JOURNAL OF PHYSIOLOGY AND PHARMACOLOGY 2009, 60, Suppl 7, 47-56
www.jpp.krakow.pl

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MUCOSAL STRENGTHENING ACTIVITY OF CENTRAL AND PERIPHERAL MELATONIN IN THE MECHANISM OF GASTRIC DEFENSE

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This review summarizes the involvement of centrally and peripherally applied melatonin, a major hormone of pineal gland, in the mechanism of gastric mucosal integrity, gastroprotection and ulcer healing. Melatonin was originally shown to attenuate gastric mucosal lesions but the controversy exists in the literature as to whether melatonin derived from the pineal gland, considered as the major source of this indole or rather that locally generated from L-tryptophan within gastric mucosa, plays predominant role in the mechanism of gastrointestinal integrity. Both, intragastric (i.g.) and intracerebroventricular (i.c.v.) administration of melatonin and its precursor, L-tryptophan to rats without or with removed pineal gland by pinealectomy attenuates in the dose-dependent manner the formation of on gastric lesions induced by topical irritants and water immersion restraint stress (WRS). Melatonin accelerated the gastric ulcer healing and this was accompanied by the rise in gastric blood flow (GBF), the plasma melatonin and gastrin levels, the mucosal generation of PGE_2 and luminal NO content. Pinealectomy, which suppresses the plasma melatonin levels, markedly aggravated the gastric lesions induced by WRS. Concurrent supplementation of pinealectomized animals with melatonin or L-tryptophan, the melatonin precursor, attenuated the lesions induced by WRS. Treatment with luzindole, an antagonist of Mel_2 receptors, or with L-NNA, the NO-synthase inhibitor, significantly attenuated melatonin- and L-tryptophan-induced protection and the acceleration of ulcer healing and the accompanying increase in the GBF and luminal content of NO. We conclude that 1) exogenous melatonin and that released from the L-tryptophan attenuate lesions induced by topical irritant such as ethanol and WRS *via* interaction with MT_2 receptors and due to an enhancement of gastric microcirculation, probably mediated by NO and PG derived from cNOS, iNOS and COX-2 overexpression and activity, and 2) the pineal gland plays an important role in the limitation of WRS-induced gastric lesions and acceleration of ulcer healing *via* releasing melatonin predominately at night time, that exerts gastroprotective and ulcer healing actions.

Key words: *melatonin, L-tryptophan, gastric blood flow, active oxygen metabolites, nitric oxide, gastroprotection, prostaglandins, sensory nerves, luzindole, brain-gut axis*

INTRODUCTION

Melatonin (5-methoxy-N-acetyltryptamine) which was discovered in 1958 by Lerner in the extract of the pineal gland (1), is an indole produced from L-tryptophan, an amino acid precursor *via* serotonin metabolic pathway. Biosynthesis of melatonin involves the four-steps process of tryptophan hydrolysis and acetylation of 5-hydroxytryptamine (serotonin) to form melatonin. A specific enzyme, N-acetyltransferase (NAT) is considered as a rate-limiting enzyme for melatonin synthesis (2). Synthesis of melatonin and its release from the pineal gland into the blood-stream undergoes a circadian rhythm with maximal levels observed during the darkness and the lowest plasma concentrations during the day (3). Because of its rhythmic diurnal/nocturnal fluctuations, melatonin is believed to synchronize circadian activities with ambient photoperiods and hormonal hypothalamic-pituitary axis (4-6) (*Fig. 1*). Besides its recognized circadian rhythmicity, melatonin is also known as a potent scavenger of reactive oxygen species (ROS) and highly effective protector of various tissues against the deleterious effect of ROS (7-10). Under physiological conditions, small amounts of ROS are produced from molecular oxygen in mitochondria and immediately inactivated by a system of natural scavengers such as melatonin and other antioxidants. During inflammatory, neoplastic or neurodegenerative diseases the massive production of ROS exceeds the capacity of intrinsic defense mechanisms and results in the accumulation of ROS in the damaged tissues (11, 12). Melatonin acts not only a non-enzymatic scavenger, but also enhances the activity of antioxidative enzymes such as superoxide dismutase (SOD), catalase (CAT), or glutathione peroxidase (GPx) (13-14). This indole stabilizes lipid membranes and defends them from peroxidation particularly due to its high lipophilic properties allowing for the easy entrance into the cells to protect their subcellular compartments (15).

SIGNIFICANCE OF EXTRA-PINEAL SOURCES OF MELATONIN IN PHYSIOLOGY AND PATHOPHYSIOLOGY OF GASTROINTESTINAL TRACT (GIT)

Following discovery of melatonin in the pineal gland, subsequent studies showed that this indole is widely distributed in many extrapineal tissues including retina, Harderian gland, placenta, kidneys, respiratory tract and GIT (16-19). It was revealed that total amount of melatonin generated in the GIT may be 400 - 500 times larger than those present and secreted by the pineal gland (20) and it is found in all portions of GIT, particularly in the stomach, ileum and colon of all species tested including humans (21-23).

Mitochondrial oxidative stress is found to be central to the pathogenesis of many degenerative diseases. Under experimental conditions, the treatment of rat gastric mucosal cells *in vitro* initiates oxidative stress in the mitochondria of these cells and pretreatment with melatonin prevents mitochondrial-mediated oxidative stress. Since, melatonin is a small molecule, which is lipophilic in nature, it is distributed through out the cell with significant deposition within the mitochondria. Melatonin protects the mitochondria from ischemia-reperfusion and indomethacin-induced damage due to its radical scavenging action and restores normal function of these organelles (24). Melatonin was shown to protect the indomethacin-induced activation of the mitochondrial pathway of apoptosis. Previous studies revealed that the attenuation of Bax and Bak expression and upregulation of Bcl-2 and BclxL inhibited the indomethacin-induced activation and mitochondrial translocation of Bax (24). As a result, the opening of MPTP was significantly prevented by melatonin and subsequently, the release of cytochrome c was suppressed from gastric mucosal cells (24).

