

T BRZOZOWSKI¹, K. ZWIRSKA-KORCZALA², P.C. KONTUREK³, S.J. KONTUREK¹,
Z. SLIWOWSKI¹, M. PAWLIK¹, S. KWIECIEN¹, D. DROZDOWICZ¹,
M. MAZURKIEWICZ-JANIK¹, W. BIELANSKI¹, W.W. PAWLIK¹

ROLE OF CIRCADIAN RHYTHM AND ENDOGENOUS MELATONIN IN PATHOGENESIS OF ACUTE GASTRIC BLEEDING EROSIONS INDUCED BY STRESS

¹Department of Physiology, Jagiellonian Medical College, Cracow, Poland,

²Department of Physiology, Silesian Medical University, Zabrze, Poland,

³Department of Medicine, Erlangen-Nuremberg University, Erlangen, Germany

Stress that appears as a consequence of burns, surgical trauma and life threatening conditions is a serious clinical entity, can result in acute gastric mucosal lesions. Such stress lesions can develop in response to the imbalance between the aggressive factors promoting mucosal damage and the gastric mucosal defense mechanisms including predominantly gastric blood flow (GBF), biosynthesis of gastroprotective prostaglandins (PG) and enhanced mucus/bicarbonate secretion. Melatonin, a major hormone of pineal gland, whose activity is also abundant in the gastrointestinal tract, was shown to inhibit gastric acid secretion, augment GBF and scavenge free radicals, resulting in the attenuation of stress-induced gastric lesions. Melatonin is released during the night but little is known about the effect of circadian rhythm and day/night alterations in melatonin secretion on the formation of stress-induced gastric lesions. Using rats with intact pineal glands and those with removed pineal glands (pinealectomy) exposed to water immersion and restraint stress (WRS) at both, day and night hours, we studied the effect of light and nocturnal melatonin on the formation of these lesions, and accompanying changes in GBF and plasma melatonin levels. It was found that the gastric mucosa exposed to WRS of various time duration's lasting 1.5, 3 and 6 h, time-dependently increased the number of gastric lesions and this effect was accompanied by the time-dependent fall in the GBF and an increase in the plasma and luminal melatonin levels. Pinealectomy augmented WRS-induced lesions at each time intervals of WRS and produced a marked fall in the GBF and plasma and luminal melatonin levels at each time interval of WRS tested. WRS lesions were significantly reduced at night hours and showed circadian variations in plasma levels melatonin with significantly higher plasma melatonin levels at night than in the day and with a greater magnitude of damage induced in the daily hours than at night hours. WRS-induced gastric mucosal lesions were markedly enhanced in pinealectomized rats, both at day and night, and this was accompanied by a significant fall in plasma melatonin levels with a pronounced reduction in mucosal generation of PGE₂ and GBF and by a small increase in plasma melatonin levels during the dark phase. We conclude that 1) stress-induced gastric bleeding erosions exhibit circadian rhythm with an increase in the day and attenuation at night and that these fluctuations in the formation of stress-induced gastric damage may depend upon the melatonin synthesis 2) the progressive increase in plasma melatonin in pinealectomized animals exposed to various time intervals of WRS suggests that extra-pineal melatonin possibly that derived from gastrointestinal tract, play an important role in the gastric mucosal defense against stress-induced gastric damage.

Key words: *circadian rhythm, pineal gland, stress gastric lesions, melatonin, tryptophan, prostaglandins*

