Melatonin is more effective than ascorbic acid and β-carotene in improvement of gastric mucosal damage induced by intensive stress

Aysin Akinci[1], Mukaddes Esrefoglu[2], Asli Cetin[3], Burhan Ates[4]

[1]Dogu Fertil Tup Bebek Merkezi, Malatya, Turkey
[2]Department of Histology and Embryology, Medical Faculty, Bezmialem Vakif University, Istanbul, Turkey
[3]Department of Histology and Embryology, Medical Faculty, Inonu University, Malatya, Turkey
[4]Department of Chemistry, Faculty of Science and Art, Inonu University, Malatya, Turkey

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Corresponding author:
Prof. Mukaddes Eşrefoğlu
Department of Histology and Embryology
Medical Faculty
Bezmialem Vakif University
34093, Istanbul, Turkey
Phone: +90 532 3465239
Fax: +90 212 5336855
E-mail:
drmukaddes@hotmail.com,
mdrmukaddes@yahoo.com

Abstract

Introduction: Oxidative stress has been considered to play a primary role in the pathogenesis of stress-induced gastric damage. The aim of this study was to investigate the effects of melatonin, ascorbic acid and β-carotene on stress-induced gastric mucosal damage.

Material and methods: Fifty-six male Wistar albino rats were divided into control, stress, stress + standard diet, stress + saline, stress + melatonin, stress + ascorbic acid and stress + β-carotene groups. The rats from stress groups were exposed to starvation, immobilization and cold by immobilizing for 8 h at +4°C following 72-hour food restriction. Following stress application, melatonin, ascorbic acid and β-carotene were administered for 7 days. Specimens of gastric tissue were prepared for microscopic and biochemical examinations.

Results: Mean histopathological damage scores and mean tissue malondialdehyde levels were significantly decreased but mean tissue glutathione levels and glutathione peroxidase and superoxide dismutase activities were increased in treatment groups vs. stress groups in general. Mean histopathological damage scores of the stress + Mel group was lower than those of stress + D, stress + S, stress + β-car ($p < 0.05$) and stress + Asc groups ($p < 0.005$). Additionally, mean tissue catalase activity of the stress + Mel group was higher than that of stress + S ($p < 0.005$), stress + D ($p < 0.05$) and stress + β-car groups ($p < 0.05$).

Conclusions: Melatonin is more effective than ascorbic acid and β-carotene in improvement of gastric damage induced by intensive stress. We suggest that as well as the direct antioxidant and free radical scavenging potency of melatonin, its indirect effect via the brain-gut axis might account for its greater beneficial action against stress-induced gastric damage.

Key words: ascorbic acid, β-carotene, melatonin, stress.

Introduction

It has been shown that reactive oxygen species (ROS) have significant roles in the pathogenesis of stress-induced gastric damage [1] by attacking important cellular components such as lipid, protein, carbohydrate and DNA [2]. The maintenance of gastric mucosal integrity depends on a vari-

ety of factors and physiological mechanisms, such as in particular the maintenance of microcirculation, mucus-alkaline secretion, activity of anti-oxidizing factors, such as superoxide dismutase (SOD), glutathione peroxidase (GPx) and the reduced form of glutathione (GSH) [3]. These factors were also implicated in the gastroprotection against exogenous and endogenous irritants [2, 4, 5]. The endogenous antioxidants involving intra-cellular antioxidant enzymes and hormones such as melatonin, and exogenous antioxidants such as ascorbic acid and β-carotene, are important protective factors against oxidative stress [2, 6, 7].

Although the most common experimental ulcer models are immobilization, cold-exposure and water-immersion methods [8–10], the most effective stress model with regard to protein oxidation, lipid peroxidation and anti-oxidant defense has been found different for different tissues. For instance, cold stress was found to be the most effective stress model for liver, whereas immobilization-cold stress was found to be the most effective for kidney and heart [8]. The model of water immersion and restraint stress proposed by Takagi *et al.* [11] seems to be most useful in testing of various physiological and pharmacological factors involved in the formation of gastric damage and those known to exert protection against these lesions [10]. In fact, when ulcerative factors, including starvation, immobilization, environmental change and drugs, are used in combination, the severity of gastric ulcers might increase. In the present study, we exposed rats to immobilization, cold and starvation in order to achieve intensive stress.