Food intake and a long term food deprivation induce the tissue and plasma melatonin concentrations. Interestingly, high concentrations of this indole have been detected in the bile, particularly in the gallbladder concentrated bile (22,25). Of note is that the amount of the melatonin in the jejunum and ileum of the rats exceeded the corresponding hormone levels in the serum of these animals by 12-15 folds (26). Because of the fact that its precursor L-tryptophan is easily available in the gut due to the meal consumption and subsequent protein digestion and that melatonin enzymes such as NAT and hydroxyindole-O-methyltransferase (HIOMT) are detected in the GIT, it was reasonable to assume that gastrointestinal melatonin is generated locally in the GI system, and this has been well documented by immunochemical as well as by radioimmunoassay (RIA) studies (20,21). However, in terms of melatonin localization in the gut, large variations between species have been observed (19-22). Furthermore, the removal of the pineal gland by pinealectomy failed to affect GIT melatonin but affected the night-time blood level of this indole, which is markedly reduced in pinealectomized animals (26). Gene expression for NAT and HIOMT, two key enzymes involved in the synthesis of melatonin from serotonin could be traced in the gut and in the pancreas (23,27-29). These observations support the notion that melatonin is produced in the GIT and that high content of gastrointestinal melatonin is in part, independent of the pineal production.

Melatonin is probably synthesized in the enterochromaffin cells (EC) of the GIT mucosa, after oral or parenteral administration of its substrate, L-tryptophan, but the digestive tract may take up also additional amounts of pineal melatonin from the circulation. It is of interest that melatonin has been detected also in the gut lumen and this luminal melatonin probably originates from the GIT mucosa, from the bile secreted into the duodenum and from ingested food (23,25,29,30). The fourth source of gastrointestinal melatonin is that originating from the mother's milk (31). There was, however, a speculation based on the experimental animal observations suggesting that, at least, a

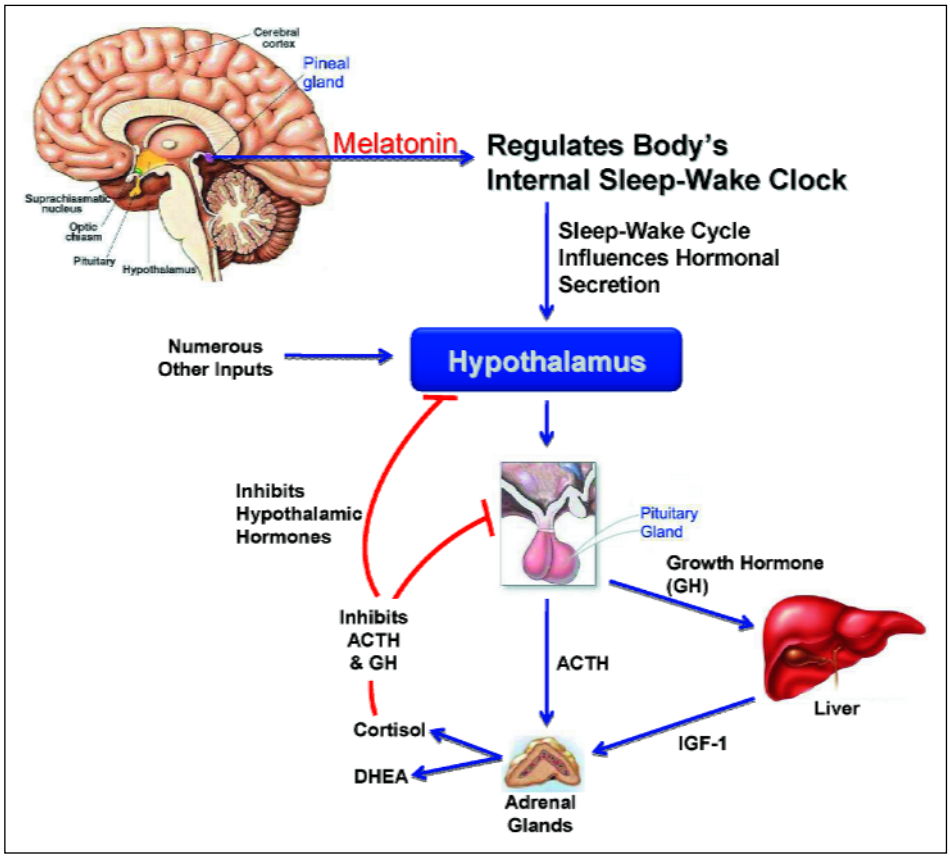

Fig. 1. Melatonin, a major pineal gland hormone exerts the pleiotropic influence on human body. This pineal indoleamine regulates "sleep-wake clock" influencing the hormonal secretion from hypothalamic-pituitary axis *via* negative feedback mechanism.

portion of melatonin which could occur in the digestive system represents that of pineal origin. Messner *et al*. (28) proposed that the digestive tract might act as a sink for pineal-derived melatonin, released in high concentrations, especially during the night phase.

IS THE PINEAL GLAND, A MAJOR SOURCE OF MELATONIN BIOSYNTHESIS, ESSENTIAL FOR THE GASTRIC SECRETORY FUNCTION AND GASTRIC PROTECTION EXHIBITED BY THIS HORMONE?

The question remains whether the presence of pineal gland and its hormonal activity are absolutely essential for the circadian rhythm of the formation of acute gastric lesions induced by various stressors and damaging agents and for the reduction in stress-induced lesions observed at the dark phase. The circadian rhythm of the formation of gastric lesions has so far been studied mainly in rats with intact pineal gland and little attempts were made to check whether this circadian rhythm of stress-induced gastric is affected in pinealectomized animals.