INTRODUCTION

Previous studies revealed that melatonin and its precursor, L-tryptophan, when applied exogenously, are highly effective in prevention of the formation of acute gastric lesions induced by ethanol, stress, aspirin and ischemia-reperfusion (1-3). The mechanism of melatonin's beneficial action has been attributed to the ability of this indole to stabilize lipid cell membranes and to the antioxidizing activity of this compound, particularly due to its high lipophilic properties allowing for the rapid entrance into the cells to protect their subcellular compartments (4). Moreover, this hormone exhibits immunomodulatory action by influencing the activity of nitric oxide synthase (NOS) and cytokine production during inflammatory and carcinogenic processes (5-8). Our recent studies (9-11) fully confirmed previous observations that both, melatonin and its precursor, L-tryptophan, 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 water immersion and restraint (WRS), representing typical oxidative stress-induced gastric disorder. These protective effects were accompanied by gradual increase in plasma melatonin levels suggesting that topical melatonin exerts a local protective action on gastric mucosa, acting *via* circulation following its absorption from the gut. Furthermore, L-tryptophan as highly hydrophobic substance, easily penetrates the gastrointestinal barrier to be quickly transformed into melatonin within gastrointestinal mucosa, showing the same activity as oral melatonin to achieve the same plasma indole levels. Our and others previous studies demonstrated that liver causes marked inactivation of melatonin on its way to pass from the gut lumen into the circulation (10).

The mechanism of the gastroprotection afforded by melatonin and its precursor, L-tryptophan, has been attributed to the stimulation of cyclooxygenase (COX)/PG system and the scavenge of free radicals as described before (1-3, 9, 11-15). These beneficial effects of melatonin released from the gastrointestinal (GIT) mucosa were supported by the finding that pinealectomy, that is to deplete the major source of melatonin in the body, reduced plasma levels of this indole but failed to affect melatonin contents in the GIT (16). Also, it was shown that pinealectomy in rats (17) which resulted in strong attenuation of diurnal melatonin level and suppressed the night surge of this hormone reversed, in part, the stress-induced gastric lesions, suggesting that normally the nocturnal melatonin limits to some extent of stress-induced gastric injury. However, during the day, pinealectomized rats were more vulnerable to stress-induced gastric lesions and the supplementation of these rats with melatonin or its precursor L-tryptophan reversed the stress-induced gastric ulcerogenicity in these pinealectomized animals (16, 17). 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 be protective against the mucosal lesions even after pinealectomized rats. Indeed, pinealectomy significantly reduced the basal plasma levels of melatonin and enhanced gastric ulcerogenicity of stress but failed to prevent the gastroprotective activity of exogenous melatonin and its precursor, L-tryptophan (9, 10). The question remains whether the presence of pineal gland and its hormonal activity are really relevant to the circadian rhythm of the formation of stress-induced gastric lesions 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.

MATERIAL AND METHODS

This study was approved by the Committee of Animal Research of Jagiellonian University and run in accordance to Helsinki Declaration.

Induction of WRS lesions in rats with intact pineal gland and after pinealectomy

Male Sprague-Dawley rats weighing 220-250 g were maintained on Purina rat chow and tap water *ad libitum* and were placed in room with an automatically regulated lighting cycle with a 12 h light/12 h dark cycle (light on at 08.00 h). The animals were fasted for 24 h prior to experiments but allowed free access to water preceding the beginning of each experimentation. The studies were performed at 3 h after lights on and off. For the diurnal studies, light intensity in experimental room was 600 lux, sufficient to inhibit endogenous pineal melatonin synthesis. Nocturnal experiments were carried out using a photo safe dim red light source while the animals were handled.

In separate group of 60 rats, the pineal gland was removed (pinealectomy) according to the procedure described previously by Kato *et al.* (18). Briefly, under the pentobarbital anesthesia,

