

## Protective effects of an interaction between vagus nerve and melatonin on gastric ischemia/reperfusion: the role of oxidative stress

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### ABSTRACT

**Objectives:** Vagal pathways in gastrointestinal tract are the most important pathways that regulate ischemia/reperfusion (I/R). Gastrointestinal tract is one of the important sources of melatonin production. The aim of this study was to investigate probable protective effect of the interaction between vagus nerve and melatonin after I/R.

**Materials and methods:** This study was performed in male rats that were divided into six groups. Cervical vagus nerve was cut bilaterally after induction of I/R and the right one was stimulated by stimulator. Melatonin or vehicle was injected intraperitoneally. The stomach was removed for histopathological and biochemical investigations.

**Results:** A significant decrease in infiltration of gastric neutrophils and malondialdehyde (MDA) level after I/R was induced by melatonin and was disappeared after vagotomy. The stimulation of vagus nerve significantly enhanced these effects of melatonin. However, a stimulation of vagus nerve alone increased neutrophils infiltration and MDA level. Melatonin significantly increased the activities of catalase, glutathione peroxidase (GPx), superoxide dismutases (SOD). Unlike stimulation of vagus nerve, vagotomy decreased these effects of melatonin.

**Conclusion:** According to these results, it is probable that protective effects of melatonin after I/R may be mediated by vagus nerve. Therefore, there is an interaction between melatonin and vagus nerve in their protective effects.

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### Introduction

The protective effects of vagus nerve during ischemia/reperfusion (I/R) have been suggested (1). Vagus nerve stimulation decreases the inflammation and the injuries resulted from I/R through affecting neutrophils while vagotomy increases these processes (1). Moreover, it has been observed that stimulation of vagus nerve can cause protective effects on gastric injury through releasing prostaglandins, nitric oxide (NO) and calcitonin gene related peptide (CGRP) (2). It is probable that vagus nerve exerts its gastric protective effects through releasing the aforementioned mediators. According to a report, vagus nerve acts through hypothalamic-

pituitary-adrenal axis. Also, it has been reported that cholinergic activity of the efferent vagus nerve can participate in immunity modulation (3). Another study has shown that nicotine in monocytes not only decreases the production of pre-inflammatory cytokines, but also changes the response to IL-10, an anti-inflammatory cytokine (4, 5). Cho *et al* have shown that alternative stimulation of cervical vagus nerve increases intra-gastric pressure and also causes bleeding ulcers in mucosal glands of stomach, and these effects can be prevented by atropine administration or sub-diaphragmatic vagotomy (6). It has been reported that gastric vagotomy is effective in treating gastric and duodenal ulcers (7).

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Melatonin as a neurohormone is basically produced in the pineal gland and also in epiphysis, gastrointestinal (GI) mucosa and other organs (8-10). Melatonin has several physiological activities such as controlling circadian rhythm, sleep induction, regulation of seasonal reproduction, and improvement of immunity. The most important effect of melatonin is an anti-oxidant effect that protects living organisms against oxidative stress (11).

In several studies, the ability of melatonin in decreasing molecular injury has been shown during I/R. Melatonin prevents myocardial infarction, necrotic cell death and renal abnormality after I/R (8, 12), reduces brain edema in ischemia (12), and prevents acute gastric ulcers resulted from stress. These effects can be done through direct reactive oxygen species/ reactive nitrogen species (ROS/RNS) detoxification, increase in anti-oxidative enzymes, and the prevention of electron leakage in mitochondrial inner membrane (11, 13).

Interaction between melatonin and vagus nerve has also been reported in other organs. Melatonin has a role in expression and function of nicotinic acetylcholine receptors and increases the efficacy of beta bungarotoxin-sensitive acetylcholine receptors (14). It has been recognized that intracerebral injection of melatonin is effective in prevention of acid and pepsin secretion through cholinergic activity (15). It has been identified that 5HT<sub>3</sub> and 5HT<sub>2</sub> receptors are involved in stimulatory effects of melatonin in releasing pancreatic enzymes (11).

