CHAPTER IV

ROLE OF MELATONIN PRECURSOR, L-TRYPTOPHAN ON REFLUX ESOPHAGITIS
8. CHAPTER IV: RESULTS AND DISCUSSION

In view of the earlier reports regarding the role of melatonin precursor L-tryptophan on circulating melatonin level and our own finding of the occurrence of melatonin synthesizing enzymes in esophagus, we hypothesized that L-tryptophan supplementation can be used to improve the melatonin local deficiency in RE-rats. Thus, the 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. In the current study we also focused an important issue that the effect of L-tryptophan on the melatonin levels in the esophagus is subjected to change under pathological conditions such as RE when the esophagus mucosal tissue become injured.

8.1 Effect of L-tryptophan on RE-induced esophageal damage

In order to assess the effect of L-tryptophan on RE model, we performed pilot studies using graded doses of L-tryptophan (100-400mg/kg, p.o.). As represented in Figure 8.1, RE-control rats exhibited formation of macroscopically evident lesions (107.0±11.2 sq. mm) along the entire esophagus during the 5h duration. Pre-treatment with L-tryptophan (100, 200 and 400mg/kg), dose dependently reduced the area of lesions (80.8±4.31, 52.5±4.15 and 42.82±4.04 sq. mm. respectively). L-tryptophan significantly (P<0.001) reduced the area of lesions at 200 and 400mg/kg when compared to RE-control group. Omeprazole also significantly (P<0.001) protected the esophagus damage (39.6±3.3 sq. mm) as compared to RE-control group. From the above observations, it become apparent that 200mg/kg and 400mg/kg doses of L-tryptophan produced the most significant effect over the RE-induced haemorrhagic lesion, based on which we selected 200mg/kg dose as the lowest effective one for further studies.

Administration of Indomethacin caused a significant (P<0.05) increase of RE-induced oesophageal injury (162.6±17.37 sq. mm.). L-tryptophan (200mg/kg) pre-treatment produced no significant alteration in the indomethacin exacerbated esophageal damage in RE-rats (131.5±15.69 sq. mm.).
As shown in Figure 8.2, LUZ+RE group exhibited a non significant increase (133.0±23.84 sq. mm) in the area of haemorrhagic lesions compared to RE-control group (107.0±11.2 sq. mm). Administration of L-tryptophan dose dependently reduced the area of lesion (98.8±7.41, 63.08±5.98 and 52.9±4.8 sq. mm. respectively). It significantly reduced the area of lesions at 200mg/kg (P<0.01) and 400 mg/kg (P<0.01) doses compared to LUZ+RE group.
8.2 Effect of L-tryptophan loading on the esophageal melatonin and L-tryptophan level

Estimation of esophageal melatonin revealed that induction of RE in rats significantly (P<0.001) lowered the melatonin content of esophagus in comparison to normal control group, as shown in Figure 8.3A. L-tryptophan failed to reverse this RE-induced deficit of melatonin (Figure 2A) while its own endogenous concentration increased significantly in a dose-dependent approach (Figure 8.3B). In L-200+RE and L-400+RE groups, the esophageal L-tryptophan level increased significantly (P<0.05 and P<0.001 respectively) in comparison to RE control group, however at 100mg/kg the increase was insignificant. Contrastingly, L-200 and L-400 perse group showed significantly (P<0.01 and P<0.001 respectively) elevated
levels of esophageal melatonin compared to normal control group (Figure 8.4A) while exhibit no alterations in esophageal L-tryptophan contents over normal control values (Figure 8.4B respectively).

Figure 8.3: A) Alterations in the esophageal melatonin levels in Normal Control, RE Control, and L-tryptophan pretreated RE rats (L-100+RE, L-200+RE and L-400+RE). Results were represented as means±SEM, with six rats (n=6) in each group. ***P<0.001. B) Changes in the esophageal L-tryptophan content in normal control rats and L-tryptophan pretreated RE rats (L-100+RE, L-200+RE and L-400+RE). Results were represented in means±SEM, with six rats (n=6) in each group. *P<0.05 and ***P<0.001 respectively.

Figure 8.4: A) Alterations in the esophageal melatonin levels in Normal Control and L-tryptophan pretreated normal rats (L-100 persec, L-200 persec and L-400 persec respectively). Results were represented as means±SEM, with six rats (n=6) in each group. **P<0.01 and ***P<0.001 respectively. B) Changes in the esophageal L-tryptophan content in Normal Control rats and L-tryptophan pretreated normal rats (L-100 persec, L-200 persec and L-400 persec respectively). Results were represented in means±SEM, with six rats (n=6) in each group.
8.3 Effect of L-tryptophan on gene expression of AA-NAT and HIOMT

As represented in Figure 8.5, infliction of RE significantly (P<0.05 and P<0.001 respectively) repressed the gene expression of both melatonin synthesizing enzymes, AA-NAT and HIOMT in comparison to normal control rats. L-100-400+RE group exhibited significantly (P<0.05) increased expression of AA-NAT compared to RE-rats. Further, L-100-400+RE groups showed no significant reversal of RE-suppressed expression of HIOMT gene. Further as represented in Figure 8.6, L-100-400 *perse group exhibited insignificant alteration in the expression of both AA-NAT and HIOMT genes compared to normal control group.