For assessment of biological effects of ROS, the tissue levels of malondialdehyde (MDA), as an indicator of lipid peroxidation, and the levels/activities of antioxidant enzymes including SOD, GSH, GSH-Px, and catalase (CAT) are useful. We detected tissue MDA and GSH levels and SOD, CAT and GSH-Px activities in addition to histopathological examination. We aimed to compare the effects of melatonin, β-carotene and ascorbic acid on the intensive stress-induced mucosal alterations by microscopic and biochemical methods. To the best of our knowledge, limited studies on experimental stress models have investigated the effects of melatonin and ascorbic acid on eliminating oxidative damage induced by intensive stress, and moreover the effect of β-carotene has not been evaluated.

Material and methods

Animals and experimental protocol

Fifty-six male Wistar albino rats weighing 340–360 g were used. Animals were fed with standard rat chow and tap water ad *libitum*. They were maintained on a 12 h light/12 h dark cycle at 21°C.

The rats were randomly divided into 7 groups, each consisting of 8 animals. The first group represented intact controls (control group). The rats from all of the other groups were exposed to starvation for 72 h. Later they were immobilized and kept at 4°C for 8 h. The rats from the 2nd group were exposed to stress as mentioned above (stress group). The rats from the 3rd group received a standard diet after stress exposure (stress + D), and those from the 4th group received daily intraperitoneal injections of 0.5 ml of 0.9% saline for 7 days (stress + S group). The rats from the 5th group (stress + Mel group) received 20 mg/kg body weight (bw)/day melatonin intraperitoneally (Merck Chemicals, Darmstadt, Germany) for 7 days after stress exposure. Melatonin was dissolved in absolute ethanol and further dilutions were made in saline with 1% final concentrations of ethanol. The rats from the 6th group (stress + Asc group) received 5 mg/kg bw/day ascorbic acid (L(+) ascorbic acid, Carlo Erba, Rodano, Italy) dissolved in saline. Finally, the rats from the 7th group (stress + β-car group) received 5 mg/kg bw/day β-carotene by oral gavage (MP Biomedicals, Illkirch, France). The rats from the 1st and 2nd groups were sacrificed on the day after stress exposure, while those from the 3rd–7th groups were sacrificed on the 8th day by cervical dislocation.

The experiment was performed in accordance with the guidelines for animal research from the National Institute of Health and was approved by the Committee on Animal Research at Inonu University, Malatya, Turkey.

Histological evaluation

At the end of the experiment, stomachs were rapidly removed. The first part of the samples was placed in 10% neutral formalin and prepared for routine paraffin embedding. Sections stained with hematoxylin-eosin (HE) were examined and scored by a blind observer using a Lyca DFC280 light microscope and a Leica Q Win and Image Analysis system (Leica Micros Imaging Solutions Ltd., Cambridge, UK). By assessment of tissue alterations in 20 different fields for each section, histopathological damage was evaluated. Gastric mucosal damage was scored by grading damage in epithelium, congestion, hemorrhage, cellular infiltration and glandular dilatation. Each alteration was scored as: 0 = absent or very rare; 1 = mild; 2 = moderate; 3 = severe, with a maximum score of 15.

Biochemical analysis

The other part of tissue samples was stored at –80°C for the determination of MDA, SOD, CAT, GSH, GSH-Px. After cutting into small pieces on ice, tissues were homogenized in 1/5 (w/v) phos-

phate buffer saline. Homogenates were divided into two portions; one part was directly used for MDA measurement. The second was sonicated four times for 30 s with 20 s intervals using a VWR Bronson scientific sonicator (VWR Int. Ltd, Merch House Pool, UK). Then, homogenates were centrifuged at 20 000 g for 15 min in a Beckman L-8-70M (Check correct name.) ultracentrifuge (Rotor SW-28; Beckman L8-70M Ultracentrifuge, Munich, Germany). Supernatants were separated and kept at –40°C until enzyme activity measurements were performed. Care was taken to keep the temperature at +4°C throughout the preparation of homogenates and supernatants.