Protective effects of melatonin are not only due to antioxidant activity, but it also activates capsaicin-sensitive afferent fibers (1, 16). Melatonin may have a potential impact on the treatment of peptic ulcer via significant increase in ghrelin expression (17). Melatonin increases the release of pancreatic amylase and proteins through vagus nerve (18). The effects of melatonin on pancreatic enzymes are reversed by vagotomy and administration of capsaicin (18, 19). Intra-lumen administration of melatonin increases plasma cholecystokinin (CCK) and antioxidants (11, 19, 20). It has been shown that sensory nerve fibers are involved in protective effects of melatonin in the healing of acute gastric injury and peptic ulcer (16).

It has been reported that vagus nerve increases the secretion of bicarbonate from duodenum mucosa through an increase in melatonin secretion (21). Also, vagus stimulation can affect the secretion of melatonin (22).

Vagal pathways are the most important regulatory pathways in the gastrointestinal tract which is one of the most important sources of

melatonin production. In previous studies, both protective and harmful effects of the stimulation of vagus nerve in digestive system have been reported. The role of sensory neurons in protective effects of melatonin in the healing of acute gastric injury and peptic ulcer has been reported. There is no study about the combined effects of melatonin administration and intervention of vagus nerve during gastric I/R injury. Probably, vagus nerve and melatonin have interactions in their protective effects during gastric I/R events so, the present study was performed to examine this interaction during gastric I/R.

## Materials and Methods

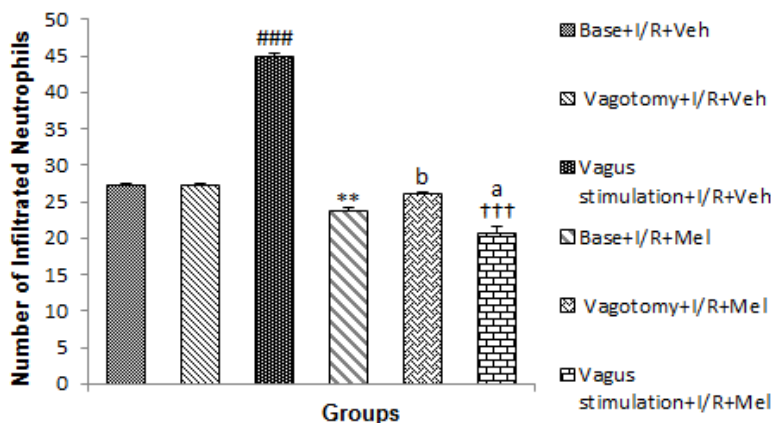
This study was performed in 42 male Wistar rats weighing 180-220 g. Animals were kept at room temperature 20-22 ° C and 12 hr-12 hr light-dark cycle. Animals had free access to water and food and were randomly divided into 6 groups of 7 rats. Rats were fasted for 12 hr prior to the experiment but had access to water. Experimental groups were: 1) Base+I/R+vehicle; 2) Base+I/R+melatonin; 3) Vagotomy+I/R+vehicle; 4) Vagotomy+I/R+melatonin; 5) Vagus nerve stimulation+I/R+vehicle; 6) vagus nerve stimulation+I/R+ melatonin. Base, means condition that neither vagus nerve was stimulated nor vagotomy was performed.

### Vagus nerve stimulation

Animals underwent tracheostomy and were cannulated (in order to prevent probable airway occlusion) under anesthesia induced by intraperitoneal pentobarbital (50 mg/kg). After local shaving, a small incision was made in cervical area and branches of cervical vagus nerve were carefully dissected from carotid artery and cut (1). It was then covered with normal saline-dipped cotton. After dissection of vagus nerve, distal end was covered with mineral oil and stimulated with bipolar electrode of a stimulator using 10 volt/msec pulses at the frequencies of 0.625, 1.25, 2.5, 5 or 7.5Hz for 30 sec. Stimulations were performed with 2-min intervals (23). In order to ensure the stimulation of vagus nerve, electrocardiogram was recorded by power lab instrument and saved each 10 sec (24).

