

## Mechanisms involved in gastric protection of melatonin against oxidant stress by ischemia-reperfusion in rats

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### Abstract

The generation of oxygen-derived free radicals has been suggested to be significantly responsible for ischemia-reperfusion injury in gastrointestinal tissues. Biochemical mechanisms include the xanthine-oxidase-derived oxidants mainly the superoxide anion. Both in vitro and in vivo studies have demonstrated that the pineal hormone melatonin possesses free radical scavenging and antioxidant properties. The indolamine has been effective in reducing the induced-oxidative damage in several tissues and biological systems. The aim of this study was to elucidate additional antioxidant mechanisms responsible for the gastroprotection afforded by the indolamine in ischemia-reperfusion gastric injury. Therefore, changes of related enzymes such as xanthine-oxidase, superoxide dismutase, glutathione reductase and total glutathione were investigated. Our results showed that treatment with 5, 10 or 20 mg kg<sup>-1</sup> of melatonin, administered i.p., clearly diminished the percentage of damage to 49.56 ± 17.20, 37.54 ± 11.40 and 26.70 ± 8.12 respectively. Histologically there was a reduction of exfoliation of superficial cells and blood cell infiltration. These protective effects were related to a significant reduction of xanthine-oxidase activity (2.23 ± 0.38 U/mg prot × 10<sup>-4</sup> with the highest tested dose of melatonin) and significant increases in superoxide dismutase reaching a value of 6.20 ± 0.56 U/mg prot with 25 mg/Kg of melatonin and glutathione reductase activities (417.44 ± 29.72 and 649.43 ± 81.11 nmol/min/mg prot with 10 and 20 mg/Kg of melatonin). We conclude that the free radical scavenger properties of melatonin mainly of the superoxide anion, probably derived via the xanthine-oxidase pathway, and the increase of antioxidative enzymes significantly contributes to mediating the protection by the hormone against ischemia-reperfusion gastric injury. © 2001 Elsevier Science Inc. All rights reserved.

*Keywords:* Melatonin; Oxidative damage; Gastric injury; Xanthine-oxidase; Antioxidant; Rats

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## Introduction

Melatonin, the principal secretory substance of the pineal gland, has been detected in the digestive tract of several species [1]. The stomach has the highest level of melatonin [2] and the most probable site of melatonin synthesis is the enterochromaffin cells of the gut. In rats, this indol was found in different alimentary segments such as the glandular portion of the gastric wall. In addition, melatonin binding sites have been identified in the gastrointestinal tract of mammals and non mammalian vertebrates and it has been suggested that the hormone could act as a protecting agent of the gut [3, 4].

Both *in vitro* and *in vivo* studies have demonstrated that melatonin is a direct free radical scavenger and indirect antioxidant [5,6]. In terms of its scavenging activity this indol has been shown to quench the hydroxyl radical, superoxide anion radical, singlet oxygen, peroxy radical and the peroxy nitrite anion [6–10]. In addition, it stimulates mRNA levels for superoxide dismutase (SOD) and the activities of antioxidative enzymes such as glutathione peroxidase (GSH-px), glutathione reductase (GSSG-rd) and glucose-6-phosphate dehydrogenase. These effects have explained, in part, the protection afforded by the hormone against oxidative stress caused by different chemicals in several tissues and biological systems [11–13].

Studies of ischemia-reperfusion (I-R) injury in the intestine and stomach implicate both xanthine-oxidase (XO)-derived oxidants and neutrophils in the pathogenesis of this disorder [14,15]. XO-derived oxidants, mainly the superoxide anion, seem to elicit the production of inflammatory mediators which attract and activate neutrophils into postischemic injury.

Previous works in our laboratory [16,17] and by other investigators [18–21] have shown a significant protection by melatonin against the damage induced by I-R, ethanol, non-steroidal anti-inflammatory drugs and stress induced-gastric ulcers. These findings have illustrated that the mechanisms mediating the antiulcer effects could be due, at least in part to its radical scavenging activity. In I-R gastric injury, melatonin was able to reduce neutrophil-infiltration and inhibited lipid peroxidation. In addition, the indol completely protected the gastric mucosa against the loss of GSH-px activity [16].

The present study was undertaken in order to further elucidate additional antioxidative mechanisms responsible for melatonin-induced gastroprotection in I-R injury. Therefore the activities of certain enzymes such as XO, a producing oxidants enzyme, total glutathione levels (GSH) and the antioxidant enzymes SOD and GSSG-rd were investigated. Furthermore, histological analysis of the lesions was also studied.