Protein determination in supernatants was done according to Lowry *et al.* [12] using BSA as a standard. A Shimadzu 1601 UV/Vis spectrophotometer (Shimadzu, Kiyoto, Japan) with a connected PC and a Grand LTD 6G thermo stability unit adjusted to 37 ±0.1°C was employed for all spectrophotometric assays.

Catalase activity was measured in supernatants by the method of Luck [13]. The decomposition of the substrate H_2O_2 was monitored spectrophotometrically at 240 nm. Specific activity was defined as micromole substrate decomposed per minute per milligram of protein. CAT levels were expressed as micromoles per milligram of protein (U/mg protein).

GSH-Px activity measurements were conducted according to Lawrence and Burk [14]. 1.0 ml of 50 nM PBS solution (pH 7.4) including 5 mM EDTA, 2 µM NADPH, 20 µM GSH, 10 µM NaN_3 and 23 mU of glutathione reductase was incubated at 37°C for 5 min. Then 20 µl of 0.25 mM H_2O_2 solution and 10 µl of supernatant were added to the assay mixture. The change in absorbance at 340 nm was monitored for 1 min. A blank with all ingredients except supernatants was also monitored. Specific activity was calculated as micromoles of NADPH consumed per minute per milligram of protein (i.e. U/mg protein), using an appropriate molar absorptivity coefficient (6220 M^{-1} cm^{-1}). GSH-Px levels were expressed as micromoles per milligram of protein (U/mg protein).

SOD (Cu, Zn-SOD) activity was measured in the supernatant fraction using the xanthine oxidase/cytochrome c method according to McCord and Fridovich [15] where 1 unit of activity is the amount of enzyme needed to cause half-maximal inhibition of cytochrome c reduction. The amount of SOD in the extract was determined as ng of enzyme per mg protein, using a commercial SOD as the standard. SOD levels were expressed as micromoles per milligram of protein (U/mg protein).

The formation of 5-thio-2-nitrobenzoate (TNB) is followed spectrophotometrically at 412 nm [16]. The amount of GSH in the extract was determined as nmol/mg protein using a commercial GSH as the standard. GSH levels were expressed as nanomoles per milligram of protein (nmol/mg protein).

The level of MDA in tissue homogenate was determined using the method of Uchiyama and Mihara [17]. Half a milliliter of homogenate was mixed with 3 ml of H_3PO_4 solution (1% v/v) followed by addition of 1 ml thiobarbituric acid solution (0.67% w/v). Then the mixture was heated in a water bath for 45 min. The colored complex was extracted into n-butanol and absorption at 532 nm was measured using tetramethoxypropane as the standard. MDA levels were expressed as nanomoles per milligram of protein (nmol/mg protein).

Statistical analysis

Statistical analysis was carried out using the SPSS 13.0 statistical program (SPSS Inc., Chicago, Ill., USA). All data are expressed as arithmetic mean ± SE. For the analysis of tissue enzyme levels and histopathological damage scores Kruskal-Wallis variance analysis and the Mann-Whitney *U* test were used. Values of $p < 0.05$ was considered statistically significant.

