administration of H$_2$RAs or PPIs, there is still a need of alternative treatment strategies because of their limitations such as prolonged administration, failure to provide complete symptom relief, various side effects, drug interactions and frequent relapses following drug withdrawal. The other limitations of current therapy are associated to its inability to address other important pathogenic factors of GERD like oxidative stress or inflammation.

Apparently GERD is a disorder having multifactorial aetiology rather than a single cause. Besides gastric acids, the other pathological factor contributing significantly to aetiology of GERD, in both experimental models and human subjects, are the ROS. Administration of various free radical scavengers have been shown to prevent esophageal mucosal damage signifying a role of oxidative stress in the pathogenesis of RE. Moreover, alterations in specific cytokine and chemokine profiles leading to inflammatory responses are also associated with the development and progression of RE. Thus, therapeutic agents which possess the properties to scavenge toxic free radicals and modulate inflammatory response might have a significant effect in ameliorating tissue destruction induced by RE.

One important finding in the search for alternative approaches for GERD treatment is the discovery of protective influences of melatonin in GERD patients. Melatonin which has been primarily linked to the pineal gland, also been noticed to present widely in GIT. The concentration of melatonin in the gastrointestinal tissues surpasses blood levels by 10–
100 times and there is at least 400 times more melatonin in the GIT than in the pineal gland. The declined levels of circulating melatonin are also noticed to be associated with esophageal erosions in GERD, signifying its role in the protection of esophageal integrity. The above observations along with demonstration of the clinical applicability of melatonin in the treatment of GERD patient and its possible role in the maintenance of esophageal integrity intrigues us to define in great details the actual role of melatonin in this esophageal disorder using various biochemicals, molecular and functional methods, on which our present study was designed.

Prior to evaluation of any future therapeutics for GERD, it is essential to device a tool which can largely replicate the pathological changes of human esophagitis. Thus, we start our study with the standardization of acute reflux esophagitis models in rats for which we adopted the simultaneous ligation method proposed by Nakamura. The reflux esophagitis was induced in rats by concurrently ligating both the pyloric end and the fore stomach. The animals were sacrificed at different time hours (2.5, 5 and 10) and their oesophagi were examined for the developed area of lesions under stereo zoom microscope. We observed that the area of lesions in the esophagus of RE-operated rats increased with increasing time. At the 2.5 postoperative h, almost negligible sign of haemorrhagic lesions were observed. Increasing the time of ligation to 5 h produced haemorrhagic lesions at more homogeneity. Subsequently increasing the time to 10 h resulted in poor rate of survival due to which 5h time was realized as the most effective period for the induction of RE in rats. Further experiments were carried out to inspects the validity of our RE model by examining the drug responsiveness. We validated our model using classical proton pump inhibitor omeprazole, as the reference drug. Omeprazole dose dependently reduced the area of lesions, elevated the pH and concurrently reduced the total volume and acidity of gastric juice in RE-operated rats. Omeprazole act to inhibit the luminal gastric secretions and thereby offered protection in this RE-model suggesting and validating the appropriateness of this model in studying the potential therapeutic agents.

In the light of latest investigations revealing the importance of both oxidative stress and inflammation in the symptoms of GERD in humans, we next evaluated whether our RE-model can reproduce these changes. Our data showed that esophageal injury in RE rats significantly increased the mucosal oxidative load characterized by high lipid peroxidation
and is accompanied by a clear pattern of antioxidant activity signified by a profound decrease in SOD activity, GSH content and GPx activity. In addition we established increased mucosal inflammation following RE infliction in rats reflected via greatly increased MPO activity, gene expression of pro-inflammatory cytokines like TNF-α, IL-1β and IL-6 and anti-inflammatory cytokine, IL-10. Similar to other inflammatory diseases, COX-2 and its byproduct PGE₂ was also found to be significantly upregulated in our study.

Further we observed that administration of melatonin caused dose dependent amelioration of the area of lesion in the esophageal mucosa of RE-operated rats. Our pilot investigation with graded doses of melatonin revealed 20mg/kg, i.p. dose of melatonin to be most effective and therefore used throughout. In our study SOD and GPx enzyme activities were also noticed to decline markedly following RE-establishment in rats. Melatonin apparently augments the activity of SOD and GPx helping esophageal mucosa to regain back the normal redox state. Treatment with melatonin also replenished the depleted levels of GSH in RE-operated rats. Our results thus signified that apart from melatonin’s ability to act as free radical scavenger, it considerably improves the esophageal defences against free radical attack by facilitating anti-oxidant enzyme activities required to metabolize these radicals and their products to innocuous agents. In addition our data revealed that melatonin significantly repressed RE-induce increase in TNF-α, IL-1β and IL-6 expressions but failed to attenuate the expression of IL-10 in RE-rats. These results inferred that melatonin exerts it immunosuppressive effects mainly by inhibiting the TH-1 specific cytokines expression. The increase expression of IL-10 can be perceived as an adaptive response by Treg cells to overcome the imbalance between TH1/TH2 subsets which rapidly occur following RE elicitation. However melatonin apparently maintains this imbalance by suppressing the TH-1 representative cytokines, independently of IL-10.