rats were placed in a prone position and mounted in a head holder. A sagittal incision was made, and the parietal and interparietal bones were drilled with a dental burr, allowing visualization of the sagittal and transverse sinus confluence. The pineal gland was removed by aspiration *via* a specially constructed 22-G needle, which reached the pineal gland through the sagittal sinus. Sham-operation consisted of insertion and withdrawal of needle without applying suction. Rats were used in experiments after 1 week recovery from the pinealectomy. Complete recovery was confirmed based on locomotor's activity compared to that of normal rats. In addition, the brain specimens were collected at the day of rat autopsies to confirm the successful removal of the pineal gland by histology determination. Acute gastric stress lesions were induced by exposure of rats with their intact pineal gland or in those with pineal gland surgically removed to **water immersion restraint stress** (WRS) method as described before (9). Briefly, the animals were placed in restraint metal cages and immersed vertically to the level of the xyphoid process into a water bath of 23 °C for 3.5 h. The rats were mounted in individual restraint cages for 3.5 h at two points in the light/dark cycle (from 10.00 a.m. to 2 p.m. and from 10.00 p.m. to 2.00 a.m.). After the end of 3.5 h of WRS, the rats were lightly anesthetized with ether, the abdomen was opened and the stomach was exposed. GBF was measured in the oxyntic gland area of stomach by means of local H₂-gas clearance method using an electrolytic regional blood flow meter (Biomedical Science, Model RBF-2, Osaka, Japan) as described before (19). The measurements were made in the three areas of the mucosa and the mean values of the measurements were calculated and expressed as percent changes of those recorded in the rats not exposed to WRS (control group) saline-treated animals. The stomach was then removed, opened along the greater curvature and placed flat to count the number of gastric lesions by two investigators, unaware of the treatment given as described in our previous studies (1-3). The stress lesions were defined as round or linear mucosal defects of at least 0.1 mm in diameter (*Fig. 1*). Blood samples of about 1.5 mL were withdrawn from the jugular vein under ether anesthesia 3.5 h after the initiation of WRS and at the corresponding time before and after stress exposure during the light and dark phases for measurement of plasma melatonin in the non-stressed group samples serving as control values for the measurement of blood plasma immunoreactive melatonin. After counting the number of lesions in each stomach, the corpus mucosa samples (about 100 mg) were excised from each stomach for the assessment of the generation of PGE₂.

Measurement of PGE₂ generation in the gastric mucosa exposed to WRS at the day and night

Corpus mucosa samples were obtained separately during the light and dark phases from control groups and those subjected to WRS. Mucosal samples were obtained immediately after removal of the stomach, the corpus mucosa being stripped, weighed and processed for *ex vivo* PGE₂ measurement as previously described (2). Briefly, the tissue was minced with scissors for 1 min in microfuge plastic tubes containing 1 mL of phosphate buffer (pH 7.4) and centrifuged in a fixed-speed bench centrifuge at 15 000 r.p.m. for 30 s. After the supernatant was discarded, the tissue was suspended in 1 mL of phosphate buffer and mixed by vortex for 1 min at room temperature. Then, 10 µL of indomethacin was added to each sample to inhibit further formation and release of PGs. The samples were centrifuged for 1 min and the supernatant stored at -20 °C until assay using radioimmunoassay kit (PGE₂ ¹²⁵I-PGE₂ kit, NEN Life Science Products Inc. Boston, MA, USA). The assay medium was 0.0255 mL/L phosphate buffered medium azide (0.05%), pH 6.8. Generative capacity was expressed as nanograms of PGE₂ per gram wet tissue weight (ng/g). Sensitivity of the assay was 1 pg/mL. Recovery as determined by adding 25 and 50 pg of PGE₂ to the samples was about 50 ± 7% (mean ± S.E.M, n = 10).

Determination of plasma and gastric luminal concentration of melatonin

At the termination of experiments with exogenously administered melatonin at the day and night in animals with or without pinealectomy, the blood samples (about 3 mL) were taken from

the *vena cava* (into tubes containing 2500 U Trasylol, Bayer, FRG and 0.5 mg/ml of EDTA) and plasma was separated. The plasma samples were stored at -20°C until radioimmunoassay (RIA) of melatonin using commercially available kit purchased from DRG Instruments GmbH (Marburg, Germany) and described in detail previously (9).

Statistical analysis

Results are expressed as means \pm SEM. Statistical analysis was performed using Mann-Whitney and Friedman two-way analysis of variance. Differences with $p < 0.05$ were considered as significant.

RESULTS

Effect of various time durations of WRS on the WRS lesions and changes in the GBF in rats without or with pinealectomy

As presented in *Fig. 1A* and *B*, removal of pineal gland by pinealectomy aggravated the number of WRS-induced gastric lesions.