Figure 8.5: Histogram representing the changes in the gene expression of AA-NAT and HIOMT in Normal Control rats, RE Control group and RE group pretreated with L-tryptophan at graded doses (L-100+RE, L-200+RE and L-400+RE respectively). Results were expressed as means±SEM with three rats (n=3) in each group. The gel image provided were representative of experiments performed in triplicates. *P<0.05 and ***P<0.001 respectively.
8.4 Effect of L-tryptophan on gastric secretions

As summarized in Table 8.1, the gastric secretion study showed that L-tryptophan pre-treatment significantly reduced the accumulated volume of gastric juice and total acidity in a dose dependent manner with respect to RE-control group. L-200+RE and L-400+RE group showed significantly lowered gastric volume (P<0.001) and acidity (P<0.05) compared to RE-group, whereas L-100+RE group failed to produce significant suppression of RE-induced increase in gastric volume and acidity. Omz+RE group displayed the significant reduction in the gastric juice volume (P<0.001) and total acidity (P<0.05) over RE-group. Also, gastric juice pH increased significantly in the L-200+RE and L-400+RE group (P<0.01).
and P<0.001 respectively) compared to RE-control group. Moreover, L-200+RE and L-400+RE group showed significantly (P<0.05 and P<0.001 respectively) elevated the mucin level compared to RE-control rats.

<table>
<thead>
<tr>
<th>Group</th>
<th>Gastric volume (mL)</th>
<th>Total acidity (μEq/ml)</th>
<th>pH</th>
<th>Mucin content (μg/ml of gastric juice)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RE Control</td>
<td>4.41±0.50</td>
<td>157.20±15.85</td>
<td>2.14±0.23</td>
<td>548.10±58.87</td>
</tr>
<tr>
<td>L-100+RE</td>
<td>3.76±0.45</td>
<td>133.30±29.94</td>
<td>1.91±0.27</td>
<td>656.00±75.14</td>
</tr>
<tr>
<td>L-200+RE</td>
<td>2.12±0.38***</td>
<td>73.67±5.20*</td>
<td>3.37±0.26**</td>
<td>894.6±77.66*</td>
</tr>
<tr>
<td>L-400+RE</td>
<td>1.27±0.18***</td>
<td>58.79±5.46*</td>
<td>4.53±0.42***</td>
<td>1455.00±135.40***</td>
</tr>
<tr>
<td>Omz+RE</td>
<td>0.80±0.12***</td>
<td>54.78±6.09*</td>
<td>5.20±0.31***</td>
<td>919.60±83.65*</td>
</tr>
</tbody>
</table>

Table 8.1: Alterations in the total gastric volume, acidity, pH and mucin contents in RE Control, RE groups pretreated with L-tryptophan at the doses of 100-400mg/kg (L-100+RE, L-200+RE, L-400+RE respectively) and omeprazole (Omz+RE) at the dose of (10mg/kg). Results were expressed as mean±SEM with n=6 rats in each group.*P<0.05, **P<0.01 and ***P<0.001, in comparison to control.

Further, as shown in Table 8.2, IND+RE group exhibited significantly reduced gastric juice volume (P<0.05), acidity (P<0.01), pH (P<0.05) and mucin (P<0.05) content over RE-control values. These parameters did not change significantly in L-200+IND+RE group compared to IND+RE group.

<table>
<thead>
<tr>
<th>Group</th>
<th>Gastric volume (mL)</th>
<th>Total acidity (μEq/ml)</th>
<th>pH</th>
<th>Mucin content (μg/ml of gastric juice)</th>
<th>Gastric PGE2 level (pg/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal Control</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>4487±662.30</td>
</tr>
<tr>
<td>RE Control</td>
<td>4.41±0.50</td>
<td>157.20±15.85</td>
<td>2.14±0.23</td>
<td>548.10±58.87</td>
<td>2569±459.40^</td>
</tr>
<tr>
<td>L-200+RE</td>
<td>2.12±0.38***</td>
<td>73.67±5.20*</td>
<td>3.37±0.26**</td>
<td>894.6±77.66*</td>
<td>9981±764.00***</td>
</tr>
<tr>
<td>IND+RE</td>
<td>5.93±0.35#</td>
<td>260.60±42.13##</td>
<td>1.00±0.07#</td>
<td>253.00±31.92#</td>
<td>1229±194.90#</td>
</tr>
<tr>
<td>IND+L-200+RE</td>
<td>4.96±0.42</td>
<td>238.90±28.56</td>
<td>1.84±0.35</td>
<td>428.5±93.52</td>
<td>1715±286.50</td>
</tr>
</tbody>
</table>