In our further studies we detected that COX-2 proteins were overly-expressed in the esophageal mucosa of RE-rats. This increased COX-2 levels was also noted to accompany high PGE₂ content and MPO activity in RE-rats. Interestingly, inhibition of COX-2 by celecoxib reversed this RE-induced increase in both PGE₂ level and MPO activity, suggesting its proinflammatory nature. To support, our postulation that COX-2 is the proinflammatory mediator during RE, we examined the role of PGE₂ analogue dmPGE₂ over RE-induced injury and MPO activity. It was noted that dmPGE₂ intake in RE-rats amplified the MPO
activity and tissue damage compared to untreated RE-rats which complements our interpretations that COX-2 activation resulting in increase PGE₂ production, in fact, augments esophageal inflammation and adds to tissue injury. Further, estimation of PGE₂ levels after dmPGE₂ systemic administration in RE-group revealed that dmPGE₂ actually increased its own synthesizing inducible COX-2 expression and thereby PGE₂ level in oesophagus signifying that COX-2 activation may underlie the mechanism via which it aggravated tissue damage in RE-group. As esophagitis mucosa has compromised vascular permeability, it might be possible that dmPGE₂ administration may increase the vascular permeability and thereby caused more migration of inflammatory cells to the damaged area which adds to injury. Our results with dmPGE₂ allowed the assumption that endogenous PGE₂ are not the mediator of esophageal mucosal protection, in fact, they may be detrimental.

Although the mechanism of COX-2 induced increase in MPO activity is unclear from current results, however the relationship can be explained considering the fact that overproduction of PGE₂ in RE, increase the peptic activity in refluxed material which in turn cause damage and further, since injured epithelia have high propensities for inflammatory cells, it may also result into increased MPO activity. With our study with melatonin we observed that melatonin profoundly repressed the overexpression of COX-2 level during RE that coincides with the decreased PGE₂ level, MPO activity and tissue injury. Since COX-2 appeared to be responsible for the induced PGE₂ production during RE, it was likely that the repression of COX-2 induction via melatonin apparently reduces the inflammatory PGE₂ synthesis and thus dramatically reduce the MPO activity. Meanwhile, melatonin failed to reproduce its protective effect in RE-rats when administered along with dmPGE₂. The possible rationale might be the inadequacy of selected dose of melatonin to buffer entirely the augmented PGE₂ level resulting from additional supplementation of its synthetic analogue during RE. To further address this issue we examined the effect of melatonin at higher dose of 40mg/kg on COX-2 expression and PGE₂ levels after dmPGE₂ intake in RE-rats. We found that melatonin used at this dose significantly normalized the dmPGE₂ augmented COX-2 expression and PGE₂ level in RE rats. Also, melatonin at this higher dose significantly protect against damage caused by systemic administration of dmPGE₂ in RE rats. Thus our results suggested that effect of melatonin on COX-2 expression and PGE₂ is dose related and their inhibition underneath the protective functions of melatonin against RE
induced damage. Moreover our results also demonstrated that suppression of acid secretions via proton pump inhibition was not adequate to bring effective reversal of RE-induced COX-2 expression and a consequent PGE2 level. In contrast, melatonin more efficiently suppressed COX-2 expression and protein, bringing down its level to normal control values. These data suggested that while suppression of acid can reverse the esophageal inflammation to some extent complete reversal may not be achieved, which may underlie the failure of proton pump inhibitors to offer complete symptomatic relief in GERD patients.