## Material and methods

### *Animal groups and drug preparation*

Male and female Wistar rats (8–10 each group), 180–200 g, supplied by Animals Service, University of Seville, were placed singled in cages with wire-net floors in a controlled room (temperature 22–24 °C, humidity 70–75%, lighting regimen of 12L/12D) and were fed a normal laboratory diet. Rats were deprived of food for 24 h before experimentation but allowed free access to tap water throughout. In previous experiments undertaken in our laboratory, the sex had not influence in the incidence and severity of I-R-induced gastric injury. On the basis of these data, in the present study both male and female rats were mixed. Experiments fol-

lowed a protocol approved by the local animal Ethics Committee and the Local Government. All experiments were in accordance with the recommendations of the European Union.

Melatonin (Sigma, St. Louis) was freshly dissolved in absolute ethanol + 0.9 % NaCl (1:5) and was injected IP 30 min before experimentation. Control rats received the vehicle only in comparable volume also by parenteral route. The animals were randomly divided into five groups: control (non-ulcerated), ischemia-reperfusion (I-R) ulcerated control and melatonin (5, 10 and 20 mg Kg<sup>-1</sup> B.W.) groups.

### *Gastric injury*

Rats were anesthetized by intraperitoneal injection of sodium pentobarbital at a dose of 50 mg kg<sup>-1</sup> B.W. The left side of the abdomen was shaved and a 3 cm incision was made from the midline to below the ribcage using a diathermy. The celiac artery was dissected free of excess fat and clamped for 30 min approximately 0.5 cm from its origin from the aorta, using an atraumatic microvascular clamp. Reoxygenation was allowed by removal of the clamp for 60 min [22]. At the end of the experimental period, the animals were killed by exsanguination via the abdominal aorta. The stomach of each rat was removed and opened along the greater curvature and any lesions were examined macroscopically; the number and area of gastric lesions were determined using a planimetry (Morphomat, Carl Zeiss, Berlin, Germany) by one investigator who was unaware of the treatment given. The length and width of the ulcers were measured on a cold stand and the sum of the damaged areas was calculated. Results were expressed as percentage of damage. Then, the mucosa was scrapped off by means of two glass slides on ice, weighed and frozen at -70 °C until biochemical determinations. In 6 rats of each group, samples of macroscopically normal and ulcerated stomachs were processed by routine methods for subsequent histological evaluation.

### *Xanthine-oxidase activity (XO)*

XO is the enzyme responsible for the conversion of xanthine and hypoxanthine to uric acid. The enzyme occurs in two forms: the NAD<sup>+</sup>-dependent dehydrogenase, which reduces NAD<sup>+</sup> to NADH, and NAD<sup>+</sup>-independent oxidase, which reduces molecular oxygen to superoxide. The tissue was homogenized in buffer consisting of Tris-HCl, EDTA, phenylmethylsulfonyl fluoride (PMSF), dithiothreitol and leupeptine, pH=8.1. The homogenate was centrifuged and the supernatant was separated by Sephadex (G-25) column. Xanthine was used as substrate for XO activity studies. XO activity was assayed as uric acid production by the increase in absorbance at 294 nm in the absence of NAD<sup>+</sup>. One unit of XO activity corresponds to the formation of 1 μM of uric acid per minute [23]. Results were expressed as U/mg protein.

### *Superoxide dismutase activity (SOD)*

The enzymatic activity of SOD is based on the inhibition of the reduction of cytochrome C according to the method of McCord and Fridovich (1969) [24]. Samples of gastric mucosa were homogenized in a mixture of PBS and EDTA. The homogenate was supplemented with 0.1% Triton. The assay method used ferricytochrome c, xanthine, as source of O<sub>2</sub><sup>-</sup>, and sufficient milk XO to give a rate of increase in absorbance of 0.025/min at pH 7.8 and 25 °C. The

reaction kinetic was measured in spectrophotometer at 550 nm at a rate of 0–80 seconds. Results were expressed as U/mg protein. One unit of SOD is defined as the amount of enzyme that causes 50 % inhibition of cytochrome C reduction.

#### *Glutathione reductase activity*

Glutathione reductase (GSSG-Rd) reduces the oxidized glutathione (GSSG). Its activity was measured by the method of Worthington et al (1974) [25], following the decrease in absorbance at 340 nm induced by oxidized glutathione in the presence of NADPH in PBS buffer, pH 7.8. Results were expressed as nmol/min/mg protein.

