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Ghrelin gene expression in rats with ethanol-induced gastric ulcers: a role of melatonin

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Objectives. The aim of the present work was to reveal the mechanisms of melatonin treatment on ethanol-induced gastric mucosal lesions in rats, including its role in the induction of ghrelin biosynthesis.

Methods. Sixty male Wistar rats were divided into 3 groups (20 in each group): a) control group, b) ulcer group (100% ethanol was given intragastrically (i.g.) in a dose of 1 ml/100 g of body weight), and 3) melatonin-treated group, which received a single dose (25 mg/kg) of melatonin (Biovea) i.g. 30 min before ulcer induction with ethanol. Reduced glutathione (GSH) and malondialdehyde (MDA) were measured in tissues and ghrelin levels determined in the serum. RNA isolation and RT-PCR expression of ghrelin were performed. Both macroscopic and microscopic examinations of gastric mucosa were done in all groups.

Results. Significant decrease in ghrelin levels and mRNA expression and reduced levels of GSH were observed in ulcer group of rats in comparison with controls. All parameters studied were significantly increased after treatment with melatonin in comparison with ulcer bearing group of rats. On the other hand, the tissue levels of MDA were significantly increased in ulcer group of rats in comparison with controls and significantly decreased after melatonin treatment in comparison with the ulcer group of rats. Histological examinations revealed severe mucosal lesions induced by ethanol which were significantly improved by melatonin administration.

Conclusions. The present data indicate that melatonin may have a potential impact in the treatment of peptic ulcer not only via its known antioxidant effect but also via induction of the ghrelin biosynthesis, as it was documented by significant increase in ghrelin mRNA expression.

Key words: gastric ulcers, melatonin, ghrelin, rat

Peptic ulcers occur in that part of the gastrointestinal tract which is exposed to gastric acid and pepsin, i.e. the stomach and the first part of the small intestine, duodenum. However, the etiology of peptic ulcers is not clearly known (Panda and Sonkamble 2012).

Reactive oxygen species (ROS) play an important role in gastric ulceration, induced by several kinds of stresses. They also decrease the level of antioxidants, such as reduced gluthathione (GSH) and ascorbate, which make mucosa more prone to oxidative damage (Sudjarwo 2005).

Melatonin has been reported to protect the gastric mucosa against acute lesions and to influence the gastrointestinal motility and secretions. The melatonin protection mechanisms have been attributed to its scavenging of ROS and its ability to enhance the gastric blood flow to attenuate lipid membrane peroxidation and hydroxyl radical damage, neutrophil-induced infiltration of the gastric mucosa, and oxygen intermediates cytotoxicity (Otsuka et al. 2001).

Melatonin is not only a non-enzymatic scavenger, but also an inducer of antioxidative enzymes such as

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superoxide dismutase (SOD), catalase (CAT), and gluthatione peroxidase (GSH-PX). It also stabilizes lipid membranes and defends them from peroxidation, particularly due to its high lipophilicity and easy entry into the cells to protect their subcellular compartments (Costa et al. 1995).

Ghrelin, a 28-amino acid peptide, isolated from rat and human stomach, possesses strong growth hormone-releasing activity and plays central as well as peripheral roles in food intake, gastric motility, and acid secretion (Nakazato et al. 2001). The majority of circulating ghrelin appears to be of gastric origin. Following gastrectomy, the plasma concentration of ghrelin falls to about 35% of regular levels (Ariyasu et al. 2001).

The aim of this work was to reveal the underlying mechanisms of melatonin in treatment of gastric ulcers induced by ethanol and its relation to ghrelin biosynthesis in rats.

Materials and Methods

Animals. Sixty male Wistar rats (obtained from Faculty of Medicine, Minia University, Egypt), weighing 175-200 g, were used in this study. The experiment was approved by the Institutional Animal Ethics Committee (IAEC). The animals were housed in stainless steel cages and kept under constant environmental conditions for acclimatization. Food (ground wheat, soybean-meal-based diet) and water were provided ad libitum. One week before the experiment the rats were subjected to light and dark regimen and 18 h before experiment the food was withheld in all groups.