Up to this point we established that melatonin’s anti-oxidant abilities and its suppressive effects over pro-inflammatory molecules specially the TH-1 subset dominant cytokines and COX-2 driven PGE2 served for its protective influences in RE. Several effects of melatonin in mammalian tissues are demonstrated to be mediated via two specific high-affinity G-protein coupled cell surface receptors, termed MT-R and MT-2. Our interesting findings with melatonin pre-treatment in RE-rats prompt us to examine the possible role of its putative receptors in these protective functions. Both MT-1 and MT-2 receptors were detected in rat esophagus. Further, MT-2 protein showed distinctly higher levels after RE-induction implying it as a major melatonin receptor involved in pathogenesis of RE. Interestingly melatonin treatment was recorded to produce significant amplification of MT-2 and not MT-1 expression in RE-rats indicating that enhanced MT-2 levels may aid in the protective process via anti-oxidative and anti-inflammatory actions of this indole. Further high MT-2 level after melatonin supplementation appeared as an adaptation to cope with the increased melatonin contents following its administration. In our study with MT-2 antagonist luzindole, we noticed that it aggravated the haemorrhagic lesions induced by RE, however not at the statistical significant level. Further luzindole was not able to block the melatonin offered protection against RE-injury, offering an interpretation that exogenous melatonin might mediate its action against RE-induced injury in a receptor-independent approach. Nevertheless, the role of MT-2 receptors in RE cannot be completely ruled out as we are getting significant changes in their level. So we move further with our experiments and decided to monitor the changes in the endogenous level of melatonin relative to their receptor expression.

Significant decline in the local levels of melatonin was detected in RE-rats from our HPLC data. Melatonin exogenous administration notably increased its endogenous levels, an
observation reported by different individual studies. One interesting observation with our study was that, blocking of MT-2 receptor drastically augmented the tissue melatonin levels. Further melatonin administration after luzindole treatment also brings significant increase in melatonin level in RE-rats. Our these findings contradicts our hypothesis that elevation of endogenous melatonin leads to protection against RE, as luzindole aggravated the esophageal injury even though it increased the melatonin level in RE-rats. To clarify this issue we examine the changes in the expression level of melatonin synthesizing enzymes, AA-NAT and HIOMT to find out whether antagonist treatment actually increases the melatonin biosynthesis or the increased melatonin levels merely reflects their accumulated amount, freed from its receptor due to possible reduction in their receptor affinities on luzindole treatment.

Establishment of RE caused significant decline in the gene expression of both AA-NAT and HIOMT enzymes. The possible explanation of suppressed melatonin level in our study might be related to the insufficient cholinergic stimulus of inflamed mucosa which may cause proteosomal degradation of AA-NAT and as a consequent depressed melatonin synthesis. Further we noticed that melatonin or luzindole administration did not significantly enhance the AA-NAT or HIOMT expression in RE-group. This result reflected that increased melatonin levels particularly after melatonin administration was not because of its increased synthesis but due to its passive accumulation. Similarly, increased melatonin level after luzindole treatment rather reflects the accumulated melatonin molecules which were freed from their receptors due reduced affinity for ligands after antagonist treatment. Thus it seemed that activation of these receptors by binding to melatonin molecules is essential for melatonin to exert its beneficial effect against RE-induced injury.

However an unclear issue in our study is the finding of increased melatonin level and subsequent protection against RE-injury in rats receiving both melatonin and luzindole treatments. Luzindole had been demonstrated to compete for the same receptor sites as melatonin, thus increasing the concentration of its ligand can effectively reverse its antagonistic effect on receptors. We think this to be the possible explanation for getting protection in both melatonin and luzindole treated rats compared to luzindole alone treated rats, as increased melatonin levels following its exogenous administration amplified the competition for luzindole for receptor sites, making them more approachable to melatonin
molecules and thus initiating signalling events which served for melatonin mediated protective functions. We also observed significantly decreased expression of both MT-1 and MT-2 after luzindole treatment. MT-2 protein was recorded to show more significant repression compared to MT-1 confirming the specificity of luzindole to MT-2 receptors. The decrease receptors density after luzindole treatment might be due to the interference of luzindole with receptor synthesis, causing reduced gene transcription or post-transcriptional events like mRNA destabilization. The finding of increased RE-induced injury following luzindole treatment can also be explained considering our finding with low receptor expression on its treatment which may be responsible for the decline in receptor messages and due to which the injury aggravated. Melatonin and luzindole both simultaneous treatment revealed high MT-2 expression and levels in RE-rats further suggesting that melatonin through its receptors activation can initiate signals which render the esophageal mucosa resistant to injury.