Table 8.2: Changes in the total gastric volume, acidity, pH, mucin contents and PGE2 level in RE Control, RE group pretreated with L-tryptophan (L-200+RE), indomethacine (IND+RE) and indomethacin+L-200 simultaneously (IND+L-200+RE). Results were expressed as mean±SEM with n=6 rats in each group.*P<0.05, **P<0.01 and ***P<0.001, in comparison to RE control. ^P<0.05, with respect to normal control group. #, ##P<0.05 and 0.01 respectively, in comparison to RE Control group.
8.5 Effect of L-tryptophan on H+K+ATPase activity

The effect of L-tryptophan on the \textit{in-vitro} H\(^+\) K\(^+\)-ATPase activity was examined. As shown in Figure 8.7, L-tryptophan in the dose range (10–100 \(\mu\)g/ml) failed to inhibit the gastric H\(^+\) K\(^+\)-ATPase activity compared to control values. In contrast, omeprazole (10–50 \(\mu\)g/ml), used as a positive control, significantly reduced the H\(^+\) K\(^+\)-ATPase activity with an IC\(_{50}\) value of 30.24 \(\mu\)g/ml.

![Figure 8.7: Effect of L-tryptophan and standard drug omeprazole on H+K+ATPase activity in the rat gastric microsomes. Dots and lines are mean ± S.E.M. of experiments performed in triplicates.](image)

8.6 Effect of L-tryptophan on gastric PGE\(_2\) level

As represented in Table 8.2, significant reduction in the gastric PGE\(_2\) (P<0.05) level was observed in RE-group compared to normal control rats. L-200+RE group exhibited significantly (P<0.001) elevated gastric PGE\(_2\) level compared to RE-control rats. IND+RE group showed significantly (P<0.05) reduced gastric PGE\(_2\) level compared to RE-control groups. The gastric PGE\(_2\) level did not change significantly in L-200+IND+RE group compared to IND+RE group.

8.7 Effect of L-tryptophan on gastric cyclooxygenase (COX) 1 and 2 activities

As shown in Figure 8.8, significant reduction in the COX-2 activity (P<0.05) was observed in RE-group compared to normal control rats with no significant change in the
activity of COX-1 isoform in these groups. L-200+RE group showed significantly (P<0.01) elevated COX-2 activity compared to RE-control rats; however COX-1 activity remained unaltered. IND+RE significantly (P<0.05) reduced the activity of both COX-1 and COX-2 enzymes as compared to RE-control groups. However in L-200+IND+RE group there was no significant change in the activities of both COX-1 and COX-2 when compared with IND+RE group.

![Figure 8.8: Changes in the activities of cycloxygenase (COX) 1 and 2 in Normal Control group, RE Control, RE group pretreated with L-tryptophan (L-200+RE), indomethacine (IND+RE) and indomethacin+L-200 simultaneously (IND+L-200+RE). Results were represented as means±SEM, with six rats (n=6) in each group. *P<0.05 and **P<0.01.](image)

**DISCUSSION**

Discovery of GIT as the major extra-pineal source of melatonin has added new dimensions to the understanding of its diverse biological functions. GIT synthesized its own melatonin from precursor amino acid L-tryptophan, independent of pineal gland (Bubenik, 1980). Various studies shows that L-tryptophan administration can improve the circulating melatonin levels produced largely from GIT (Esteban et al., 2004; Hajak et al., 1991; Huether et al., 1992; Yaga et al., 1993). It is also shown that L-tryptophan supplementation in combination with melatonin and vitamins significantly reduced the GERD associated symptoms (Pereira Rde, 2006) in patients observed separately to have poor melatonin levels (Klupinska et al., 2006). However the study deciphering the exact role of L-tryptophan in
reflux esophagitis is largely unknown, we therefore aim to investigate the role of L-tryptophan in experimental RE and its contribution in the esophageal level of melatonin.

Present study supported the previous notion that establishment of RE caused a deficit in local production of melatonin (Klupinska et al., 2006). Our hypothesis that L-tryptophan treatment would improve this loss of esophageal melatonin level accompanying RE-induction failed, as various doses of L-tryptophan were not being able to overcome this deficit. L-tryptophan is a substrate of two important biosynthetic pathways, one leading to the synthesis of serotonin and melatonin (Wurtman et al., 1969) and other to the production of niacin (Ikeda et al., 1965). It is reported that chronic immune activation pushes L-tryptophan toward niacin production (Schrocksnadel et al., 2006). Furthermore, previous report demonstrates that esophagitis imposed esophageal mucosa to massive inflammatory and oxidative load (Yoshida, 2007). Thus here it can be hypothesized that L-tryptophan entering into GIT could be converted into niacin rather than melatonin, causing a decreased levels of melatonin during RE. 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.