The animals were divided into 3 groups (n=20/group): 1) control group - animals in this group did not receive any medication and were fed with standard laboratory diet, 2) ulcer group - animals in this group received a single dose of 1 ml of 100% ethanol/100 g of b.w. after 18 h of fasting. Ethanol was administered i.g., by means of a metal orogastric tube (Khosla et al. 2004), and 3) melatonin-treated group - animals in this group received i.g. a single dose of melatonin (25 mg/kg, Biovea) 30 min before ulcer induction with ethanol (Bubenik 2006).

The animals were sacrificed 60 min after ulcer induction. Blood samples were taken and immediately centrifuged. The sera were collected and kept at -80°C until used.

Determination of gastric ulcer. Stomach tissues were excised, washed in ice-cold saline, opened along the greater curvature, and re-washed with cold saline to reveal the macroscopical mucosal lesions (Valcavi et al. 1982). Then, the tissue was divided into two parts; one was kept frozen for biochemical assays of GSH and MDA in the scraped mucosa and the other one was kept in 10% formalin for histological examinations.

Measurement of tissue reduced GSH and MDA was performed by colorimetric method according to manufacturer's instructions (Bio Diagnostic assay kit).

Tissue samples of the stomach mucosa (100-150 mg) were homogenized in a solution containing ice-cold 50 mM Tris HCl, pH 7.4, 320 mM sucrose, 0.1 mM EDTA, 1 mM dithiothreitol, and 10 mg/ml trypsin inhibitor (Buege and Aust 1978), for 20 sec.

Principle of GSH measurement. The method is assembled based on the reduction of 5,5` dithiobis (2 - nitrobenzoic acid) (DTNB) with GSH to produce a yellow compound. The reduced chromogen is proportional to GSH concentration and its absorbance was measured at 405 nm. The reduced GSH concentration in tissue was calculated as follows:

$$[GSH]\ (mg/g) = A_{sample} \times 66.66\ (mg/dl)/g\ tissue\ used.$$

Principle of MDA measurement. Thiobarbituric acid (TBA) was reacted with MDA in acidic medium at temperature of 95°C for 30 min to form thiobarbituric acid reactive product. The absorbance of the resultant pink product was measured at 534 nm. The MDA concentration in the tissue was calculated as follows:

$$[MDA]\ (mg/g) = A_{sample}/A_{standard} \times 10/g\ tissue\ used.$$

Measurement of ghrelin. Determination of ghrelin serum levels was carried out by rat/mouse ghrelin ELISA KIT 96-Well Plate (Cat. No. EZRGRA-90K, USA) according to manufacturer's instructions. Absorbance was read at 450 nm in a plate reader within 5 min.

Total RNA isolation. Total RNA was extracted from 1 g gastric mucosa of stomach tissues in RiboZol solution (Amresco, Solon, USA) according to the manufacturer's instructions. RNA samples were stored at -80°C until being analyzed.

Reverse transcriptase-polymerase chain reaction for ghrelin (RT-PCR). RT-PCR was performed according to manufacturer's instructions (Qiagen one step). Reaction tube of RT-PCR contained 5 µl of RT-PCR buffer 5×, 10 pM of specific primers for ghrelin or 18 S, 5 µg of total RNA template, 1 µl dNTPs mix (100 mM), 1 µl enzyme mix (Hot Star Taq DNA polymerase, Omniscipt and Sensiscript reverse transcriptases).