### Ischemia/reperfusion

Gastric I/R injury was induced by dissecting celiac artery from the surrounding tissues in rats anesthetized with 50 mg/kg pentobarbital, 30 minutes after dissection of vagus nerve. Then, celiac artery was closed with a microvascular clamp and returned to its place and the area was sutured using 4-0 silk. Occlusion was continued for 30 min (18) followed by a 3-h circulation (reperfusion). Melatonin (10 mg/kg) was injected



**Figure 1.** The number of neutrophils in gastric tissue of the study groups (n=7). \*\* $P < 0.01$ : Base+I/R+Mel group vs. Base+I/R+Veh group. ### $P < 0.001$ : Vagus stimulation+I/R+Veh group vs. Vagotomy+I/R+Veh group. ††† $P < 0.001$ : Vagus stimulation+I/R+Mel group vs. Vagus stimulation+I/R+Veh group. a  $P < 0.05$ : Vagus stimulation+I/R+Mel group vs. Base+I/R+Mel group. b  $P < 0.05$ : Vagotomy +I/R+Mel group vs. Base+I/R+Mel group. Base: condition that neither vagus was stimulated nor vagotomy was performed; I/R: ischemia/reperfusion; Mel: melatonin; Veh: vehicle

intraperitoneally before reperfusion in groups 5, 6 and 7 (18, 25). Melatonin was dissolved in 1% ethanol, diluted with 0.9% saline, and injected at a final volume of 0.3 ml (18).

#### Measuring malondialdehyde (MDA) level, and glutathione peroxides (GPX), catalase and superoxide dismutase (SOD) activities

The samples of stomach tissue were taken from fundus region of anesthetized rats. One part was kept in 10% formalin solution for histopathological assessment and the rest was kept at  $-70^{\circ}\text{C}$  for evaluation of oxidant and antioxidant factors (18, 25). MDA level was measured according to Hiroshi Ohkawa method via reaction with thiobarbituric acid and color formation. Optical absorbance was determined at 532nm and MDA level was reported as nmol/mg protein (26). Glutathione peroxides activity was determined by the method described by Valentine and Paglia method (20), and enzyme activity was reported as U/mg protein. Superoxide dismutase activity was determined using Ransod kit (Randox, UK) and enzyme activity was reported as U/mg protein (19). Catalase activity was determined by the method described by Mari and reported as U/mg protein (20).

#### Infiltration of neutrophils

Two paraffin blocks of gastric tissue were selected for neutrophil counting. The H & E-stained sections were examined under low power (40x) to identify the areas of neutrophil aggregates within all tissue blocks. The number of neutrophils was calculated in a semiquantitative manner using the

mean value of 20 non-overlapping high power fields (HPF; magnification of 400x;  $0.08\text{ mm}^2$ ) by using a 40x objective and a square grid mounted in a 10x microscopic eyepiece.

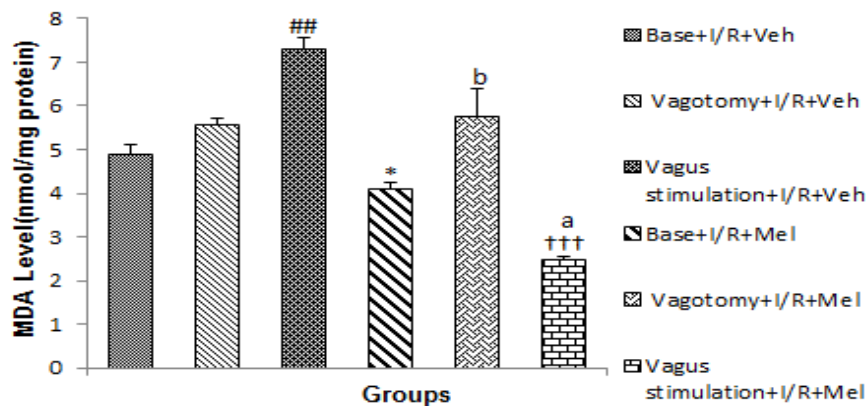
#### Statistical analysis

Values were presented as mean±SEM. Comparisons among different groups were made by one-way and two-way ANOVA followed by *post hoc* Tukey's test. *P*-value considered significant if was  $< 0.05$ .