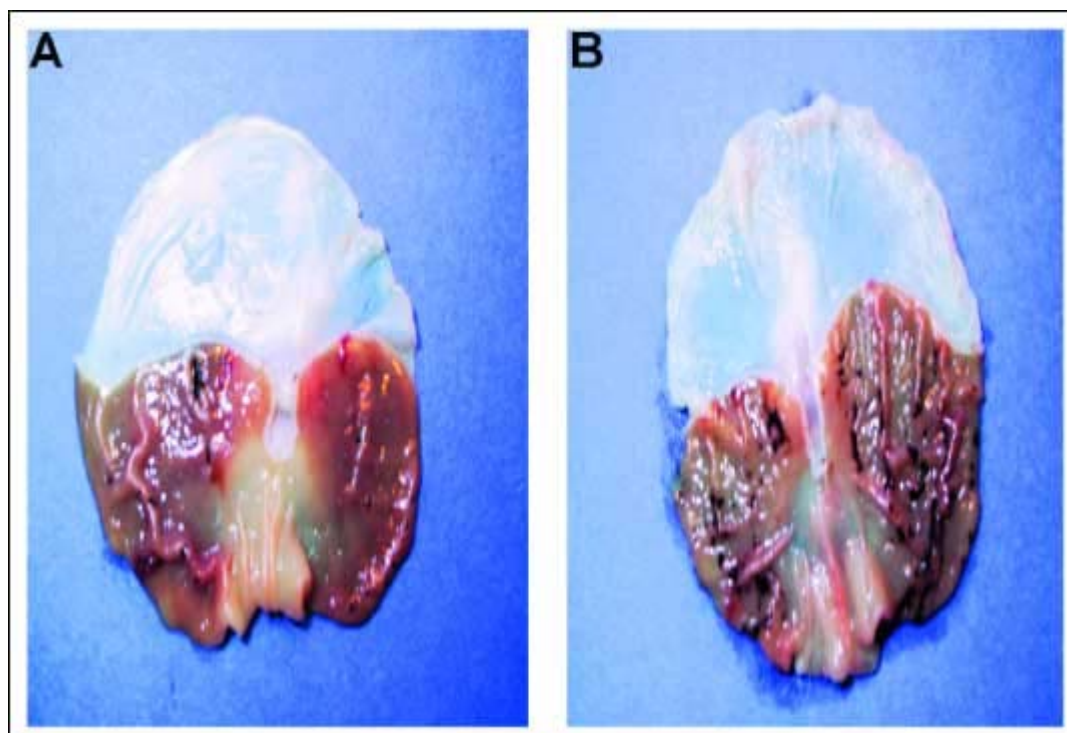


Fig. 1. Representative photomicrograph of gastric mucosa of rat with intact pineal gland exposed to 3.5 h of WRS (A) and that from the pinealectomized animal also exposed to 3.5 h of WRS (B). Note that the number of WRS-induced erosions is enhanced in the rat subjected to pinealectomy as compared to that observed in rat with intact pineal gland.

Fig. 2 (upper and lower panel) shows the effect of various time exposures to WRS on the number of WRS lesions, alterations in the GBF in rats with intact pineal glands and in those subjected to pinealectomy and plasma levels and luminal concentrations of melatonin. Exposure to 1.5 h of WRS resulted in the formation of gastric lesions and produced a significant decrease in the GBF as compared to non-stressed controls (*Fig. 2*, upper panel). When the WRS was extended up to 3 h and 6 h, the number of gastric erosions was significantly increased and this effect was accompanied by the greater fall in the GBF as compared to the respective values obtained in animals exposed to WRS at 1.5 h. Pinealectomy augmented the WRS-induced gastric erosions and significantly decreased the GBF as compared to the values achieved in non-pinealectomized animals (*Fig. 2*, upper panel). Plasma

and luminal concentration of melatonin in pinealectomized rats were markedly lower than those in animals with intact pineal gland, but these values exhibited a small, though significant increase in response to each time extension of WRS exposure (*Fig. 2*, lower panel).