#### *Total glutathione*

For glutathione GSH and GSSG estimates, a portion of gastric mucosa was homogenized in a solution of trichloroacetic acid (1:20 wt/vol). The homogenate was centrifuged in a Beckman desk-top centrifuge at 9.500 *g* for 5 min at 4°C. The supernatant was used to measure total GSH and GSSG using the method DTNB-Recycling Assay described by Anderson (1985) [26].

#### *Protein assay*

Proteins were determined using the Bradford procedure, with albumin as standard [27].

#### *Statistical analysis*

Values are given as arithmetic means  $\pm$  SEM. The significance of differences between means was evaluated by the Anova's test followed by the Fisher test and the Mann-Whitney U-test. The level of significance was accepted at  $P < 0.05$ .

## **Results**

In the stomach, macroscopically, clamping the celiac artery during 30 min and reperfusion it for 60 min caused important lesions. Damage was predominantly observed in the gastric corpus and few or no lesions appeared in the antrum. Fig. 1a; Fig. 1b

The lesions were oedematous and haemorrhagic and occurred in a linear fashion. Fig. 1c

Histologically, the damaged areas included exfoliation and necrosis of superficial cells, structural alterations on the 2/3 parts of the glandular pits, generalized blood cell infiltration and evident signs of reperfusion in comparison with non-ulcerated gastric mucosa (Fig. 1a). Treatment with either 5, 10 or 20 mg  $\text{kg}^{-1}$  of melatonin clearly diminished the percentage of damage to  $37.6 \pm 11.3$  and  $26.7 \pm 8.1$  respectively (Fig. 2).

There was a reduction of hyperaemia, petechiae, exfoliation of superficial cells and blood cell infiltration (Fig. 1c). Fig. 2

As shown in Table 1, XO activity was significantly increased after I-R from a basal activity of  $2.98 \pm 0.36$  to  $4.68 \pm 0.60$  U/mg protein  $\times 10^{-4}$  ( $p < 0.05$ ). This increase was significantly reduced by treatment with the hormone. In melatonin-treated non-ulcerated rats, XO activity remained unchanged. Table 1

Changes in SOD and GSSG-Rd activities are also shown in Table 1. The activity of SOD in the gastric mucosa of rats treated with the vehicle was  $5.01 \pm 0.47$  U/min/mg protein. Our

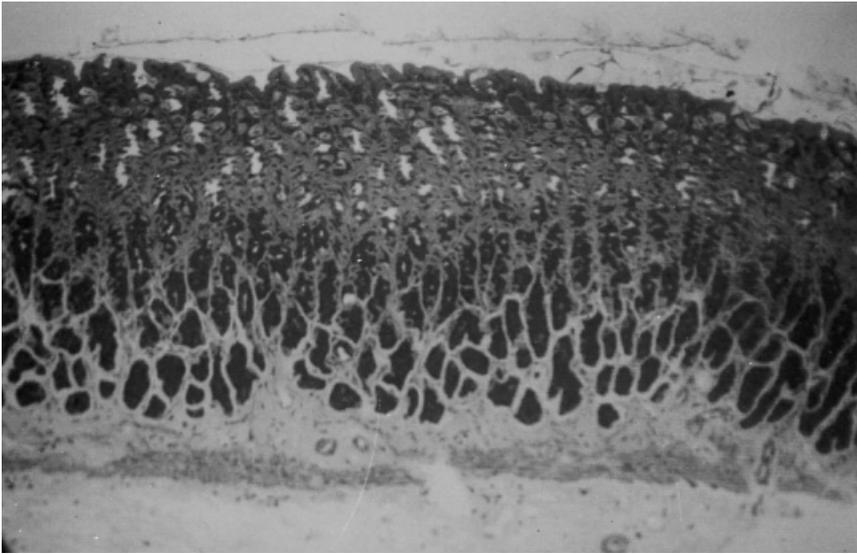


Fig. 1a. Gastric mucosa non-ulcerated control. Haematoxylin and eosin, magnification 150 $\times$ .