Results

Microscopic findings

By naked eye, the outer surface of the stomach showed hyperemia and small hemorrhagic areas (Figure 1). Mucosal damage involving epithelium and connective tissue was observed in the stress group. Degeneration of the surface epithelium and that of the glands, vascular congestion, hemorrhage (Figure 2) and cellular infiltration were obvious. Glandular dilatation and sometimes vacuolization were detected within the epithelium of the glands. Histopathological alterations were clearly improved in the antioxidant-administered groups. The histological picture of the stress + Mel group was almost normal in appearance except for mild epithelial degeneration (Figure 3). Degeneration of the surface epithelium and that of the glands, glandular dilatation, and congestion were still sometimes observed in the sections from the stress + Asc and stress + β-car groups (Figures 4 and 5, respectively). Mean histopathological damage scores were 1.50 ±0.22 in the control group, 7.00 ±0.57 in stress, 4.00 ±0.17 in stress + D, 4.00 ±0.00 in stress + S, 2. 33 ±0.40 in stress + Mel, 5.33 ±0.33 in stress + Asc, and 4.00 ±0.36 in stress + β-car groups. Mean histopathological damage scores of the stress group was higher than that of the control group ($p < 0.005$). However, MHDSs of stress + D ($p < 0.005$), stress + S ($p < 0.005$), stress + Mel ($p < 0.005$), stress + Asc ($p < 0.05$) and stress + β-car groups ($p < 0.05$) were all lower than that

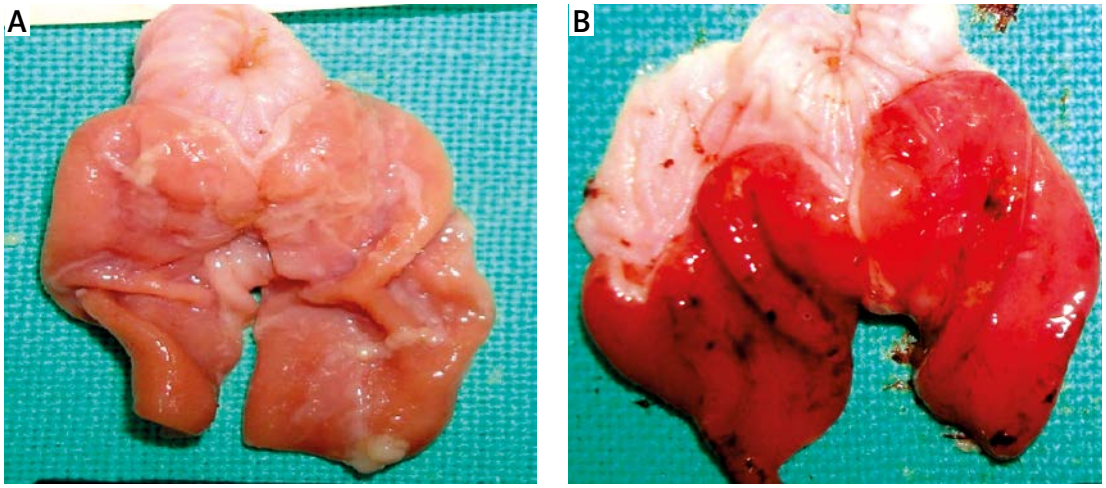

Figure 1. Outer surface of the stomach from control (**A**) and stress groups (**B**) is shown. Note the hyperemia and small hemorrhagic areas on the outer surface of the stomach of the rat exposed to intensive stress

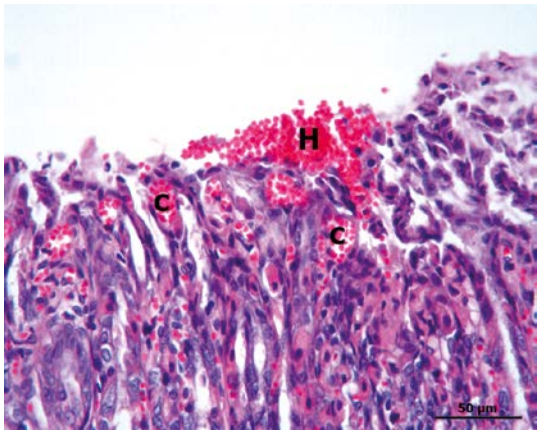

Figure 2. Microscopic appearance of rat stomach from stress group. Degeneration of the surface epithelium and that of the glands, vascular congestion (c), and hemorrhage (H) are obvious. HE 40×