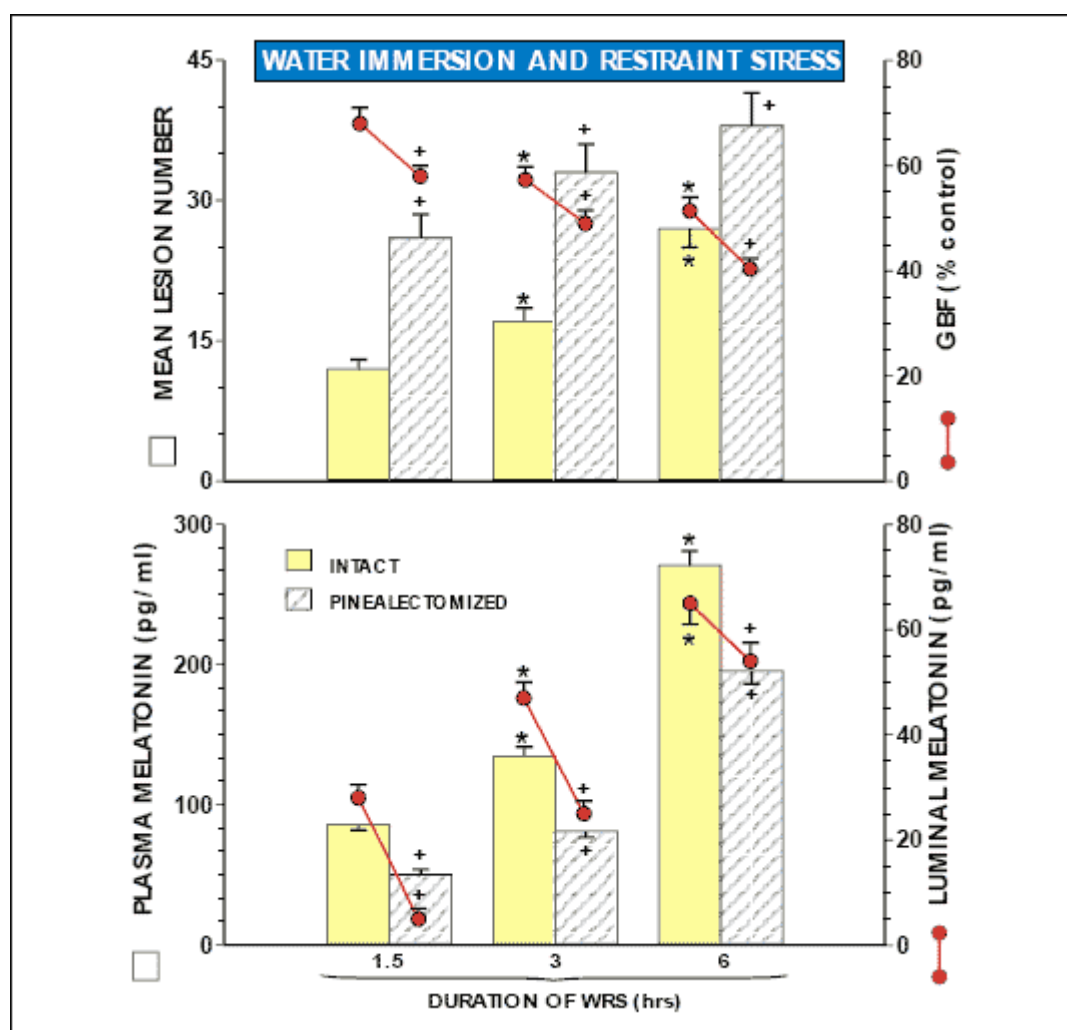


Fig. 2. The number of water immersion and restraint stress (WRS) lesions and alterations in the gastric blood flow (GBF) (upper panel) and mucosal and luminal concentrations of melatonin (lower panel) in rats exposed to various time durations of WRS ranging from 1.5 h up 6 h. Asterisk indicates significant change as compared to the value obtained at 1.5 h of WRS. Cross indicates significant change as compared to the respective values obtained in animals with intact pineal gland.