Actually, there is not so many informations available on the physiological role of melatonin in digestive system with particular respect to the maintenance by this indoleamine of gastroduodenal integrity. Recent studies of Flemstrom's group have shown that luminal melatonin is a potent stimulant of duodenal bicarbonate secretion in response to gastric acid entering the duodenum (30). Also melatonin has been implicated in the regulation of interdigestive motility patterns and is able to accelerate intestinal transit after the feeding (32). These findings led to the conclusion that melatonin could be a humoral mediator of hepatic- and gastrointestinal communication. Although, melatonin binding sites were detected in the gut (33), only a few attempts were made to determine the contribution of melatonin and its rate-limiting precursor, L-tryptophan (34,35) to the mechanism of gastric mucosal integrity, gastroprotection against the damage induced by various irritants and healing of chronic gastric ulcerations.

Melatonin was originally implicated in the mechanism of gastric mucosal integrity and in gastroprotection against various irritants because intracerebroventricular (i.c.v.) or intragastric (i.g.) pretreatment with this indole or its precursor, L-tryptophan, applied exogenously, greatly attenuated the formation of acute gastric lesions induced by ethanol, stress, nonsteroidal anti-inflammatory drug (NSAID) such as aspirin and ischemia-reperfusion (36-40). The mechanism of this protection by melatonin has been attributed to the its scavenging of ROS and its ability to enhance gastric blood flow, to attenuate lipid membrane peroxidation and hydroxyl radical damage, neutrophil-induced infiltration of the gastric mucosa and oxygen intermediates cytotoxicity (41).

This beneficial effects of exogenous melatonin and that released from the GIT mucosa were supported by the observation that pinealectomy, which resulted in the removal of only one source of melatonin, reduced serum levels but failed to affect melatonin contents in the upper and lower GIT (26,39). Another study documented that pinealectomy in rats (42), which resulted in almost complete elimination of the circulating melatonin, markedly worsened the stress-induced gastric lesions in the dark phase suggesting that the nocturnal increase in melatonin influences the extent of stress-induced gastric injury. Thus, these data indicate that the circadian diurnal-nocturnal rhythm may influence the extent of stress-induced gastric mucosal lesions. Moreover, the gastric lesions induced by 4hr of rats exposure to WRS during the dark phase were significantly lower than that the light phase controls. It was reported that even during the day, when circulating melatonin levels are low, pinealectomy which eliminated circulating melatonin, augmented the gastric lesions induced by WRS in the dark phase (39,43,44), and data presented in this review are in keeping with these findings (*Fig. 2*).

These data emphasize a direct role for the pineal gland in gastric mucosal protection and further suggest that an intact pineal gland is important for this protective activity of melatonin in dark phase. These findings are also corroborative with the previous report by Tan *et al*. (45), who also showed that an intact and functional pineal gland is important for protecting hepatic DNA from naturally occurring chemical carcinogens such as safrole. We found that the supplementation of pinealectomized rats with melatonin or L-tryptophan reversed the stress ulcerogenesis in these pinealectomized animals with the extent similar to those with intact pineal glands treated with this indoleamine and its precursor (*Fig. 2*). This observation supports the notion of direct involvement of a pineal gland in gastroprotection and appears to be consistent with another observation that melatonin applied i.c.v. afforded significant protection against stress-induced damage and reduced the severity of these lesions caused by a thyrotropin-releasing hormone (TRH) analogue *via* interaction with its specific receptors localized in central nervous system (CNS). This protective activity of melatonin could be demonstrated not solely

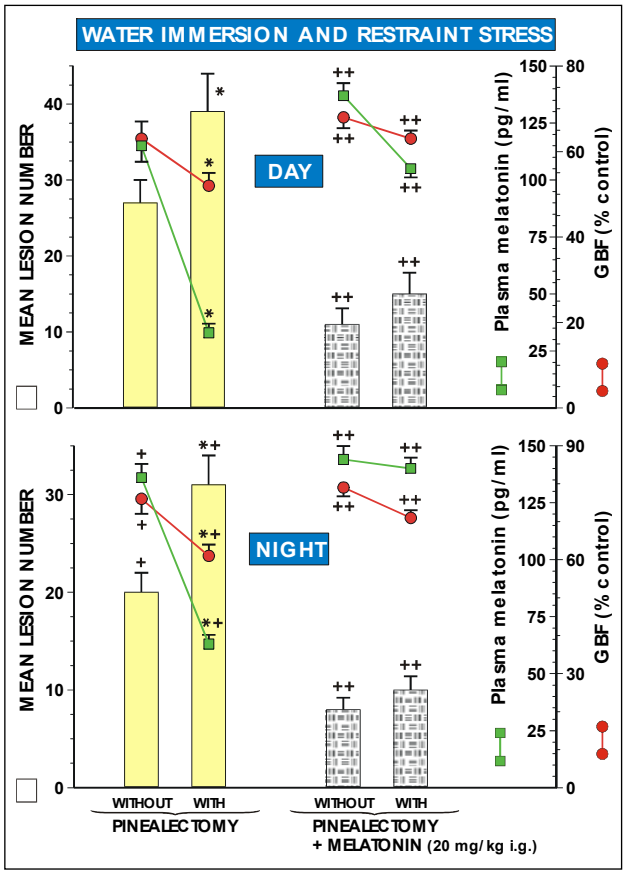

Fig. 2. The number of WRS-induced gastric lesions, plasma melatonin levels and gastric blood flow (GBF) in rats with or without pinealectomy or pinealectomy combined with melatonin treatment (20 mg/kg i.g.) and exposed to 3.5 h of WRS during the day or at night. Mean±SEM of 6-8 animals. Asterisk indicates a significant change as compared to the values obtained in animals without. pinealectomy. Cross indicates a significant change as compared to the values obtained in rats subjected to WRS during the light phase. Asterisk and cross indicate a significant change as compared to the values obtained in rats with pinealectomy subjected to WRS during the light phase. Double crosses indicate a significant change as compared to the values obtained in non-pinealectomized and pinealectomized animals without melatonin treatment.

in the gut but also in the CNS, since this indole was shown to ameliorate the neurological damage in experimental models of stroke *via* suppression of apoptosis and attenuation of ROS-mediated brain infarct damage (46).