RT PCR protocol for ghrelin was carried out as follow: 50°C, 30 min for first strand synthesis, 95°C, 15 min for RTase Blend inactivation and initial denaturation, followed by 33 cycles of 94 for 1 min., 64°C for 45 s and 72°C for 2 min. Final extension was done at 72°C for 10 min. The 2 sets of primers used were as follow, ghrelin sense: 5'-TTG AGC CCA GAG CAC CAG AAA-3' and antisense 5'-AGT TGC AGA GGA GGC AGA AGC-3'. 18 S sense: 5'TTGACGGAAGGGCACCACCAG3' and antisense 5' GCACCACCACCCACGGAATCG 3'. The protocol of RT-PCR for rat 18 S was carried out as described for ghrelin RT-PCR except that the number of cycle 30 and the annealing temperature was set to 55°C. The PCR was done in a thermal cycler (Progene, Techne, Cambridge, LTD., UK).

Detection of PCR product. Polymerase chain reaction products (5 µl) were detected by electrophoresis on a 1.5% agarose gel containing ethidium bromide. Location of a predicted product was confirmed by using 100 bp ladder as a standard size marker. The density of PCR product was measured using Scion Image J Soft Ware (Scio Cooperation, Fredrick, Maryland). The negative control tube contained all components as in RT-PCR reaction tube except the total RNA template. All primers were obtained from Richard V. Case, Ph.D. Midland Certified Reagent Company Inc. of Midland, Texas.

Histopathological examination. Tissue specimens were taken from the stomach tissue mucosa in the 3 groups of rats and then fixed in 10% formalin for 24 h. Trimming was done on the fixed tissue specimens washed in tap water for 12 h. Serial ethyl alcohols were used for dehydration of the tissue samples. Tissue specimens were cleared in xylene and embedded into paraffin. The paraffin blocks were cut into 3 µm thick sections by sledge microtome. Then, the sections were collected on glass slides and stained by heamatoxylin and eosin (H&E). Histopathological evaluations were performed by the light microscope (Gridley 1960).

Statistical analysis. The data were expressed with SPSS version 13 soft ware as the mean ± SE, analysis of variance (one way ANOVA) indicated a significant at ($p<0.05$). Independent t-test was used for parametric quantitative data. Analysis of variance (one way ANOVA) was used to compare the expression of ghrelin mRNA.

Results

Effect of pretreatment with melatonin on gastric mucosal level of MDA, reduced GSH, and ghrelin serum level. Table 1 shows a significant decrease in ghrelin level in ulcer group of rats in comparison with controls (2276 ± 120 vs. 3011 ± 140 pg/ml; $p<0.05$), and a significant increase after melatonin treatment in comparison with ulcer group (2654 ± 124 vs. 2276 ± 120 pg/ml; $p<0.05$). The MDA level in tissues was significantly increased in ulcer group of rats in comparison with controls (66.8 ± 9.1 vs. 37.4 ± 3.6 mg/g tissue; $p<0.05$), and significantly decreased after melatonin treatment in comparison with ulcer group of rats (34.03 ± 3.6 vs. 66.8 ± 9.1 mg/g; $p<0.05$). Table 1 also shows a significant decrease in reduced GSH level in ulcer group of rats in comparison with controls (0.670 ± 0.055 vs. 1.250 ± 0.076 mg/g tissue; $p<0.05$) and a significant increase after melatonin treatment in comparison with ulcer group of rats (0.99 ± 0.08 vs. 0.67 ± 0.055 mg/g tissue; $p<0.05$).

Mean expression of ghrelin mRNA. Table 2 shows a significant decrease in ghrelin level in ulcer group of

Table 1

Ghrelin, MDA and reduced glutathione levels in different groups

Parameter	Control	Ulcer group	Treated group	P1	P2	P3
Ghrelin (pg/ml)	3011±140	2276±120	2654±124	0.001*	0.001*	0.005*
Mean expression of ghrelin mRNA	56.2±0.2	25±0.3	34±0.5	0.001*	0.001*	0.001*
MDA (mg/g tissue)	37.4±3.6	66.8±9.1	34.03±3.6	0.001*	0.001*	0.001*
Reduced GSH (mg/g tissue)	1.25±0.076	0.67±0.055	0.99±0.08	0.001*	0.001*	0.001*

P1= Control vs. ulcer group
P2= Control vs. treated group.
P3= Ulcer vs. treated group
(*) p value significant at < 0.05

Table 2

Rat ghrelin mRNA expression in mucosa of stomach tissue of different groups

Group	%
Control	100
Treated	65
Ulcer	45

rats in comparison with controls (25.0 ± 0.3 vs. 56.2 ± 0.2; $p<0.05$), and a significant increase after melatonin treatment in comparison with ulcer group of rats (34.0 ± 0.5 vs. 25.0 ± 0.3; $p<0.05$) as detected by RT-PCR method (Fig. 1).