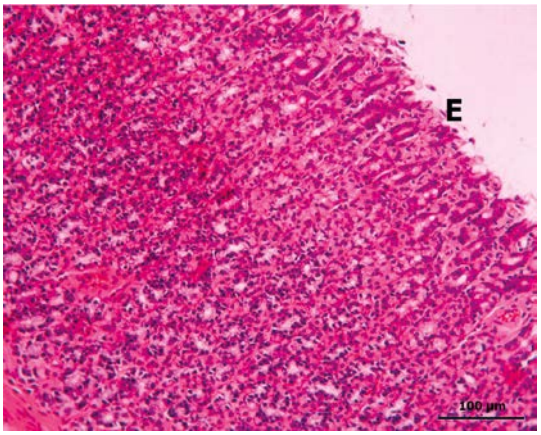

Figure 3. Microscopic appearance of rat stomach from stress + Mel group. It seems nearly normal except mild epithelial degeneration. HE 20×

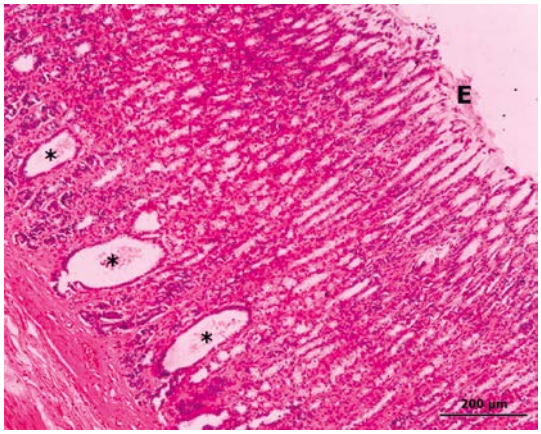

Figure 4. Microscopic appearance of rat stomach from stress + Asc group. Note the epithelial degeneration and glandular dilatation. HE 10×

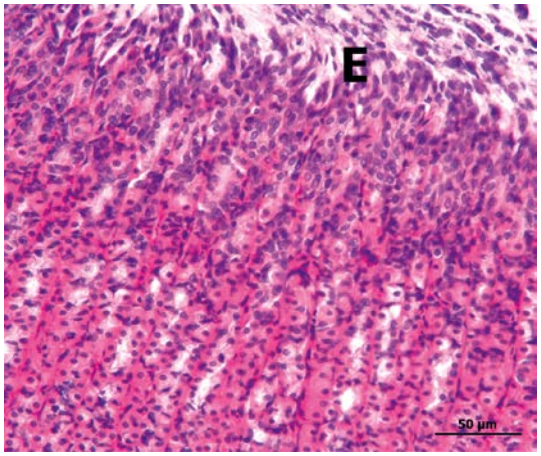

Figure 5. Microscopic appearance of rat stomach from stress + β-car group. Note the epithelial degeneration and desquamation. HE 40×

of the stress group. Moreover, no significant difference was found between stress + Mel and control group. Mean histopathological damage scores of the stress + Mel group was also lower than those of stress + D, stress + S, stress + β-car ($p < 0.05$) and stress + Asc groups ($p < 0.005$). Mean histopathological damage scores of all groups are summarized in Table I.

Significant differences were detected in mean tissue MDA levels among groups. Mean tissue MDA level of the stress group was higher than that of the control group ($p < 0.05$). MDA levels of stress + S, stress + Mel, stress + Asc ($p < 0.05$), and stress + β-car ($p< 0.005$) were lower than that of the stress group. MDA levels of antioxidant-administered groups were also lower than that ofstandard diet groups. However, no significant difference was found between stress + D and stress + S groups, and antioxidant-administered groups. Mean tissue MDA levels of all groups are summarized in Table I.