To date, there have been no reports on the functional consequences of melatonin receptor activation in isolated esophageal cells invitro. Thus we next switch our study from invivo to invitro and carried out few experiments on isolated esophageal cells in order to find out the possible intracellular signalling mediated through MT-2. We observed that the isolated cells expressed both MT-1 and MT-2. Melatonin significantly increased $[\text{Ca}^{2+}]_i$ level over basal value. Meanwhile luzindole significantly antagonized the melatonin elicited increase in $[\text{Ca}^{2+}]_i$ level reflecting that melatonin increases $[\text{Ca}^{2+}]_i$ levels receptor dependently. Further we found that melatonin could not bring increase in $[\text{Ca}^{2+}]_i$ level in presence of BAPTA-AM however it significantly increases the $[\text{Ca}^{2+}]_i$ in Ca$^{2+}$ free medium containing EGTA which chelates the extracellular Ca$^{2+}$ suggesting that through its receptor activation melatonin mobilizes Ca$^{2+}$ ions release from the intracellular stores. Further quantification of cAMP level revealed that melatonin significantly lowered the cAMP levels following stimulation via forskolin. The inhibition of forskolin elicited cAMP level with melatonin was completely abolished by luzindole suggesting that melatonin mediated inhibition of forskolin stimulated cAMP level is receptor dependent. Further invitro investigations with melatonin revealed that melatonin reduced the intracellular ROS level in a receptor independent manner but its effect on intracellular stores of GSH and GPx activity is receptor dependent.
Thus our studies hitherto showed that RE-induction in rats created a significant deficit in melatonin production which can be improved by melatonin exogenous supplementation. Melatonin administration caused significant increase in its own esophageal levels independently of its synthesis and required increased receptor density to communicate the messages that may culminate into activation of unknown signal transduction pathways which make esophageal mucosa resistant to gastric acid induced injury. MT-2 seemed to play a key role in these physiological functions of melatonin as blocking its activation by luzindole not only reduced the affinity of these receptors for melatonin they also appear to delay the messages conveyed by melatonin that were translated into its protective functions against RE. Melatonin treatment with luzindole appeared to overcome these shortcomings possibly by reversing the changes in the receptor density and its functional responses produced via luzindole treatment. The physiological consequences of MT-2 receptor activation appeared to be associated with increased \([\text{Ca}^{2+}]\) release, inhibition of cAMP activation, increased antioxidative enzyme GPx activity and intracellular GSH level serving the protective functions of melatonin against RE.

In view of our finding of declined melatonin level following RE-induction in rats we next examined whether L-tryptophan supplementation can be used to improve the melatonin local deficiency in RE-rats. Thus, the next study was performed to understand the intricate relationship between L-tryptophan and melatonin system in the protection of esophageal integrity and to elucidate their mode of function by deciphering their synergism or independent action. Our hypothesis that L-tryptophan treatment would improve the loss of esophageal melatonin level accompanying RE-induction failed, as various doses of L-tryptophan were not being able to overcome this deficit. Paradoxically, increasing the doses of L-tryptophan in RE-rats resulted into significant enhancement of its own endogenous levels which were not seen in L-tryptophan perse group. The possible explanation for these discrepancies could be that during normal condition L-tryptophan that reaches the GIT may get metabolized into the melatonin, and because of the large size of GIT it may get consumed rapidly to convert into the melatonin. While during esophagitis since L-tryptophan may not enter the pathway to produce melatonin, it could be less efficiently used in GIT, consequently its concentration may build up with the increasing doses. Further it was noted that L-tryptophan administration in RE rats significantly upregulated the expression of AA-NAT enzyme however it was not able to counteract the RE-repressed level of HIOMT enzymes.
Thus possibly the insufficient cholinergic stimulus due to the esophagus injury may cause proteosomal degradation of AA-NAT, and despite of the L-tryptophan mediated increase in its expression we observed decreased melatonin synthesis in RE-rats. Altered melatonin synthesis in RE model can also be attributed to the decline in the expression of HIOMT enzyme, which L-tryptophan failed to reverse.

Apart from these results, our observation of L-tryptophan *per se* effect showed that in normal control rats the melatonin level improved with increasing L-tryptophan doses reflecting that it possibly participates in the mechanism of esophageal melatonin synthesis. These results are in agreement to a study showing the role of L-tryptophan in GIT synthesis of melatonin. However in RE this pathway may get hampered due to the injured mucosa, which can be associated with reduced cholinoreceptor signals during esophageal inflammation which regulate the activity of melatonin synthesizing enzyme. Moreover the intake of L-tryptophan in normal control rats did not change the normal expression of AA-NAT and HIOMT gene, showed that esophagus have the ability to produce melatonin under normal condition, this notion is in agreement with other studies also.