Histological evaluations. Macroscopic study showed normal mucosal pattern in control group, extensive hemorrhage and ulcer occurrence in ulcer group, and healing of ulcers and some hemorrhage in treated group of rats (Fig. 2). Pathological study showed regular integrity of epithelium in control group, severe congestion of vessels in ulcer group, and reduced congestion and fewer inflammatory cells in treated group of animals (Fig. 3).

Discussion

The diversity of etiological factors underlying gastric ulcers and the complex nature of pathways participating in healing always make the peptic ulcer treatment a complicated challenge. Maintaining the equilibrium between aggressive and defensive factors is always a critical objective in the peptic ulcer management (El-Moselhy et al. 2009).

The physiological role of melatonin generated in the digestive system has been shown and the literature data indicate that luminal melatonin is a potent stimulant of the duodenal bicarbonate secretion in response to gastric acid entering the duodenum. Melatonin has been also implicated in the regulation of interdigestive motility patterns demonstrating that it is able to accelerate intestinal transit after the feeding (Drago et al. 2002).

Stress induced gastric ulceration is associated with the increase of lipid peroxide and depletion of endogenous GSH due to increase formation of ROS, a condition suitable for the generation of H_2O_2 (Das and Banerjee 1993). Melatonin displays antioxidant activity, protecting cells against reactive oxygen species. It has been previously shown that melatonin may decrease the oxidative DNA damage in gastrocytes (Klupinska et al. 2009). It may prevent the development of gastric ulcers by enhancing the protective properties of the mucosal barrier. Melatonin manifests in anti-inflammatory activities by inhibiting the production of pro-inflammatory cytokines. It also elicits an immunomodulatory activity. Moreover, it displays a relaxation action on smooth muscles in the gastrointestinal tract. The indole structure blocks the