Stress resulted in decreases in mean tissue GSH level and SOD and GSH-Px activities and an increase in CAT activity. Mean tissue CAT activities were 5.92 ±0.58 in the control group and 14.69 ±0.99 in the stress group ($p < 0.005$). It was noted that CAT activity of the stress + Mel group (13.39 ±1.57) was higher than those of control and stress + S (both $p < 0.005$), stress + D ($p < 0.05$), stress + Asc, and stress + β-car groups ($p < 0.05$; both). CAT activities of stress + Asc and stress + β-car groups ($p < 0.005$) were also higher than that of the control group.

Mean tissue SOD activity was significantly lower in the stress group than that of the control group ($p < 0.005$). The lowest SOD activity was found in the stress group. Mean tissue SOD activities of stress + Mel and stress + Asc groups were higher than that of the stress group ($p < 0.05$). However, there was no significant difference between stress + D, stress + S and antioxidant-administered groups. Mean tissue CAT and SOD activities of all groups are summarized in Table I.

Stress also caused a significant reduction in GSH level and GSH-Px activity in comparison to the control group ($p < 0.05$). GSH values of stress + Asc and stress + β-car groups were higher than those of stress + D and stress groups. A statistically significant difference was found between the tissue levels of GSH of stress + Asc and stress groups ($p < 0.05$). Mean tissue GSH-Px values of stress + D, stress + S and antioxidant-administered groups were all higher than that of the stress group. GSH-Px activities of stress + Asc and stress + β-car groups were higher than those of standard diet and saline groups but with no statistical significance. Mean tissue GSH levels and GSH-Px activities of all groups are summarized in Table I.

Discussion

After removal of the rat stomach from the stress group, the most striking observation was the presence of hyperemia and small hemorrhagic areas on the outer surface of the stomach. Aziz Ibrahim *et al.* [18] observed petechial bleeding, most often 1–2 mm in size, in the stomach of rats subjected to water-immersion restraint stress for 3.5 h. In the present study, intensive stress resulted in microscopic changes including disintegration of epithelium and lamina propria, cellular infiltration, congestion, hemorrhage and submucosal edema [19–21]. In the present study, stress-induced alterations were reduced in saline, standard diet and antioxidant-administered groups. Although no therapeutic agent was administered to stress + S and stress + D groups, MHDS decrease may be related to the regeneration capability of gastric mucosa within 7 days following stress. Differences between these two groups and the antioxidant-administered group are of special importance. In this

Table I. Mean MHDSs, mean tissue MDA and GSH levels and SOD, CAT, and GSH-Px activities of all groups

Groups	MHDS (n = 8)	MDA [nmol/mg] (n = 8)	CAT [U/mg] (n = 8)	SOD [U/mg] (n = 8)	GSH-Px [U/mg] (n = 8)	GSH [nmol/mg] (n = 8)
Control	1.50 ±0.22	0.73 ±0.05	5.92 ±0.58	16.68 ±0.57	49.50 ±4.66	61.44 ±5.06
Stress	7.00 ±0.57[a]	0.88 ±0.02[i]	14.69 ±0.99[a]	12.77 ±0.61[a]	32.92 ±2.94[i]	38.20 ±4.02[i]
Stress + D	4.00 ±0.17[a,b]	0.78 ±0.05	7.71 ±0.65[b]	14.29 ±1.01	38.48 ±2.97	48.69 ±3.48
Stress + S	4.00 ±0.001[a,b]	0.69 ±0.05[e]	6.33 ±0.40[b]	13.89 ±0.72[i]	35.16 ±1.63[i]	48.13 ±3.78
Stress + Mel	2.33 ±0.40[b,c,d]	0.76 ±0.03[e]	13.39 ±1.57[a,d,j]	14.93 ±0.42[e]	36.75 ±3.52	39.19 ±4.13[i]
Stress + Asc	5.33 ±0.33[a,c,d,e,f]	0.67 ±0.04[e]	8.03 ±0.67[b,g]	17.18 ±1.21[e]	45.96 ±4.41	58.56 ±5.27[e]
Stress + β-car	4.00 ±0.36[a,e,g,h]	0.73 ±0.02[b]	8.28 ±0.41[a,b,g]	14.08 ±0.96	42.23 ±4.19	51.68 ±4.01