## Results

#### Infiltration of neutrophils

Neutrophils infiltration in study groups is shown in Figure 1. Mean number of neutrophils in base+I/R+ melatonin group ( $23.8 \pm 0.37$ ) was lower than that in base+I/R+ vehicle group ( $27.2 \pm 0.37$ ) ( $P < 0.01$ ). Also, this parameter in vagus stimulation + I/R+ vehicle group ( $45 \pm 0.32$ ) was higher than that in base+I/R+ vehicle and vagotomy+I/R+ vehicle groups ( $27.2 \pm 0.36$ ) ( $P < 0.001$ ). A significant decrease in neutrophils count was indicated in vagus stimulation+I/R+melatonin group ( $20.6 \pm 0.4$ ) in comparison to vagus stimulation+I/R+vehicle group ( $P < 0.001$ ). This parameter was significantly low in vagus stimulation+I/R+ melatonin group as compared to base+I/R+ melatonin group ( $P < 0.01$ ). A significant increase in neutrophils count was shown in vagotomy+I/R+melatonin group ( $26 \pm 0.32$ ) as compared to base+I/R+melatonin group ( $P < 0.05$ ).



**Figure 2.** MDA level in gastric tissue of the study groups (n=7). \* $P<0.05$ : Base+I/R+ Mel group vs. Base +I/R+ Veh group. ## $P<0.01$ : Vagus stimulation+I/R+Veh group vs. Vagotomy+I/R+Veh group. ††† $P<0.001$ : Vagus stimulation+I/R+Mel group vs. Vagus stimulation+I/R+Veh group. a  $P<0.01$ : Vagus stimulation+I/R+Mel group vs. Base+I/R+Mel group. b  $P<0.05$ : Vagotomy+I/R+Mel group vs. Base+I/R+Mel group. Base: condition that neither vagus was stimulated nor vagotomy was performed; I/R: ischemia/reperfusion; Mel: melatonin; Veh: vehicle

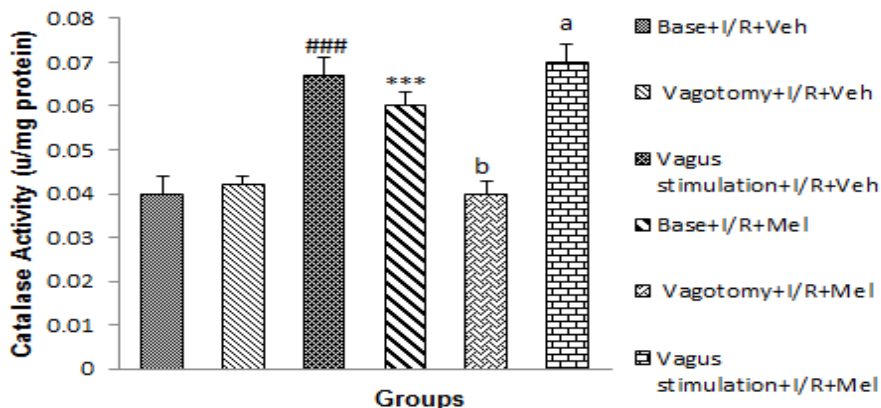
**MDA level**

MDA level in the study groups is presented in Figure 2. As it is seen, MDA level in gastric tissue was lower in base+I/R+melatonin group ( $4.09\pm 0.15$  nmol/mg protein) in comparison with base+I/R+vehicle group ( $5.53\pm 0.11$  nmol/mg protein) ( $P<0.05$ ). Moreover, a significant increase in MDA level was shown in vagus stimulation+I/R+vehicle group ( $7.31\pm 0.25$  nmol/mg protein) in comparison with base+I/R+vehicle and vagotomy+I/R+vehicle ( $5.57\pm 0.14$  nmol/mg protein) groups ( $P<0.01$ ). This parameter was reduced in vagus stimulation+I/R+melatonin group ( $2.48\pm 0.06$  nmol/mg protein) in comparison with vagus stimulation+I/R+vehicle group ( $P<0.001$ ). Also, MDA level was lower in vagus stimulation+I/R+melatonin group ( $2.48\pm 0.06$  nmol/mg protein) in comparison with base+I/R+melatonin group ( $P<0.01$ ). MDA level was higher in vagotomy+I/R+melatonin group ( $5.75\pm$