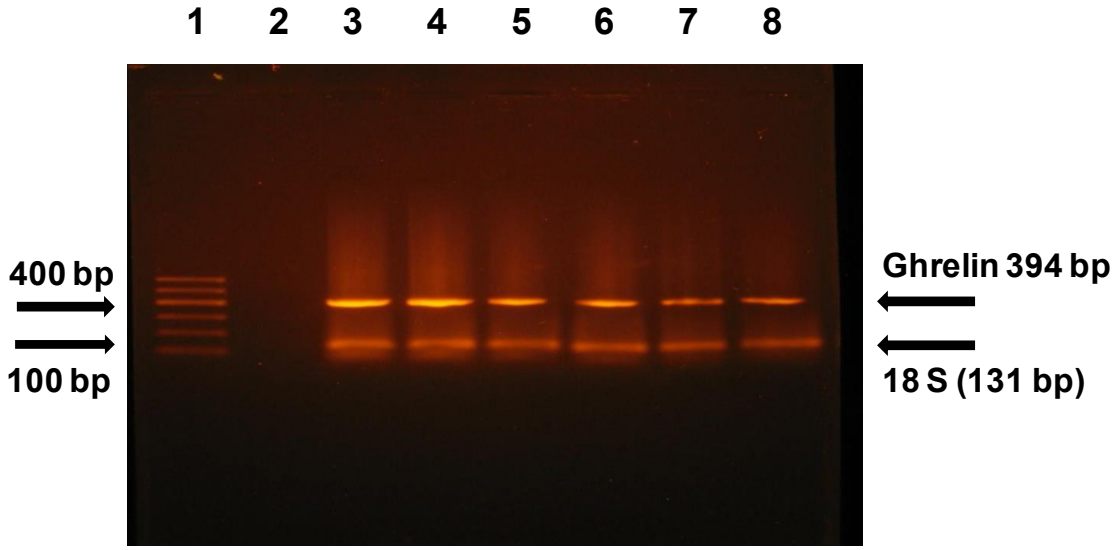

Fig. 1. Agarose gel electrophoresis of RT-PCR product showing rat ghrelin expression (394 bp) in stomach tissue of different groups.
Lane 1 - DNA ladder (100 bp ladder), lane 2 - negative control, lanes 3, 4 - the first group (control group), lanes 5, 6 – the third group (melatonin-treated group), lanes 7, 8 - the second group (ulcer group). 18 S is shown in lanes 3, 4, 5, 6, 7 and 8 at 131 bp.

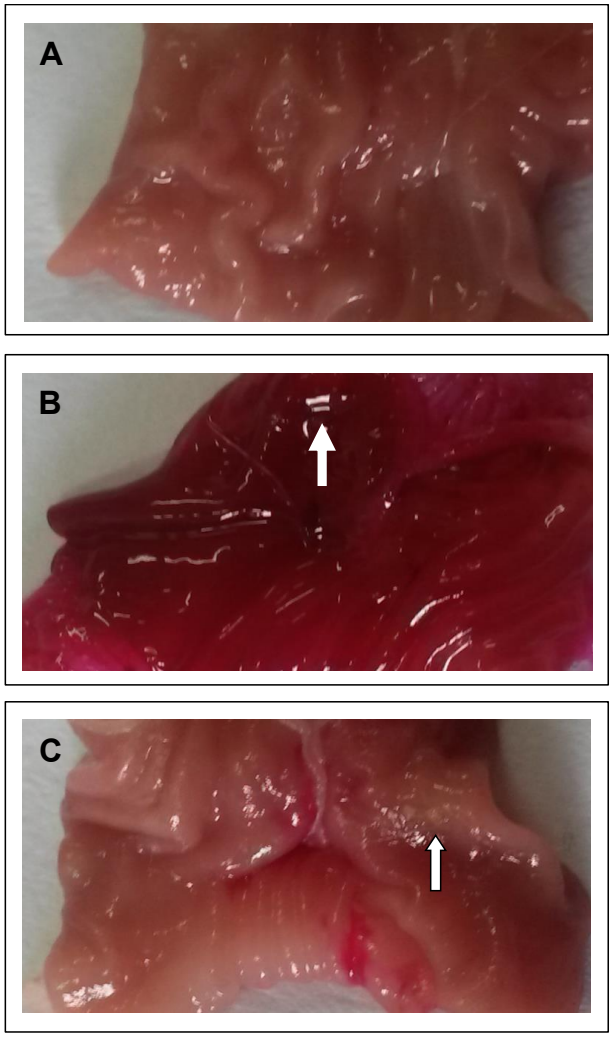

Fig. 2. A) Control group: normal macroscopic structures of examined stomach were seen without any abnormalities. Normal mucosal pattern. B) Ulcer group: the gastric mucosa was focally necrotic, ulcerated (arrow), and extensive hemorrhages. The mucosal were congested and the mucosal coat was hypertrophied. C) Treated group: mucosal congestion (arrow), slight desquamation of the lining epithelium, and few hemorrhages.

nicotinic acetylcholine receptors on the nerve endings of the submucosal plexi and activates the afferent vagal fibers via increased release of cholecystokinin and activation its receptors (Celinski et al. 2011a, b).