GASTRIC ULCER HEALING ACTIVITY OF MELATONIN DERIVED FROM L-TRYPTOPHAN. THE IMPORTANCE OF COX-PG, NOS-NO SYSTEMS AND LIPID PEROXIDATION

As documented above, melatonin and its precursor, L-Trp, applied exogenously, are highly effective in prevention of the formation of acute gastric lesions induced by ethanol, stress, aspirin and ischemia-reperfusion (47). Our recent studies (48,49) fully confirmed previous observations that both melatonin and its precursor, L-tryptophan, applied intragastrically (i.g.) dose-dependently reduced the number of acute gastric lesions, attenuated lipid peroxidation and enhanced activity of antioxidant enzymes in gastric mucosa in rats exposed to 3.5 h of WRS, representing typical oxidative stress-induced gastric disorder often leading to the microbleeding erosions in humans. These protective effects were accompanied by gradual increase in plasma melatonin levels indicating that intragastric melatonin has local protective action on gastric mucosa acting *via* circulation following its absorption form the gut. L-tryptophan, as highly hydrophobic substance, easily penetrates GIT mucosal membrane to be quickly transformed into melatonin in the GIT mucosa, showing the same activity as melatonin itself applied topically to achieve the same plasma indole levels. Finally liver causes marked inactivation of melatonin when passing from the gut lumen into the circulation (23,25).

The protective, anti-stress effects and ulcer healing efficacy of melatonin have been attributed not only to antioxidant action of this indole and restoration of microcirculation, but also to activation by this indole of mucosal COX-prostaglandin and NOS-NO systems, especially at ulcer margin as well as activation of capsaicin-sensitive afferent nerves releasing gastroprotective and vasodilatating neuropeptide CGRP (49). This notion is supported by the evidence that inactivation of these nerves by neurotoxic dose of capsaicin attenuated melatonin and L-tryptophan-induced protection and co-treatment with exogenous CGRP restored the beneficial action of melatonin and L-tryptophan in gastroprotection against mucosal injury. Topically applied melatonin and L-tryptophan applied intragastrically are known to enhance the release of gastrin that also might contribute to ulcer healing by stimulation of mucosal growth at the ulcer margin.

The mechanism of the gastroprotection afforded by melatonin and its precursor, L-tryptophan, involves the stimulation of COX-PG system, an enhancement in GBF and the scavenge of free radicals as described before (37,38,47,48). If the GIT-originated melatonin is indeed involved in the local mucosal protection, it is expected that exogenous melatonin and its precursor, L-tryptophan should also exert protection against the mucosal lesions even in rats undergoing pinealectomy. Indeed, pinealectomy significantly reduced the basal plasma levels of melatonin and enhanced gastric ulcerogenicity of stress but failed to abolish the gastroprotective activity of exogenous melatonin and its precursor, L-tryptophan (50,51). Our finding that melatonin exerts the gastroprotective activity is in keeping with another study showing that exogenous melatonin administered by the means of its central (i.c.v.) application afforded significant protection against stress-induced damage and reduced the severity of these lesions caused by a TRH analogue *via* interaction with its receptors localized in central nervous system (46).

The healing of gastric ulcers is a time-dependent process and depends upon several components including cell proliferation and differentiation at ulcer margin, an increase in the gastric blood flow at ulcer margin, formation of granulation tissue controlled by the expression of cytokines and growth factors and the formation of new vessels (angiogenesis) at the ulcer bed (52). Gastroprotection has a little in common with ulcer healing, even though ulcer healing may involve the common gastroprotective mediators that might contribute to both, protective activity and the process of repair and ulcer healing by this indoleamine. In contrast to the insight to gastroprotective mechanism of melatonin, its efficacy to influence the ulcer healing mechanism has been little elucidated.

Bubenik and coworkers were the first to observe initially the ulcer healing activity by melatonin in pigs (53) and then this effect was confirmed by our group in rats (47-50). Bubenik *et al*. (53) demonstrated that 4-week administration of melatonin in the diet significantly reduced the incidence of spontaneous (chronic) gastric ulcers in young pigs. It is of interest that the pigs with such ulcers exhibited lower contents of melatonin in the gastric mucosa and in the blood suggesting that these spontaneous ulcers originate from the local deficiency of indoleamine. They also demonstrated that coarsely ground diet, in contrast to finely ground diet, exerted stronger protective effects on the gastric mucosa by stimulating greater production of endogenous melatonin from the gastric mucosa.

Our group was particularly interested to examine whether melatonin that exerts a beneficial action against gastric injury due to the activation of the COX/PG system as well as the NOS-NO system could also accelerate healing of preexisting gastric ulcers induced by acetic acid. In previous reports from our laboratory, the suppression of COX by a non-selective COX inhibitor, *i.e*. indomethacin, attenuated the protective effects of melatonin against mucosal damage induced by stress and ischemia-reperfusion (47). Based on these observations, the hypothesis has been put forward that PG and NO play a pivotal role in the acceleration of ulcer healing by melatonin (54).