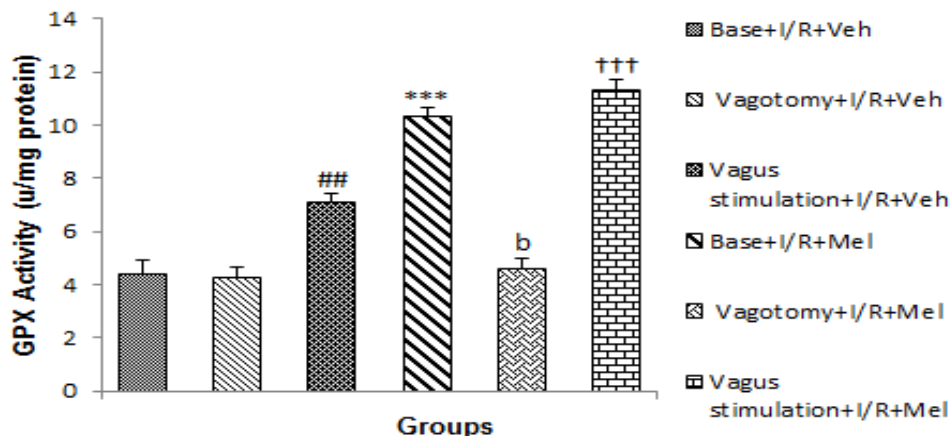
$0.63$  nmol/mg protein) in comparison with base+I/R+melatonin group ( $P<0.05$ ).

**Catalase activity**

Catalase activity in the study groups are shown in Figure 3. Catalase activity was increased in base+I/R+melatonin group ( $0.06\pm 0.003$  U/mg protein) in comparison with base+I/R+vehicle group ( $0.04\pm 0.003$  U/mg protein) ( $P<0.001$ ). A significant increase in catalase activity was shown in vagus stimulation+ I/R+vehicle group ( $0.07\pm 0.004$  U/mg protein) in comparison with base+I/R+vehicle and vagotomy+I/R+vehicle groups ( $0.042\pm 0.002$  U/mg protein) ( $P<0.001$ ). This parameter was increased in stimulation vagus+I/R+melatonin group ( $0.07\pm 0.004$  U/mg protein) in comparison with base+I/R+melatonin group ( $P<0.05$ ). Catalase activity was lower in vagotomy+I/R+melatonin group ( $0.04\pm 0.003$  U/mg protein) in comparison with base+I/R+melatonin group ( $P<0.05$ ).



**Figure 3.** Catalase activity (U/mg protein) in gastric tissue of the study groups (n=7).\*\*\* $P<0.001$ : Base+I/R+Mel group vs. Base+I/R+Veh group. ### $P<0.001$ : Vagus stimulation+I/R+Veh group vs. Vagotomy+I/R+Veh group. a  $P<0.01$ : Vagus stimulation+I/R+Mel group vs. Base+I/R+Mel group. b  $P<0.001$ : Vagotomy+I/R+Mel group vs. Base+I/R+Mel group. Base: condition that neither vagus was stimulated nor vagotomy was performed; I/R: ischemia/reperfusion; Mel: melatonin; Veh: vehicle



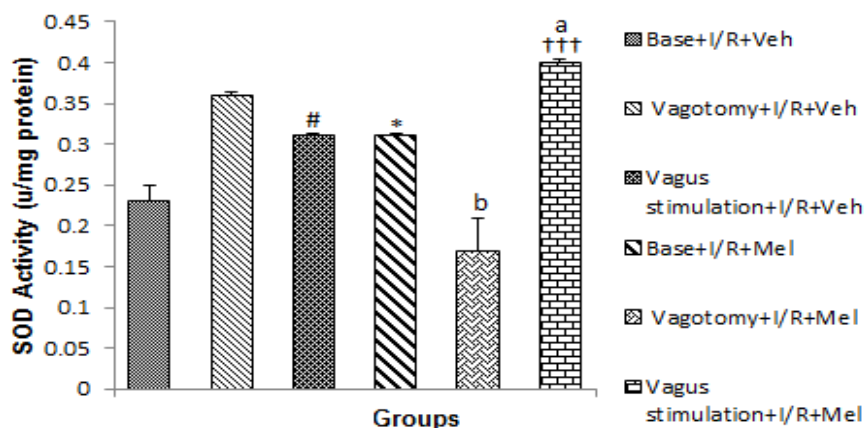
**Figure 4.** Level of GPX activity (u/mg protein) in gastric tissue of the study groups (n=7) \*\*\* $P < 0.001$ : Base+I/R+Mel group vs. Base+I/R+Veh group. ## $P < 0.01$ : Vagus stimulation+I/R+Veh group vs. Vagotomy+I/R+Veh group ††† $P < 0.001$ : Vagus stimulation+I/R+Veh group vs. Vagus stimulation+I/R+Mel group. b  $P < 0.001$ : Vagotomy +I/R+ Mel group vs. Base+I/R+Mel group. Base: condition that neither vagus was stimulated nor vagotomy was performed; I/R: ischemia/reperfusion; Mel: melatonin; Veh: vehicle