[a]P < 0.005 vs. control, [b]p < 0.005 vs. stress, [c]p < 0.05 vs. stress + S, [d]p < 0.05 vs. stress + D, [e]p < 0.05 vs. stress, [f]p < 0.005 vs. stress + Mel, [g]p < 0.05 vs. stress + Mel, [h]p < 0.05 vs. stress + Asc, [i]p < 0.05 vs. control, [j]p < 0.005 vs. stress + S.

aspect, melatonin was effective to protect gastric mucosa, since the lowest MHDS was detected in the stress + Mel group and significant differences were found between stress + Mel and stress + D, stress + S, stress + Asc and stress + β-car groups. Melatonin seems to be more effective than ascorbic acid and β-carotene in reducing stress-induced tissue alterations.

Disruption of membrane lipids by ROS is known as lipid peroxidation that causes inactivation of membrane receptors and enzymes and an increase in membrane permeability and intra-cellular calcium concentration resulting in disruption in structural and functional characteristics of the cell [22]. We detected lower tissue MDA levels, in saline, standard diet and antioxidant-administered groups compared to that of the stress group. MDA levels of the antioxidant-administered groups were also lower than that of the stress + D group; however, no significance was detected.

Aerobic cells are able to metabolize toxic reactants to less reactive or totally innocuous molecules by antioxidant enzymes including SOD, GSH-Px and CAT [2, 23]. We found decreases in tissue GSH levels and GSH-Px and SOD activities and an increase in CAT activities in groups exposed to stress. CAT may be more resistant against oxidative agents in comparison to those antioxidant enzymes. Some recent studies have reported reductions in tissue SOD activities in water-immersion, cold-restraint and ethanol-induced stress models [4, 24, 25], GSH-Px activities in cold-restraint and starvation-induced stress models [21, 26] and GSH levels in water-immersion, cold-restraint and ethanol-induced stress models [4, 27], and increases in CAT activities in a cold-restraint stress model [26]. However, in some studies, no change in SOD activity [26], an increase in SOD activity in cold-restraint stress models [28], and a decrease in CAT activity in cold-restraint and ethanol-induced stress models have been reported [27, 28].

Cerebral ischemia due to e.g. cold exposure may lead to interruption of the axis between the central nervous system (CNS) and the gastrointestinal (GI) system. This altered interrelation between the central nervous system and the GI system may cause, among other things, mainly dysphagia, dysmotility, and hemorrhage [29]. Melatonin may exert a direct effect on GI tissues, but its major influence on GI organs seems to occur indirectly, via the brain-gut axis including peripheral receptors, sensory afferent (vagal or sympathetic) pathways and CNS acting on these organs via autonomic efferents and neuromediators [30]. Brzozowski *et al.* [31] concluded that exogenous melatonin and its precursor, l-tryptophan, attenuate water immersion and restraint stress-induced gastric lesions via interaction with MT2 receptors. This protective action of melatonin is because of enhancement of gastric microcirculation, probably mediated by prostaglandin E2 derived from cyclooxygenase-2 overexpression and activity, activation of the brain-gut axis involving calcitonin gene-related peptide released from sensory nerves, and the release of gastrin. Furthermore, pinealectomy which resulted in a significant fall in plasma melatonin levels aggravated the water immersion and restraint stress-induced gastric lesions.