As shown in *Fig. 3*, the number of WRS lesions in rats with an intact pineal gland during light phase, averaged about 28 ± 3 per stomach and this was significantly higher than the number of stress lesions (20 ± 2) during the night. In these animals, the GBF and mucosal generation of PGE₂ were significantly reduced (*Fig. 3, Table 1*), while plasma melatonin concentrations showed a significant (by about 30%) rise in response to WRS as compared to non-stressed animals (data not shown). Rats subjected to WRS at night, showed the lesion number about 30% lower and the mucosal PGE₂ generation and GBF were also significantly reduced as compared with those in animals with intact pineal gland (*Fig. 3, Table 1*). Plasma melatonin levels reached significantly higher values than those obtained in rats subjected to WRS during daily hours.

Table 1. Gastric mucosal PGE₂ generation in intact rats and in those without or with pinealectomy exposed to 3.5 h of WRS at the day and night. Results are mean \pm SEM of 6-8 rats. Asterisk indicates a significant change as compared to the values obtained in intact animals. Cross indicates a significant change as compared to the values obtained in non-pinealectomized rats subjected to WRS during the day. Asterisk and cross indicate a significant change as compared to the values obtained in non-pinealectomized rats subjected to WRS during the light phase.

Mucosal generation of PGE ₂ (ng/g)	
Intact	184 ± 13
AT DAY	
Non-pinealectomized	114 ± 8*
Pinealectomized	88 ± 4 ⁺
AT NIGHT	
Non-pinealectomized	153 ± 9* ⁺
Pinealectomized	118 ± 7* ⁺