#### Glutathione peroxidase (GPX) activity

Figure 4 shows glutathione peroxidase activity in the studied groups. A significant increase in GPX activity was shown in gastric tissue of basal+I/R+melatonin group ( $10.31 \pm 0.37$ ) ( $P < 0.001$ ) in comparison with that in basal+I/R+vehicle group ( $4.42 \pm 0.57$ ). A significant increase in GPX activity was found in vagus stimulated+I/R+vehicle group ( $7.12 \pm 0.31$ ) as compared to that observed in basal+I/R+vehicle and vagotomy+I/R+vehicle groups ( $4.43 \pm 0.38$ ) ( $P < 0.001$ ). Also, a significant increase in GPX activity was indicated in vagus stimulated+I/R+melatonin group ( $11.32 \pm 0.37$ ) in comparison with vagus stimulated+I/R+vehicle group ( $P < 0.001$ ). This parameter was significantly lower in vagotomy+ I/R+melatonin group ( $4.61 \pm 0.4$ ) as compared to that in basal+I/R+melatonin group ( $P < 0.01$ ).

#### Superoxide dismutase (SOD) activity

SOD activity (U/mg protein) in gastric tissue of studied groups has been shown in Figure 5. As it is seen, this parameter in basal+I/R+melatonin group ( $0.31 \pm 0.004$ ) was significantly higher ( $P < 0.05$ ) than that in basal+I/R+vehicle group ( $0.23 \pm 0.016$ ). A significant decrease in SOD activity was shown in vagus stimulated+I/R+ vehicle group ( $0.31 \pm 0.004$ ) in comparison with that in vagotomy+I/R+vehicle group ( $0.36 \pm 0.003$ ) ( $P < 0.05$ ), but it was higher than that in basal+ I/R+vehicle group. SOD activity in vagus stimulated+I/R+melatonin group ( $0.4 \pm 0.005$ ) was significantly higher than that in vagus stimulated+I/R+vehicle and basal+I/R+melatonin groups ( $P < 0.001$  and  $P < 0.01$ , respectively). This parameter in vagotomy+I/R+melatonin group ( $0.17 \pm 0.04$ ) was lower than that in basal+I/R+melatonin group ( $P < 0.05$ ).



**Figure 5.** Superoxide dismutase activity (U/mg protein) in gastric tissue of the study groups (n=7) \* $P < 0.05$ : Base+I/R+Mel group vs. Base+I/R+Veh group. ## $P < 0.01$ : Vagus stimulation+I/R+Veh group vs. Vagotomy+I/R+Veh group. †††  $P < 0.001$ : Vagus stimulation+I/R+Mel group vs. Vagus stimulation+I/R+Veh group. a  $P < 0.01$ : Vagus stimulation+I/R+Mel group vs. Base+I/R+Mel group. b  $P < 0.05$ : Vagotomy +I/R+Mel group vs. Base+I/R+Mel group. Base: condition that neither vagus was stimulated nor vagotomy was performed; I/R: ischemia/reperfusion; Mel: melatonin; Veh: vehicle

## Discussion

The present study was performed to investigate the protective effect of interaction between melatonin and vagus nerve during gastric I/R. The results of this study showed that melatonin administration decreased neutrophils infiltration and MDA level and increased SOD, CAT and GPX activities in gastric tissue. Also, vagus stimulation increased neutrophils infiltration and MDA level in gastric tissue while melatonin administration along with vagus stimulation decreased the effect of vagus stimulation in gastric tissue. Finally, vagotomy prevented some of the protective effects of melatonin during gastric I/R.