Konturek *et al.* [32] reported that intra-cellular ROS were eliminated by melatonin. Melatonin directly scavenges oxygen species or indirectly stimulates activation of antioxidant enzymes [33, 34]. It protects cells from oxidative damage by stabilizing lipid membranes [32, 35, 36]. We found melatonin beneficial in reducing MHDSs and tissue MDA level. MHDSs of melatonin-administered rats were lower than those of stress and stress + D and stress + S groups ($p < 0.005$, $p < 0.05$). A statistically significant reduction vs the stress group was also found in MDA level ($p < 0.05$). Additionally, melatonin administration resulted in increases in SOD ($p < 0.05$) and GSH-Px activity and GSH level vs. the stress group. Melatonin significantly increased CAT activity in comparison with control, stress + S ($p < 0.005$), stress + D, stress + Asc, and stress + β-car groups ($p < 0.05$). Previous studies have demonstrated that melatonin reduces the ulcer index and tissue MDA levels and increases SOD activity and GSH levels in cold, ischemia, ethanol and aspirin-induced gastric damage [35, 36]. Antioxidant, cytoprotective and anti-inflammatory features of melatonin have been reported in various ulcer models [32, 35–37]. Melatonin has been found to play important roles in regulation of gastric blood supply, removal of acid and release of bicarbonate ion [38, 39]. Since we detected significant differences in only MHDS and tissue CAT activity between the melatonin-administered group and stress + D and stress + S groups, we suggest that melatonin at a dose of 20 mg/bw/day is not fully effective in improvement of gastric damage induced by intensive stress.

Carotenoids are the most potent superoxide suppressants [40]. β-Carotene has been demonstrated to decrease gastric mucosal damage [41]. Singh *et al.* [42] found that β-carotene at a dose of 100 mg/kg reduced the ulcer index and increased antioxidant enzymes in indomethacin-induced gastric ulcers. In the present study, the reduction in MDA level ($p < 0.005$) and MHDS ($p < 0.05$) and increase in SOD, GSH-Px and GSH levels in the β-carotene group vs. the stress group indicate the antioxidant potential of β-carotene. Higher CAT, SOD and GSH-Px activities and GSH level detected in this group in comparison with the stress + S group also indicate that β-carotene

supports the antioxidant enzyme system. Vincze *et al*. [43] found that β-carotene at a dose of 10 mg/kg prevented gastric mucosal damage, but could not increase SOD activity in a HCl-induced ulcer model. Scavenging activity of carotenoids against peroxyl radicals contributes to defense mechanisms against lipid peroxidation [41, 43]. Although oxidative stress parameters were decreased by β-carotene administration, we suggest that β-carotene at a dose of 5 mg/bw/day is not fully effective because of the absence of significant differences between stress + β-carotene, and stress + D and stress + S groups.

Ascorbic acid scavenges free radicals both alone and in combination with GSH and vitamin E [44, 45]. It was demonstrated that ascorbic acid has protective properties against ulcers induced by various factors including water immersion and indometacin [44, 46, 47]. Glavin and Lagrotteria [48] reported a beneficial effect of L-ascorbic acid in reducing incidence and severity of gastric ulcer induced by cold exposure for 2 h. Konturek *et al.* [49] demonstrated that aspirin containing ascorbic acid causes relatively little damage to gastric mucosa. Hung and Neu [50] reported that gastric damage induced by starvation for 5 days is relieved by ascorbic acid administration. In our study, MHDS and MDA levels in the stress + Asc group were significantly lower than those of the stress group ($p < 0.05$, $p < 0.005$; respectively). Moreover, the lowest mean tissue MDA value among the groups was detected in this group. SOD, GSH and GSH-Px values were similar to those of the control. Higher CAT, SOD and GSH-Px activities and GSH level, particularly in comparison with the stress + S group, indicate that this agent supports the antioxidant enzyme system. Koc *et al.* [44] found increases in SOD and GSH-Px activities and GSH levels and reductions in MDA level and ulcer index with administration of ascorbic acid. Although oxidative stress parameters were decreased by ascorbic acid administration in general, we suggest that ascorbic acid at a dose of 15 mg/bw/day is not fully effective because of the absence of significant differences between stress + Asc, and stress + D and stress + S groups.

As a consequence of our histopathological and biochemical results, we detected beneficial effects of the antioxidant agents we used, melatonin, ascorbic acid and β-carotene. Even under severe stress, all of the agents were effective, so in daily stress humans might obtain help from these agents.

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Conflict of interest

The authors declare no conflict of interest.

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