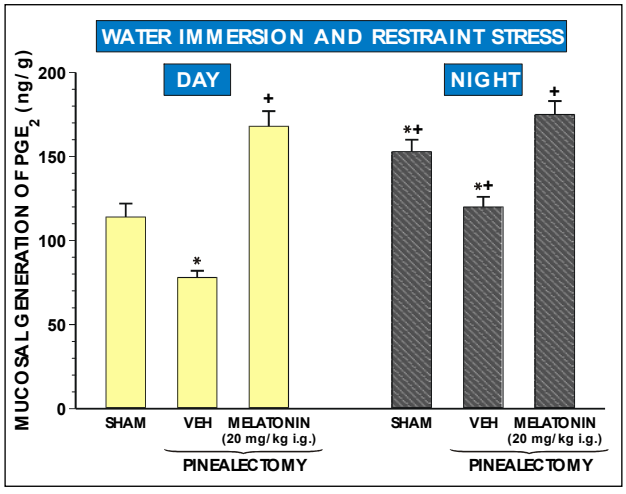

Fig. 3. Gastric mucosal PGE_2 generation in rats without or with pinealectomy and those with pinealectomy pretreated with vehicle (VEH) or melatonin (20 mg/kg i.g.) and exposed to 3.5 h of WRS at the day and night. Results are mean ± SEM of 6-8 rats. Asterisk indicates a significant change as compared to the values obtained in non-pinealectomized rats subjected to WRS during the day. Cross indicate a significant change as compared to the values obtained in non-pinealectomized and pinealectomized animals without melatonin administration.

The healing effects of melatonin involve hyperemia at the ulcer margin and this circulatory effect may be attributable to melatonin *per se* or it may also be due to a potent vasodilators such as NO or PGE_2 originating from the vascular endothelium, gastric epithelium or from the capsaicin-sensitive nerve endings releasing the potent vasodilator calcitonin gene-related peptide (CGRP) (55-57). The crucial role of NO in the action of melatonin is further supported by the observation that addition to L-NNA of L-arginine (but not D-arginine), the substrate for NOS, restored ulcer healing, luminal release of NO and the mucosal hyperemia at the ulcer margin induced by melatonin. Finally, both cNOS mRNA and iNOS mRNA were significantly upregulated at the margin of the gastric ulcer in vehicle- and melatonin-treated gastric mucosa as compared to those in intact mucosa, however, only iNOS mRNA was significantly stimulated in melatonin-treated gastric mucosa suggesting that overexpression of iNOS with subsequent excessive release of NO contributes to the acceleration of ulcer healing and the enhancement of the microcirculation at the ulcer edge (49,58).

These results remain in agreement with the existing evidence that the healing of pre-existing ulcers involves an upregulation of iNOS at the level of both mRNA and iNOS protein in the ulcer edge (59, 60). Furthermore, the importance of NO derived from the iNOS activity in a mechanism of ulcer healing was emphasized by the fact that the selective suppression of iNOS expression and activity accompanied by a reduction in NO generation, increased the number of inflammatory cells at the ulcer margin, resulting in a marked prolongation of ulcer healing (58). These observations were in contrary to the finding that the inhibition of NO biosynthesis *via* the suppression of iNOS by melatonin may contribute to the protective effect of this indole against LPS-induced endotoxemia in rats (61, 62). This possibly reflects different experimental conditions suggesting that under certain conditions, such as endotoxemia, melatonin can exert a beneficial effect due to inhibition of iNOS expression and excessive release of NO thus preventing the formation of the peroxynitrate anion, known to exhibit significant cell toxicity. An early rise in iNOS expression almost immediately after ulcer induction, probably contributed to ulcerogenesis, being the part of inflammatory response following ulcer induction. Since the treatment with melatonin and tryptophan actually significantly reduced iNOS expression as compared to that observed at early phase (day 0) of healing, it is proposed that melatonin and its precursor have inhibitory action on the expression and probably activity of iNOS, thus eliminating its noxious influence on ulcer healing (58).

Based on these findings, it was reasonable to evaluate the alterations in the gene expression of factors possibly involved in the acceleration of ulcer healing by exogenous melatonin and L-tryptophan, a major precursor of this indole. Melatonin- and L-tryptophan-induced the acceleration of ulcer healing involve an increase in the expression of specific melatonin receptors which has been supported by showing an increase in gene expression of MT_2 R and closely related enzymes, NAT and HIOMT, involved in biosynthesis of melatonin. The expression of both these enzymes was significantly increased mostly in the ulcer area and observed at late (day 8) phase of ulcer healing, suggesting that locally generated melatonin in the ulcer bed could enhance the healing rate of this ulcer. Binding of labeled melatonin (58,63) reached the highest value in the ulcer base and that this binding was greatly inhibited by administration of an excessive amounts of exogenous melatonin and L-tryptophan to the stomach with gastric ulcer. Interestingly, the administration of melatonin or tryptophan enhanced the gene expression of MT_2R especially in the ulcer area as compared to the non-ulcerated mucosa. The induction of chronic ulcer enhanced the gene expression of melatonin MT_2R being the prerequisite for the promotion of the binding of endogenous melatonin and helping healing process by exhibiting anti-oxidative and anti-inflammatory actions. This was in keeping with previous results proving the beneficial influence of melatonin and L-tryptophan on healing of chronic gastric ulcers in rats [14, 40]. It is of interest that the ulcer induction by acetic acid coincided not only with a remarkable upregulation of mRNA for MR_2R but also by the upregulation in the ulcer area of major gastroprotective and anti-ulcer system which is COX-PG system. The overexpression of COX-2 mRNA was even further increased at day 8 of ulcer healing and the co-treatment with melatonin or L-tryptophan further potentated the enhancement of COX-2 expression in the ulcer area (58).