Brzozowski et al. (2000, 2005) have also shown that exogenous melatonin and its precursor, l-tryptophan, may attenuate the water immersion and restraint (WRS) induced gastric lesions via interaction with melatonin MT_2 receptors. This protective action of melatonin is assigned to an enhancement in the gastric microcirculation, probably mediated by prostaglandin E2 (PGE2) de-

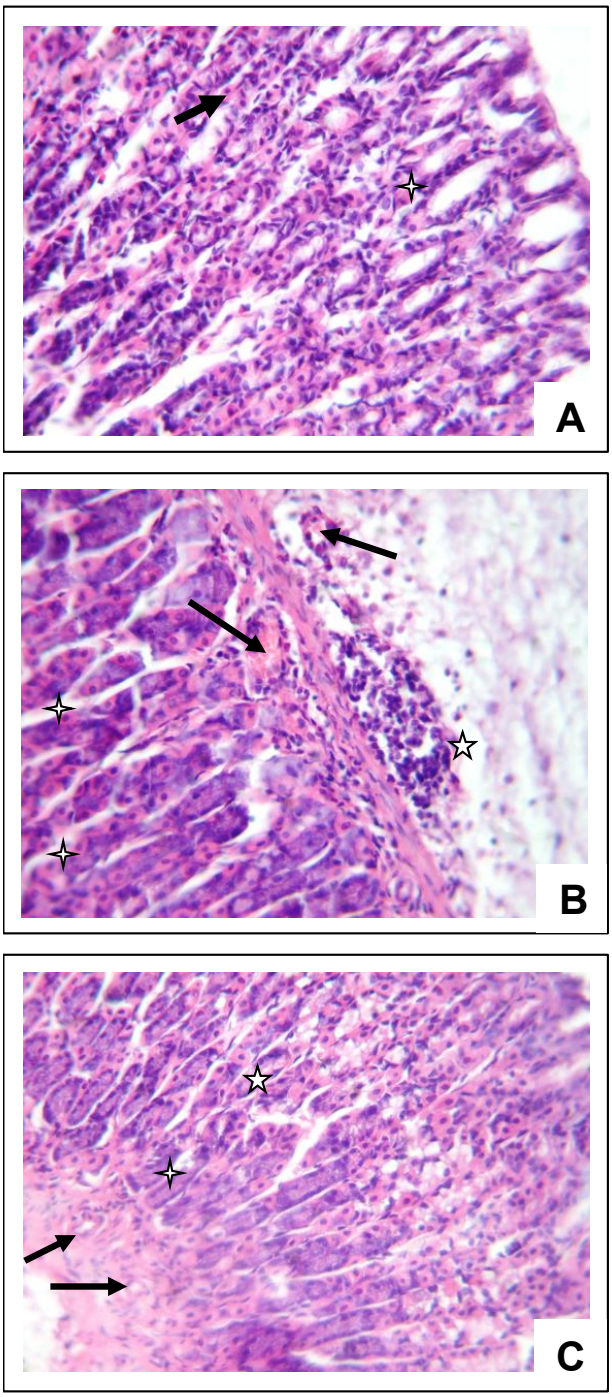

Fig. 3. A) Control group: the epithelium shows regular integrity (cross) with intraepithelial dilations (arrow). B) Ulcer group shows severe congestion of vessels in the connective tissue of both the subepithelium and lamina propria (arrow); prominent dilations in the epithelia (cross) and marked density of inflammatory cells were observed (star). C) Treated group shows congestion of vessels in the connective tissues was reduced (arrow) and fewer inflammatory cells were present (star); dilations in the surface epithelia were still ongoing (cross). H&E x 200.

rived from cyclooxygenase 2 (COX-2) overexpression, the activation of brain-gut axis, involving calcitonin gene related peptide released from sensory nerves, and the release of gastrin.

Ghrelin, a 28-amino acid peptide isolated from rat and human stomach, possesses strong growth hormone-releasing activity and plays central as well as peripheral roles in food intake, gastric motility, and acid secretion. Ghrelin has been shown to evoke weight gain by actions in the hypothalamus. It has been found that the plasma ghrelin concentrations rise before the meal and fall after the meal intake (Nakazato et al. 2001). The majority of the circulating ghrelin appears to be of gastric origin. Following gastrectomy, the plasma concentration of ghrelin falls by about 35% of normal levels (Ariyasu et al. 2001).