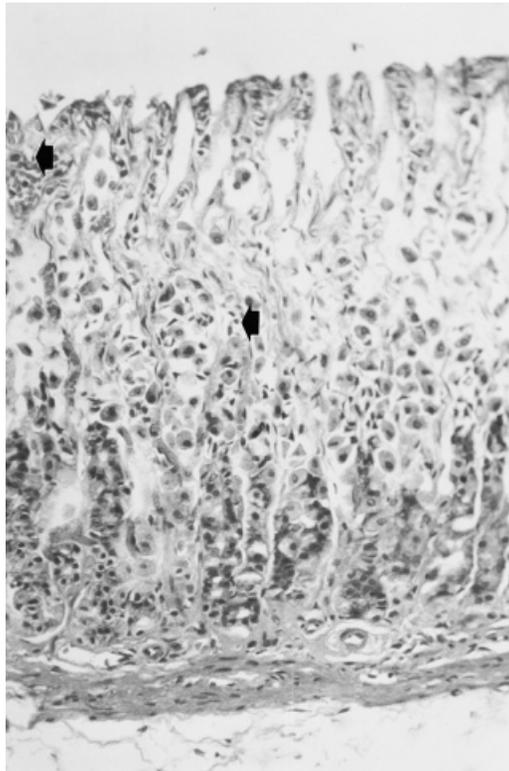


Fig. 1b. Histological appearance of a typical lesion in the oxyntic mucosa after 30 min of ischemia and 60 min of reperfusion. Besides, certain areas with superficial exfoliation is remarkable, zones with small haemorrhages and blood cell infiltration. Haematoxylin and eosin, magnification 400 $\times$ .

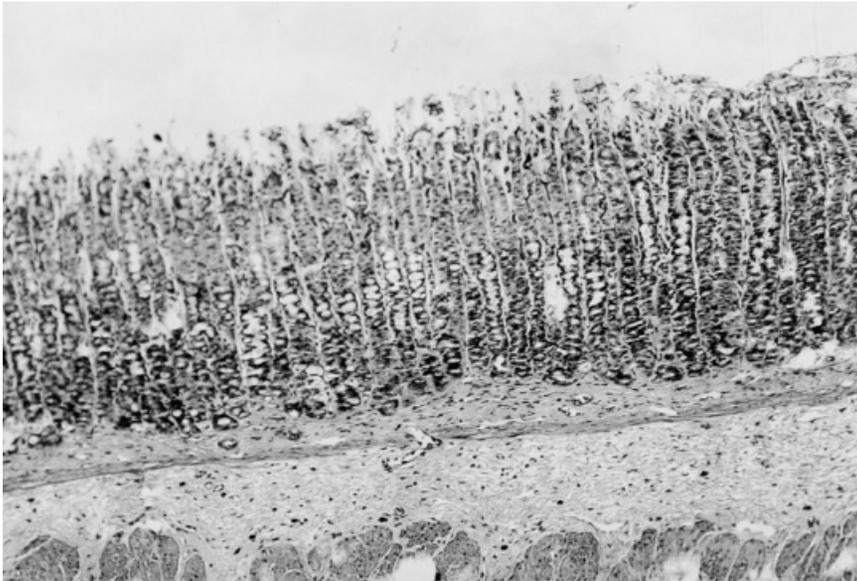


Fig. 1c. Protected I-R lesion after treatment with 20 mg kg<sup>-1</sup> b.w. of melatonin. There is a reduction of exfoliation of superficial cells, haemorrhage and blood cell infiltration. Also, is observable the recuperation of the typical structure and alignment of the gastric gland mucosa. Haematoxylin and eosin method, magnification 160×.

data showed that SOD activity was significantly decreased in gastric mucosa following gastric injury from a basal level of  $5.01 \pm 0.47$  to  $3.02 \pm 0.38$  U/mg protein ( $p < 0.05$ ). This decrease was totally reversed by all the doses assayed of melatonin ( $p < 0.005$ ). In addition, GSSG-rd activity was significantly decreased in the gastric mucosa following gastric injury ( $p < 0.05$ ), but treatment with 10 and 20 mg Kg<sup>-1</sup> of melatonin significantly protected the