The involvement of cNOS/iNOS-NO system in ulcer healing and the possible role of melatonin in this process are not quite clear from this and other studies (58, 64). Although the non-specific suppression of cNOS/iNOS-NO system by L-NNA delayed ulcer healing, there is no clear evidence whether melatonin and L-tryptophan directly affect the expression and activity of iNOS or it could be due to inflammatory conditions associated with gastric ulcer induction. The partial explanation

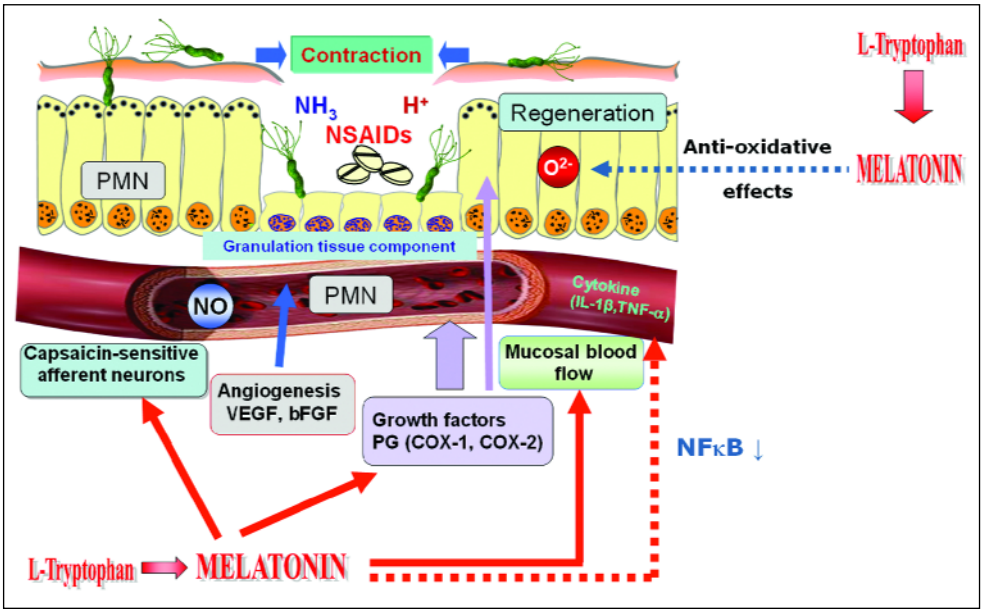

Fig. 4. Scheme of melatonin contribution to the mechanism of gastric mucosal defense. Gastrointestinal melatonin derived from amino acid L-tryptophan increases the gastric microcirculation, and inhibits the activation of neutrophils, the effect which is mediated in part by NOS/NO and PG/COX systems. In addition, melatonin exhibits antioxidative properties and inhibits the inflammatory cascade *via* downregulation of the transcription factor NFκB.

comes out from the evidence that the induction of an ulcer was accompanied by the overexpression of hypoxia inducible factor-1α (HIF-1α) that was detected almost immediately upon the application of ulcerogen, probably caused by severe tissue ischemia, resulting from the application of acetic acid (58). This effect was followed by an overexpression of proinflammatory cytokines such as TNF-α and IL-1β, and upregulation of mRNA for iNOS with excessive production of noxious NO possibly forming peroxynitrite likely contributing to early tissue damage and formation of ulceration. As reported recently by Baatar *et al*. (65) vascular injury leading to ischemia is the major factor involved in the pathogenesis of chronic tissue injury and induction of ulceration, both in the esophagus and the stomach, where the acetic acid was applied. According to these authors, the tissue ischemia and accompanying hypoxia trigger the angiogenesis and formation of network of new microvessels in the granulation tissue at the ulcer margin. These changes have been attributed to increased expression of HIF-1α that dramatically raises the expression of VEGF, activating the angiogenesis both under *in vitro* and *in vivo* conditions. Guo *et al* (64) reported an early rise in the expression on iNOS, suggesting that this expression accompanied by excessive generation of NO was probably responsible for the enlargement of ulcer crater at first days upon ulcer induction by acetic acid in rats. The expression of iNOS was observed to decline when the ulcer began to heal, indicating that NO generated by iNOS at later phase of healing , might contribute to ulcer healing by inducing apoptosis in inflammatory cells (24,58). This is supported by the observation that the overexpression of HIF-1α at early mucosal damage followed by angiogenesis in the granulation tissue as the major events contribute to the process of gastric tissue regeneration and ulcer healing. Indeed, after relative short period of time (3 h) following the application of acetic acid, a marked rise in the expression of HIF-1α and VEGF were observed and these changes were accompanied by a marked increase of expression of iNOS mRNA and overexpression of mRNA for proinflammatory cytokines, TNF-α and IL-1β that could contribute to focal tissue damage caused by acetic acid application (58).

Recently, Bubenik *et al*. (66) demonstrated that 4-week administration of melatonin in the diet significantly reduced the incidence of spontaneous gastric ulcers in young pigs. It is of interest that the pigs with ulcers exhibited lower levels of melatonin in the gastric mucosa and in the blood suggesting that the ulcers originated from the local deficiency of the melatonin synthesis. It was demonstrated that coarsely ground diet, in contrast to finely ground diet, exerted protective effects on the gastric mucosa by stimulating the production of endogenous melatonin from the gastric mucosa (66). Indeed L-tryptophan free diet delayed the healing of chronic gastric ulcers in rats and this effect was attenuated to bower plasma melatonin levels in these animals as compared with those fed with L-tryptophan rich diet (67).