According to the results of this study, melatonin can reduce gastritis during I/R, probably through decreasing the activity of oxidant enzymes and increasing the activity of anti-oxidant enzymes such as SOD, CAT and GPX. It has been reported that melatonin increases anti-oxidant activity in stomach. It has also been demonstrated that melatonin decreases MDA level (27-29). Other probable mechanisms underlying the protective effect of melatonin include an increase in gastric microcirculation (1), a reduction in acid and pepsin secretions (15), a reduction in production of inflammatory cytokines (16) and an increase in the expression of anti-oxidant gene (17, 30-32).

Moreover, in our study, a stimulation of vagus nerve increased gastritis which was associated with an increase in MDA level while vagotomy after I/R was ineffective. It is probable that the stimulation of vagus nerve following gastric I/R aggravated the condition and increased the activity of oxidant enzymes. Since vagotomy in basal condition did not have any effect, it seems that the control of gastritis and the activity of oxidant and anti-oxidant enzymes are not under the control of vagus nerve in basal conditions.

Vagus nerve probably acts through releasing acetylcholine (Ach). It has been demonstrated that the stimulation of vagus nerve increases output of gastric juice and acid (33), and decreases the activity of anti-oxidant enzymes like SOD (33). On the other hand, vagotomy showed healing effects on duodenal ulcers (7) similar to the effect of atropine (34). In a study, diaphragmatic vagotomy did not change the activity of catalase in the intestine (35) which was similar to the result of the present study.

The results of some studies are not consistent with our data. For example, it has been identified that vagal afferent fibers decrease gastrointestinal tract inflammation through nicotinic receptors leading to a decrease in inflammatory cytokines (16). Cholinergic agonists such as neostigmine decrease superoxide anion and inflammation in I/R (36). Elevation of acetylcholine transferase activity

and decrease in acetylcholine esterase inhibitors increase GPX (35, 37). Treatment with acetylcholine esterase inhibitors increases catalase half-life (38).

In the present study, melatonin administration affected the outcomes in I/R+vagus stimulation group. Melatonin decreased gastritis and MDA level, and increased SOD and GPX activity, but not in vagotomy group. These findings show that protective effects of melatonin are probably mediated through vagus nerve. Also, interaction between melatonin and vagus stimulation is suggested since these show a synergic effect in relation with anti-oxidant factors. Regarding this probable interaction, it is suggested that melatonin exerts its gastric protective effect through stimulation of GI tract neurons leading to CGRP release (1).

Intra-cerebral injection of melatonin inhibits acid and pepsin secretions through vagus cholinergic activity (15). Melatonin increases the release of amylase and pancreas proteins through vagus nerve (16). Effect of melatonin administration accompanied with vagotomy has not suggested in the release of amylase (18). Studies have shown that intra-lumen administration of melatonin or its precursor induces the release of pancreatic enzymes, while this effect is not shown in isolated pancreas. Moreover, these effects of melatonin are reversed by vagotomy and capsaicin administration (11, 18, 19).

It has been reported that melatonin effect is mediated indirectly through the release of CCK followed by vagovagal reflex (11, 19, 39). It has been shown that intra-lumen administration of melatonin increases plasma CCK level (11, 19) and CCK acts through stimulation of vagal afferent fibers in GI tract (40, 41). It has been reported that vagotomy in combination with melatonin administration inhibits the release of pancreas proteins (7, 11, 16). It has been reported that melatonin increases the expression and activity of Ach receptors, and synergistic effects are induced during vagus stimulation (42). According to a study, injection of phenylephrine causes melatonin release from mucosa enterochromaffin cells in the presence of normal vagus nerve and sympathetic paths (39). Melatonin increases the release of bicarbonate from gastric mucosa (39) and decreases the release of inflammatory cytokines of GI through increasing vagus activity (16). These studies confirm our results.

## Conclusion

It is suggested that melatonin is neuroprotective in gastric I/R probably by decreasing gastritis and MDA and increasing the

activities of CAT, SOD and GPX. These effects of melatonin are probably mediated by vagus nerve. Moreover, in gastric I/R, melatonin can reverse harmful effects of vagus stimulation. It is suggested that further studies are required to find the mechanisms involved in this interaction.

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### Conflict interest

The authors have no conflicts of interest to declare.

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