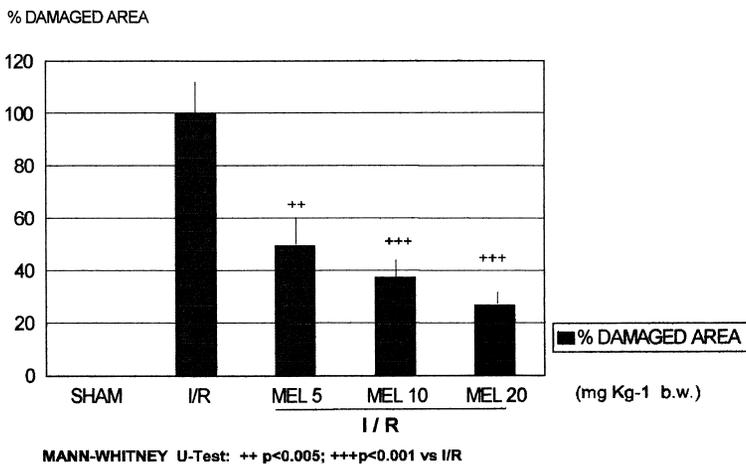


Fig. 2. Melatonin protection against ischemia-reperfusion gastric injury. The damaged area is expressed as percentage of total surface. Each values represents the mean ± S.E.M. (n = 10).

Table 1

Effects of pretreatment with melatonin on glutathione reductase, superoxide dismutase and xanthine-oxidase on I-R gastric injury

Melatonin	Glutathione reductase (nmol/min/mg prot)	Total glutathione (umol/mg prot)	Superoxide dismutase (U/mg prot)	Xanthine oxidase (U/mg prot $\times 10^{-4}$ )
Control	413.68 $\pm$ 35.52	31.47 $\pm$ 2.00	5.01 $\pm$ 0.47	2.98 $\pm$ 0.36
Mel 10 (mg/Kg IP)	431.42 $\pm$ 15.72	32.57 $\pm$ 2.72	5.48 $\pm$ 0.54	3.01 $\pm$ 0.39
I/R				
Control	333.46 $\pm$ 11.01 <sup>#</sup>	18.03 $\pm$ 3.86 <sup>#</sup>	3.02 $\pm$ 0.38 <sup>#</sup>	4.68 $\pm$ 0.60 <sup>#</sup>
Mel 5 (mg/Kg IP)	400.50 $\pm$ 22.01	13.05 $\pm$ 2.75	7.52 <sup>##</sup> $\pm$ 0.98 <sup>++</sup>	3.78 $\pm$ 0.65
Mel 10(mg/Kg IP)	417.44 $\pm$ 29.72 <sup>+</sup>	14.01 $\pm$ 2.55	6.86 <sup>##</sup> $\pm$ 1.22 <sup>++</sup>	3.73 $\pm$ 0.43
Mel 20(mg/Kg IP)	649.43 $\pm$ 81.11 <sup>+</sup>	16.43 $\pm$ 2.97	6.20 <sup>##</sup> $\pm$ 0.56 <sup>++</sup>	2.23 $\pm$ 0.38 <sup>+</sup>

Anova's Test: <sup>#</sup> p<0.05; <sup>##</sup> p<0.005 vs control.

<sup>+</sup> p<0.05; <sup>++</sup> p<0.005 vs control I-R.

stomach against the loss of the enzyme activity. GSH levels of ulcerated control gastric mucosa samples were significantly lower ( $p < 0.05$ ) when compared to non-ulcerated control group. Nevertheless, melatonin treatments did not induce significant changes in this parameter. (Table 1).

## Discussion

The results of this study clearly show a significant protection by melatonin against IR-induced gastric injury. These findings are consistent with other reports which show that melatonin readily protects the gastric and intestinal mucosa from the damage caused by a variety of agents as 100 % ethanol [20], stress [21], or ischemia-reperfusion [16,17,19,28]. In addition, pharmacological doses of melatonin prevented severe symptoms of colitis, such as mucosal lesions and diarrhea [29].

Experimental evidences suggest that the generation of oxygen-derived free radicals is significantly responsible for I-R injury in gastrointestinal tissues [11, 30]. Biochemical mechanisms which allow these radicals to be produced include the XO system, which is modified during ischemia. During this step, ATP is degraded to hypoxanthine and xanthine dehydrogenase is converted to XO. On reperfusion, XO catalyzes conversion of hypoxanthine to uric acid with the release of superoxide radicals and H<sub>2</sub>O<sub>2</sub>. These oxygen radicals may then be converted to the highly cytotoxic hydroxyl radical by the iron-catalyzed Haber-Weiss reaction. This initiates the process of lipid peroxidation and release of substances that recruit and activate polymorphonuclear leukocytes.