Another mechanisms that may play a role in reduction on stress ulcerogenesis and acceleration of ulcer healing seem to involve the activity of antioxidant enzymes and lipid peroxidation. The cold stress caused by water immersion and restraint of animals was shown to reduce the gastric mucosal PGE_2 content, the activity of SOD and GSH while increasing lipid peroxidation. Suppression by WRS of SOD activity combined with increased lipid peroxidation results in massive gastric damage most likely resulting from generation of hydrogen peroxide, the hydroxyl radical and peroxynitrite anion. Moreover, a deficit in gastroprotective PGE_2 combined with the reduction in antioxidative enxymes, enhanced lipid peroxidation and accompanying reduction in mucosal microcirculation resulted in the formation of multiple gastric erosions. This WRS-mediated ulcerogogenesis is, however, less pronounced in dark phase under stressful conditions since there is increased antioxidative enzyme activity, reduced lipid peroxidation and increased mucosal PGE_2 generation and GBF at night (*Fig. 3*). The crucial question that remains is what is the common factor that limits stress-induced ulcerogenesis at night compared with that during the day, especially in pinealectomized rats. It is suggested that this factor could be the small but significant rise at night in plasma melatonin levels, probably released from an extrapineal source *e.g.* gastrointestinal tract which in turn is responsible for an increase in PGE_2 generation in gastric mucosa (*Fig. 3*)

The crucial role of NO in the action of melatonin is further supported by the observation that addition to L-NNA of L-arginine, the substrate for nitric oxide synthase (NOS) activity, but not D-arginine, restored the ulcer healing, luminal release of NO and the mucosal hyperemia at ulcer margin induced by this hormone. The involvement of NO in the acceleration of ulcer healing is emphasized by the fact that the healing of preexisting ulcers involved an upregulation of iNOS at the level of both mRNA and iNOS protein in the ulcer edge. This remains in agreement with the finding that the suppression of iNOS expression and activity while causing a decrease in the NO generation, resulted in an increased the number of inflammatory cells at the ulcer margin and considerably prolonged ulcer healing. This suggests that under certain conditions, such as endotoxemia, melatonin can exert a beneficial effect due to inhibition of iNOS expression and excessive release of NO acting as ROS due to formation of cytotoxic peroxynitrite.

Additional evidence suggest that melatonin inhibits indomethacin and aspirin induced gastroduodenal ulcerations *via* a mechanism probably unrelated to endogenous PG is that COX-PG system was completely suppressed by the ulcerogen (40,47,48,68). In these reports melatonin caused the amelioration of mucosal sulfhydryls and scavenged ROS generated in response to indomethacin and these effects were the major factors in the protective action of the indole in animals with suppressed COX-PG system (69,70). The treatment with melatonin was shown to inhibit not only the immunohistochemical expression of the adhesion molecule, such as P-selectin, in the lower gut, but also expression of COX-2 in the rat model of experimental colitis (71). The fact that both, indomethacin and L-NNA, significantly prolonged ulcer healing also in placebo-control rats without pretreatment with melatonin or its precursor suggests that endogenous COX-PG and NOS-NO systems are definitely involved in the healing action of melatonin; however, they may not be the only mediators responsible for the promotion of ulcer healing by melatonin (*Fig. 4*).

INTERACTION OF EXOGENOUS AND ENDOGENOUS MELATONIN WITH SPECIFIC MELATONIN RECEPTORS AND GIT HORMONES IN THE MECHANISM OF ULCER HEALING

Both the protective and ulcer healing effects of melatonin in the stomach were considered to be receptor specific because centrally (i.c.v) or topically (i.g.) applied melatonin- or L-tryptophan markedly attenuated stress-damage and these effects were abolished by luzindole, a specific antagonist of melatonin MT_2 receptors (62, 73).

Since melatonin receptors were found in the vascular beds of different systems an attempt was made to determine whether systemic administration of luzindole can influence melatonin-induced protection against WRS-induced gastric lesions. We found that the gastroprotective effects of central and peripheral melatonin appear to be specifically mediated by melatonin MT_2 receptors because the antagonism of these receptors with luzindole attenuated the gastroprotective and hyperemic effect of

this indole. Luzindole was also found to counteract the protective and hyperemic effects of exogenous melatonin administered to pinealectomized animals. The ulcer healing effects of melatonin in the stomach are also considered to be receptor specific because not only melatonin-induced gastroprotection but also an acceleration of ulcer healing with an accompanying rise in the GBF in the ulcer area, were abolished by luzindole, a specific antagonist of the membrane melatonin MT_2-receptors (MT_2-R)(49).

Next, already mentioned, candidates for mediation of the ulcer healing effects of melatonin and its precursor might be gastrin and CCK. As the acceleration of ulcer healing by melatonin or L-tryptophan was accompanied by notable increase in plasma gastrin and CCK levels, it is reasonable to assume that endogenous gastrin and CCK may contribute not only to the spontaneous process of ulcer healing as originally proposed (52), but also may mediate the healing effects of exogenous and endogenous melatonin. The increase in plasma gastrin and CCK observed in rats treated with melatonin or L-tryptophan (47) or with L-tryptophan rich diet (67), could contribute to the acceleration of ulcer healing by these molecules. The increase in plasma gastrin increments in melatonin-treated rats is not fully understood but could be attributed, at least in part, to the inhibitory action of central and peripheral melatonin on gastric acid secretion but the direct action of this indole on the antral G-cells can not be excluded. Moreover, the plasma levels of gastrin was significantly diminished in rats with pinealectomy suggesting that removal of the central source of melatonin may influence "tonic" inhibitory action of this hormone on gastric acid secretion. This increase in plasma gastrin levels in animals with chronic gastric ulcers treated with melatonin were significantly attenuated by the co-treatment with luzindole, a potent antagonist of melatonin MT_2 receptors. This suggests that melatonin may promote ulcer healing *via* an increase in plasma level of gastrin due to its inhibitory effect on gastric secretion. Indeed, melatonin applied topically or injected i.c.v. to animals exerted a potent inhibitory action on gastric acid secretion while elevating plasma gastrin levels. This increase in the plasma gastrin could be secondary to the decrease in gastric luminal acidity caused by melatonin (37,38).