Our direct examination of melatonin level following L-tryptophan administration also reflected that the ameliorative effects of L-tryptophan on esophageal damage ensuing RE-induction may involve other complex mechanism and is not dependent on its conversion into melatonin. To further confirm that protective action of L-tryptophan against RE is independent of melatonin, we blocked melatonin actions using its antagonist luzindole. L-tryptophan pretreatment protected the esophagus from damage even in the presence of Luzindole further supporting our hypothesis that L-tryptophan mediated protection in RE is independent of melatonin.

In an attempt to find the possible mechanism of L-tryptophan exerted protection against RE, we noted that it offered great reduction in the total gastric output with corresponding decline in total acidity. L-tryptophan also greatly elevated the pH and mucin content of accumulated gastric juice. Our results thus inferred that L-tryptophan might protect against esophageal damage via regulating the gastric secretions and follow the mechanism that controlled the flow of gastric juice into lumen and potentiated the mucin secretions. In addition, we observed that L-tryptophan failed to inhibit the $\text{H}^+\text{K}^+$-ATPase activity under in-vitro condition compared to positive control omeprazole. However, under in-vivo
experiments, the acid suppressing effect of L-tryptophan was essentially similar to the omeprazole. The differential response of L-tryptophan on acid secretion under in-vivo and in-vitro circumstances signified that L-tryptophan did not directly influence the H^+K^+ATPase activity like omeprazole. However, unlike proton pump inhibitors, its antisecretory functions seemed to be more dependent on the actions of gastric acid secretagogue.

We also found that L-tryptophan treatment markedly augmented the gastric PGE_2 content which agrees with previous studies. The participation of PGE_2 in the anti-secretory functions in our model was confirmed by blocking it via indomethacin. Indomethacin not only lowered the gastric volume and acidity, it also lowered the pH and mucin content of gastric juice in the RE rats. However, L-tryptophan failed to overcome the changes produced by indomethacin in RE-group. This result suggested that L-tryptophan mediated augmentation of gastric PGE_2 might be the possible explanation for its antisecretory functions. In order to examine the source of the increased PGE_2 following L-tryptophan treatment we examined the changes in the activities COX 1 and 2. It was observed that L-tryptophan significantly increased the RE-lowered activity of COX-2 enzyme. However, it showed no effect on COX-1 activity. Further L-tryptophan was unable to increase the COX-2 activity when blocked by indomethacin co-administration. These results clearly showed that the increased PGE_2 obtained after L-tryptophan treatment in RE-rats was COX-2 derived. Hence the L-tryptophan imparted protection against RE seemed to be resultant of its attenuating effect on gastric acid secretions and subsequent mobilization of mucosal defensive factors such as mucin and COX-2 derived PGE_2 that counteracted the potential detrimental effects of gastric acid.

Thus from our L-tryptophan study we established the onset of RE caused impairment of melatonin synthesizing pathway via repressing the expression of rate limiting enzymes involve in its synthesis. In addition, we showed that L-tryptophan prevented the onset of RE via a mechanism that is independent of its conversion into melatonin. L-tryptophan apparently ameliorates RE-induced esophageal damage via inhibiting the gastric acid secretion as well as activating the factors such as gastric mucin and COX-2 derived PGE_2 that counterbalance its detrimental effects on esophageal mucosa.

In conclusion our study establishes that RE-onset in rats laden the mucosa with oxidative and inflammatory loads. Meanwhile melatonin exogenous supplementation help
improve the mucosal resistance by lowering the oxidative load of injured esophagus and simultaneously suppressing the inflammatory molecules viz cytokines, COX-2 and PGE_2. Melatonin appear to mediate its protective functions by enhancing its endogenous level which by binding to its MT-2 receptors communicate messages to cells aiding to maintain cellular redox status by augmenting the depleted level of intracellular anti-oxidant molecule GSH and enzyme, GPx activity. The MT-2 is believed to maintain the cellular redox status by unknown pathways induced by increase in [Ca^{2+}]_i and cAMP inhibition. Besides melatonin its precursor L-tryptophan was also found to exert protection against RE, but through a mechanism which is independent of its conversion into melatonin. L-tryptophan showed to protect against RE-induced injury via inhibiting the gastric acid secretion and simultaneous mobilization of stomach PGE_2. PGE_2 increases the luminal release of mucin thereby protecting the esophagus from harmful effects of acid and other stomach materials.

Convincingly, the findings reported herein lend support to the concept of the clinical utility of melatonin against this esophageal pathology. Thus melatonin can serve as an alternate and/or in combination with the anti-secretory regimen to ameliorate the severity of RE more effectively and may emanate as a potential drug against reflux esophagitis.