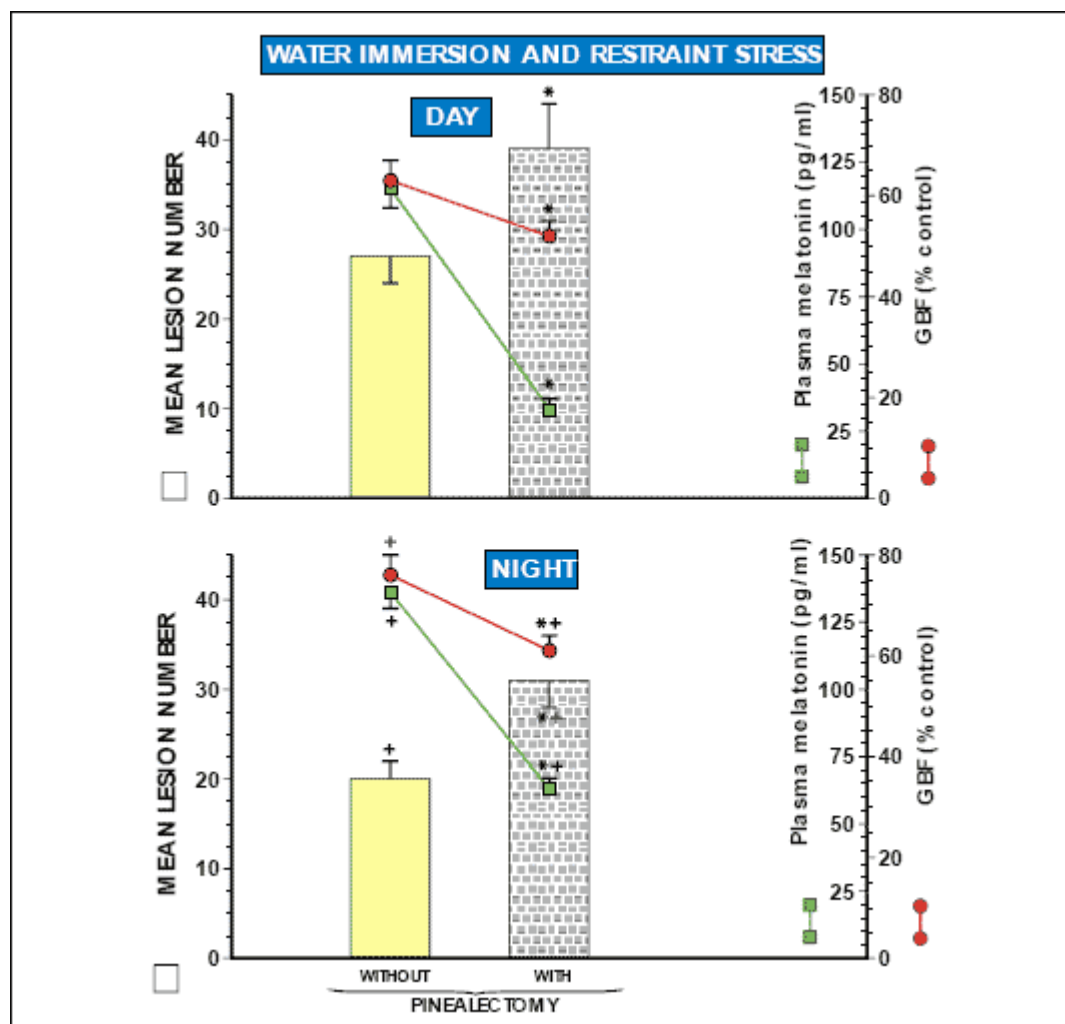


Fig. 3. The number of WRS-induced gastric lesions, plasma melatonin levels and gastric blood flow (GBF) in rats with or without pinealectomy exposed to 3.5 h of WRS during the day or at night. Asterisk indicates a significant change as compared to the values obtained in animals without pinealectomy. 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.

Following pinealectomy, the number of gastric lesions in response to WRS during the light phase was significantly higher than that in rats with an intact pineal gland (*Fig. 3*, lower panel), but these animals also developed less gastric lesions at night hours. The same tendency in GBF and PGE₂ was observed in animals subjected to WRS at night hours, though the GBF and PGE₂ generation were significantly higher as compared with respective values recorded at daily hours.

DISCUSSION

This study shows that stress ulcerogenesis involves diurnal/nocturnal rhythm in formation of gastric lesions in rats with intact pineal glands because these stress-induced gastric lesions were much more pronounced at the day than in the night suggesting that nocturnal melatonin

could contribute to the gastric mucosal defense especially at dark phase, resulting in limitation of stress-induced gastric bleeding erosions. This confirms and extends previous finding of Kato *et al.* (3) and Otsuka *et al.* (13), who described this phenomenon in rats and suggested that such a fluctuation in stress ulcerogenesis could be attributable to nocturnal melatonin. We found that various time-related exposures to WRS caused a time-dependent increase in the gastric lesion incidence and significantly decreased GBF and gastric mucosal generation PGE₂. Moreover, the removal of pineal gland by pinealectomy, augmented WRS-induced gastric lesions at each time duration of WRS determined and caused a more profound fall in the GBF than that observed in animals with intact pineal gland. This suggests that pineal melatonin and the interaction of this indole with COX/PGE₂ system could be of importance in the stress ulcerogenesis.

However, Bubenik *et al.* (17) 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 indole. They also demonstrated that coarsely ground diet, in contrast to finely ground diet, exerted stronger protective effects on the gastric mucosa by stimulating more extensively the production of endogenous melatonin from the gastric mucosa (20). Otsuka *et al.* (21) reported, however, that acute stress-induced gastric lesions in rats are accompanied by increased plasma melatonin. The proposed explanation for the rise in melatonin is that its production increases under stressful stimuli in both, experimental animals and human as suggested by Oxenkrug and McIntyre (22) and Karasek and Winczyk (23). Results of the present study remain, in part, in agreement with those studies regarding the lower gastric stress ulcerogenesis at night in animals with intact pineal gland or in those subjected to pinealectomy. Also in our hands, plasma melatonin levels accompanying a stressful procedure (WRS) showed a significant increase even in pinealectomized animals when the pre-WRS plasma levels of this indole exhibited much lower level. Our study is also corroborative with findings of Klupinska *et al.* (24) that melatonin secreted in great amounts at night attenuated dyspepsia-like- and gastric reflux episodes in patients with symptoms of GERD and NERD.