Besides these physiologic activities, ghrelin exerts a gastro-protective effect during pathological conditions. Indeed, in rats, *in vivo* administration of ghrelin attenuates the gastric mucosal lesions induced by detrimental agents, such as ethanol and indomethacin, through an increase of mucosal generation of PGE2 (Chen et al. 2010). Ghrelin is also an important regulator of nitric oxide synthase (NOS) and COX-2 (an enzyme responsible for inflammation and pain) enzyme systems (Slomiany and Slomiany 2010). Moreover, studies in different animal models revealed that ghrelin reduces the release of pro-inflammatory cytokines, such as interleukin (IL)-1β, tumor-necrosis factor-α (TNF-α), IL-6, and stimulates the expression of the anti-inflammatory cytokine IL-10 by T lymphocytes and macrophages in different mechanical or chemical-induced inflammatory conditions. Treatment of human T lymphocytes and monocytes with exogenous ghrelin inhibits the release of pro-inflammatory cytokines such as IL-1β, TNF-α and IL-6 (Paoluzi et al. 2014).

In the present work, we aimed to examine the different mechanisms of gastroprotective effect of melatonin, including the interaction between melatonin and ghrelin biosynthesis. Ethanol was used for acute ulcer production and contrarily, the external melatonin was used for the protection. Our results showed that ghrelin content in ulcer group of animals was significantly lowered in comparison with the control group. Similarly, previously published results (Sibilia et al. 2003) have shown that multiple ulcers occur in rats when their gastric mucosa is exposed to ethanol. This makes reasonable to assume that alcohol may have a toxic effect on ghrelin-secreting cells in the stomach, resulting in decreased secretion of this hormone (Sibilia et al. 2003).

Our study showed that the treatment of rat stomach with melatonin leads to a significant increase in the gastric mucosal expression of ghrelin mRNA level indicating that ghrelin, besides its recognized role in the control of appetite, energy balance, and gastric motility, appears to be an important gastroprotective factor expressed locally in the gastric mucosa in response to mucosal injury (Konturek et al. 2008). Many researchers have reported that peptic ulcer may cause a marked reduction in mRNA ghrelin levels. The alteration of plasma appetite-controlling hormones of gastric origin may contribute to the changes of appetite or dyspeptic symptoms in people with peptic ulcer (Konturek et al. 2008).

Inflammation of the gastric mucosa is also one of the important mechanisms of change in ghrelin production, greater inflammation in Helicobacter pylori - infected gastric mucosa resulted in less ghrelin production. Furthermore, it has been found that ulcer healing is an important factor in ghrelin production (Paoluzi et al. 2014).

Involvement of oxygen derived free radicals, such as the superoxide anion, hydrogen peroxide, and hydroxyl radical, is well established in the pathogenesis of ischaemic injury of gastrointestinal mucosa and in other models of mucosal damage, induced by ethanol (Mani Senthil Kumar et al. 2012). Depletion of gastric mucosal GSH may result in the accumulation of free radicals that can initiate membrane damage by lipid peroxidation (Demir et al. 2003). The present study showed lower gastric mucosal GSH levels in rats with peptic ulcer. Maity et al. (1998) have suggested that reduced glutathione may play a major role in the cytoprotection against ulceration. Our findings are in concordance with these observations. The observation that melatonin could reverse the GSH level to a great extent provides evidence for the involvement of GSH in the anti-ulcer activity of melatonin.

In conclusion, this study demonstrates that melatonin possesses a potent ulcer healing effect, which appears to be related to the MDA decrease and increase of reduced glutathione. Improvement of the antioxidant status of the stomach in addition to other protective effects of melatonin, including anti-inflammatory and immunoregulatory effects, enhancement of gastric microcirculation, and increased PGE2, help the ghrelin-secretory cells to overcome the toxic effect of ethanol and increased ghrelin expression, which add more gastroprotective effects.

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