IMPORTANCE OF BRAIN-GUT AXIS AND PINEAL GLAND IN THE GASTROPROTECTION AND ULCER HEALING BY MELATONIN AND L-TRYPTOPHAN

Another mediator of the melatonin-induced gastroprotection and ulcer healing, may be the neural component, in the particular, the gut-brain axis consisting of sensory afferent and efferent autonomic nerve fibers. To examine this possibility, animals with functionally deactivated sensory nerves using neurotoxic dose of capsaicin were used in our studies (56,57). Such capsaicin-denervated animals were previously employed and widely accepted to document the importance of sensory afferent neuropeptides in the mechanism of gastric mucosal defense and the mucosal repair from damage induced by strong irritants (56). It was shown, for instance, that functional ablation of afferent nerves delayed healing of gastric ulcers at 1 and 2 weeks after their production with acetic acid and this delay was associated with a marked and persistent decrease in tissue calcitonin gene related peptide (CGRP)-like immunoreactivity related to afferent nerve stimulation by various protective mediators, possibly including melatonin (47,48,56,57).

The hypothesis that the pineal gland and sensory nerves could interact each other contributing to gastroprotective activity of melatonin and L-tryptophan has not been extensively investigated. Pinealectomy resulted in a dramatic fall in the plasma melatonin levels and significantly augmented the WRS-induced gastric lesions. This effect was accompanied by a marked fall in the GBF, but supplementation therapy with melatonin, applied topically or injected i.c.v., restored, in part, both, GBF and plasma melatonin levels, and attenuated the increase in gastric lesions caused by WRS in pinealectomized animals. Our observation, that pinealectomy decreases plasma melatonin levels is in keeping with the previous findings that removal of pineal glands reduces plasma but not gastric tissue melatonin, and aggravates of WRS-induced gastric lesions in the day and night phases (74). It is of interest that tryptophan loading was shown to increase the daytime serum melatonin levels in intact animals as well as in those with pinealectomy suggesting release of melatonin from extrapineal sources (74). Our finding that i.c.v. administration of melatonin to pinealectomized animals counteracted in part, the aggravation of WRS lesions and the accompanying fall in the GBF caused by pinealectomy, while raising plasma levels of melatonin suggests that in addition to central melatonin, also extra-pineal melatonin modulates the gastric response against the deleterious action of stress. This could be due to interaction with the COX-products such as PG because, down regulation of COX-2 mRNA was observed in WRS-exposed animals with pinealectomy and addition of melatonin restored the expression of mRNA for COX-2 to the level observed in WRS-animals with preserved pineal glands as well as increased the mucosal generation of the major product of COX-2 that is PGE_2 (74,75) (*Fig. 3*). This finding implies that melatonin present in the gut and/or released from pineal gland interacts with COX-2 expressed in gastric mucosa and cooperates with PG generated by this enzyme to protect this mucosa against the gastric damage induced by stress. Using rats with capsaicin denervation, we have documented that ulcer healing, promoted by various anti-ulcer substances, was markedly delayed and that this was the accompanied by the fall in the microcirculation at ulcer margin (58,76). Furthermore, we found that the melatonin-induced acceleration of ulcer healing and accompanying hyperemia at ulcer margin were greatly reduced in rats with capsaicin-induced functional ablation of sensory afferents as compared to those with intact sensory nerves treated with melatonin. This suggests that capsaicin-sensitive afferent fibers and sensory neuropeptides such as calcitonin gene related peptide (CGRP) released from these fibers are essential components involved in the mechanism of ulcer healing by melatonin. When CGRP was added to melatonin and L-tryptophan in animals with capsaicin denervation, it restored the ulcer healing activity and accompanying hyperemia at the ulcer margin evoked by melatonin and its precursor.

An early rise in iNOS expression almost immediately after ulcer induction, probably contributed to ulcerogenesis, being the part of inflammatory response following ulcer induction. Since the treatment with melatonin and tryptophan actually significantly reduced iNOS expression as compared to that observed at early phase (day 0) of healing, it is proposed that melatonin and its precursor have inhibitory action on the expression and probably activity of iNOS, thus eliminating its noxious influence on ulcer healing (58,77).

Melatonin- and L-tryptophan-induced acceleration of ulcer healing involves an increase in the expression of specific melatonin receptors which have been supported by showing an increase in gene expression of MT_2R and closely related enzymes, NAT and HIOMT, involved in biosynthesis of melatonin. The expression of both these enzymes was significantly increased mostly in the ulcer area and observed at late (day 8) phase of ulcer healing, suggesting that locally generated melatonin in the ulcer bed could enhance the healing rate of this ulcer (77).

In summary, exogenous melatonin or that derived from its precursor L-tryptophan in the GIT, exhibits a potent gastroprotective activity against acute gastric lesions induced by

variety of damaging agents including ethanol, cold stress, aspirin and ischemia-reperfusion (*Fig.4*). Moreover, melatonin and L-tryptophan which raises plasma melatonin levels, accelerate the healing of chronic gastric ulcers and both, gastroprotection and ulcer healing effect of this exogenous and endogenous indoleamine involves specific MT_2-R, an increase in gastric microcirculation mediated by COX-PG and NO-NOS systems the, inhibition of HIF-1α expression and lipid peroxidation and antioxidizing activity as reflected by attenuation of proinflammatory cytokine IL-1β and TNF-α expression and their release.

Conflict of interests: None declared.

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Received: November 15, 2009
Accepted: December 20, 2009

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