Recent evidence indicates that melatonin may exert a beneficial action against gastric injury due to the activation of the COX/PG system, heat shock proteins as well as the NOS/NO system (25-28). This notion agrees with previous reports from our laboratory showing that suppression of COX by a non-selective COX inhibitor, i.e. indomethacin, and selective COX-1 and COX-2 inhibitors attenuated the protective effects of melatonin against mucosal damage induced by stress and ischemia-reperfusion (1, 2, 12). Based on these observations, the hypothesis has been put forward that PG and NO play pivotal roles in the acceleration of ulcer healing by melatonin (24). The protective and ulcer-healing effects by melatonin in the stomach are considered to be receptor specific because melatonin-induced gastroprotection and 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 M₂ receptors (26, 28).

Another mediator of the action of melatonin on gastroprotection and ulcer healing may be the gut-brain axis. To examine this possibility, animals with functionally deactivated sensory nerves using neurotoxic dose of capsaicin should be used. Such capsaicin-denervated animals were previously employed to test the mechanisms of gastric mucosal defense, the mucosal repair from damage and acceleration of ulcer healing induced by strong irritants (29-32). It was shown that functional ablation of afferent nerves delayed healing of gastric ulcers at 1 and 2 weeks after their production (by acetic acid) and this delay was associated with a marked and persistent decrease in tissue CGRP-like immunoreactivity related to afferent nerve stimulation by melatonin (32, 33).

This beneficial action of melatonin against stress ulcerogenesis seems to involve the activity of

antioxidant enzymes and lipid peroxidation as documented *in vivo* and *in vitro* conditions (12, 14, 34). It has been shown (10) that gastric stress induced by the exposing of rats to water immersion and restraint, suppressed the activity of SOD and GSH while increasing lipid peroxidation. Suppression by WRS of SOD activity combined with enhanced lipid peroxidation results in massive gastric damage most likely resulting from generation of reactive oxygen metabolites such as hydrogen peroxide, the hydroxyl radical and peroxyxynitrite anion. Moreover, a deficit in gastroprotective PGE₂ combined with the reduction in antioxidative enzymes, enhanced lipid peroxidation and accompanying reduction in mucosal microcirculation results in the formation of multiple gastric erosions. Our present study revealed that this stress-mediated ulcerogenesis is, however, less pronounced in dark phase under stressful conditions since, as shown in this report and in previous studies (12, 13, 21), there is increased antioxidative enzyme activity, reduced lipid peroxidation and increased gastric blood flow in the gastric mucosa subjected to stress at night. The crucial question remains what is the common factor that limits stress-induced ulcerogenesis at night compared with that during the day, especially in pinealectomized rats. In our opinion, this factor is the small but significant rise in melatonin at night, probably released from an extrapineal source e.g. gastrointestinal tract.

Another mediator involved in the melatonin-induced attenuation of stress ulcerogenesis observed at dark phase may be the gut-brain axis. To examine this possibility, animals with functionally deactivated sensory nerves using a neurotoxic dose of capsaicin could be used. Our preliminary observation (data not included) seems to favor the concept that the gut derived melatonin in combination with sensory nerve activation are responsible for dark phase reduction in stress ulcerogenesis in both, non-pinealectomized as well as pinealectomized animals.

Among the other possible candidates responsible for the beneficial effect of melatonin against formation of WRS-induced gastric lesions might be a major gastric hormone, gastrin, which is known to possess gastroprotective and trophic action in the stomach. Melatonin was reported to elevate plasma gastrin levels, suggesting that this hormone could not only contribute to ulcer healing process but also to the acceleration of ulcer healing by melatonin (35-38). Further studies are needed to resolve the question of the contribution of these factors in diurnal variation of gastric stress-mediated ulcerogenesis.

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Author's address: Prof. Dr Tomasz Brzozowski, Department of Physiology Jagiellonian University Medical College, 16 Grzegorzeczka Street, 31-531 Cracow, Poland;
e-mail: mpbrzozo@cyf-kr.edu.pl