Earlier immuno-histochemical studies revealed the presence of melatonin in practically all parts of rat GIT (Bubenik et al., 1977). Further detection of the melatonin synthesizing enzymes HIOMT and AANAT in GIT confirmed the occurrence of its synthesis rather than just passive accumulation (Quay et al., 1976a). In concur to this our result also showed the occurrence of both these enzymes in the esophagus of normal rat sustaining the possibility of melatonin synthesis in esophagus. Establishment of RE caused significant decline in the gene expression of both enzymes which further supported our interpretation that the process of esophageal melatonin synthesis may get blocked in RE-rats. L-tryptophan administration in RE rats though significantly upregulated the expression of AA-NAT enzyme, it was not being able to counteract the RE-repressed level of HIOMT enzymes. In
rats, biosynthesis of melatonin is positively regulated through sympathetic neurons projecting into the gland. The norepinephrine (NE) released from these nerve endings at night AA-NAT, resulting in increased melatonin levels (Klein et al., 1997; Reiter, 1991; Sugden, 1989). It is also shown that the proteolytic degradation of AA-NAT resulting from termination of NE-induced stimulation of cAMP is responsible for ending melatonin synthesis (Gastel et al., 1998). Esophagitis mucosa also showed depressed cholinoreceptor mediated responses (Harnett et al., 1999). 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. Further examination of enzyme activity is essential to test these hypotheses.

Apart from these results, our observation of L-tryptophan perse effect showed that in normal control rats the melatonin level enhanced manifold 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 (Huether et al., 1992). 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 (Schomerus et al., 2002). 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 (Esteban et al., 2004; Hajak et al., 1991; Huether et al., 1992).

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. The dose-related reduction of the volume of gastric juice indicates that this inhibition may not be due to a nonspecific effect such as a dilution effect resulting from a break in the mucosal barrier. 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. Since the scant secretion ensures the adequate time for the mucus or alkaline mucosa to act on it (Allen et al., 1993; Allen et al., 1980), the observed increase in pH after L-tryptophan might be due to neutralizing effect of mucin on accumulated gastric juice. In support of this Pavlov also stated that abundant secretion of the gastric gland have higher acidity then scant secretions due to the fact that rapid flowing of juice offer less opportunity to mucus or alkaline mucosa to neutralize it (Pavlov, 1910).

Several endogenous mediators are involved in the secretion of gastric acid, which initiate unique series of events that finally converge into activation of the enzyme $H^+ K^+$-ATPase. Thus, we next examined the effect of L-tryptophan on the $H^+K^+$-ATPase activity in gastric microsomal preparation. It was observed that L-tryptophan failed to inhibit the $H^+K^+$-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 (Larsson et al., 1983). However, unlike proton pump inhibitors, its antisecretory functions seemed to be more dependent on the actions of gastric acid secretagogue. The similar acid attenuating effect of L-tryptophan has been studied in stress induced gastric ulcer model (Brzozowski et al., 1997), where it offered gastro-protection via stimulating the function of acid secretagogue such as gastrin and PGE$_2$. In view of these literatures, we next examined the role of L-tryptophan on gastric PGE$_2$ concentration.

We found that L-tryptophan treatment markedly augmented the gastric PGE$_2$ content which agrees with previous studies (Brzozowski et al., 1997; Konturek et al., 2008). The
participation of PGE₂ in the anti-secretory functions in our model was confirmed by blocking it via indomethacin. Indomethacin not only reduced the gastric volume and acidity, it also lowered the pH and mucin content of gastric juice in the RE rats. Similar kind of association has also been noted in gastroduodenal mucosa treated with indomethacin (Sababi et al., 1995) suggesting the regulatory role played by PGE₂ in gastric secretions. However, L-tryptophan failed to overcome the changes produced by indomethacin in RE-group. This result suggested that L-tryptophan mediated augmentation of gastric PGE₂ might be the possible explanation for its antisecretory functions, a view shared by others (Ding et al., 1997; Johansson et al., 1980; Tani et al., 1997).

In order to examine the source of the increased PGE₂ following L-tryptophan treatment we examined the changes in the activities of cyclooxygenase (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 PGE₂ 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 PGE₂ that counteracted the potential detrimental effects of gastric acid.

In conclusion, we for the first time demonstrated that 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 PGE₂ that counterbalance its detrimental effects on esophageal mucosa. The major conclusion of above findings have been schematically represented in Figure 8.9.
Figure 8.9: Schematic representation of the possible role of L-tryptophan on RE-induced injury in rats.