Evidence supporting the involvement of XO as source of reactive oxygen metabolites is provided by studies in which tissue is depleted by administration of tungsten-supplemented, molybdenum-deficient diet [31, 32]. In addition, this assumption is supported by studies which show that XO inhibitors such as allopurinol or oxypurinol when used, attenuate I-R injury [33]. The results of this study clearly show that the protective effect of the hormone against I-R was linked to its ability to reduce the activity of the XO system and in consequence to a reduction of superoxide anion generation. An analogous beneficial effect of mel-

atonin was described by Cuzzocrea et al., 2000 [28] in rat ileum by clamping both the superior mesenteric artery and the celiac trunk for 45 min, followed thereafter by reperfusion.

In order to protect tissues against the deleterious effects of oxygen-derived free radicals all cells possess numerous antioxidant enzymes and free radical scavengers. Primary defenses include the enzymes SOD, catalase (CAT), GSH-px and GSSG-rd. SOD is an intracellular metalloenzyme that catalyzes the dismutation of superoxide radical with subsequent oxidative damage. In addition to removing a highly reactive species that might directly contribute to tissue necrosis, the dismutation of superoxide anion by SOD may be protective by extending the half-life of the endothelium-derived relaxing factor, nitric oxide [34].

A role for superoxide anion in the present model of gastric injury is supported by the observation that SOD activity was significantly reduced in control ulcerated stomach. These findings are in agreement with other studies on gastric and hepatic injury induced by I-R [33, 35]. In our study melatonin administration prior to I-R injury significantly increased SOD activity nearly up to the control-non ulcerated values. Similar effects have been observed by other authors in a variety of experimental models where the superoxide anion has been implicated as being in part causative of the damage and the indol has been effective in reducing oxidative damage [36, 37]. Additionally mRNA levels for SOD are stimulated following the exogenous administration of the hormone in rat brain cortex [38, 39].

GSH is an important constituent of intracellular protective mechanisms against a number of noxious stimuli including oxidative stress [40]. Additionally, GSH scavenges  $O_2^-$  and protects protein thiol groups from oxidation. This tripeptide is present in especially high concentrations in stomach [41]. The GSH redox cycle catalyzed by an endogenous antioxidative enzyme GSH-px, reduces  $H_2O_2$  thus breaking the chain reaction leading from  $O_2^-$  to the highly reactive  $\cdot OH$ . Recent reports [16, 17, 20] showed that melatonin gastroprotection was mediated by reducing the accumulation of peroxides through induction of GSH-px activity. Similar results were obtained by others investigators [42–45] in rat brain, rat lung and in several tissues of chicks. On the other hand, the antioxidant activity of GSH-px is coupled with the oxidation of GSH to GSSG, which can subsequently be reduced by glutathione reductase (GSSG-rd) with NADPH as the reducing agent [34]. Hirashi et al., 1991 [46] demonstrated that GSSG-rd was much more potent than endogenous catalase in protecting cultured gastric mucosal cells from oxidative stress revealing the importance of the GSH system as a first line of antioxidant defense in the stomach. In our study the activity of GSSG-rd and GSH content were depressed after I-R. The impairment of GSSG-rd activity which would decrease the rate of conversion of GSSG to GSH could probably contribute to GSH depletion. Melatonin counteracted these changes by restoring the normal GSSG-rd activity values, although it failed to prevent the decreased GSH levels. These findings are in part in agreement with previous observations by Melchiorri et al., 1997 [20] showing that the administration of the hormone completely or partially overcame the ethanol-induced changes in GSSG-rd activity in the gastrointestinal tract. These changes were linearly correlated with the level of protection of the hormone against ethanol-induced gastric injury. Similar results have been obtained by Pablos et al., 1997 [42] on oxidative damage in lung and brain induced by hyperbaric oxygen and by Sewerynek et al., 1996 [47] in I-R liver-induced oxidative damage.

In addition to the antioxidant effects, others mechanisms could be implicated in the antiulcerogenicity of melatonin. In this context, several studies indicated that the gastroprotective

effects afforded by the hormone could be mediated by the stimulation of prostaglandins or to the increase in the gastric blood flow [19,48]. Because melatonin easily penetrates the blood-brain barrier, it has also been reported a central effect of the hormone against stress-induced gastric ulcers in rats [21].

Using the radiolabelled melatonin agonist, 2-[<sup>125</sup>I] iodomelatonin, and melatonin binding sites have been identified in the gut of several species [49], including the glandular portion of the gastric wall in the rat [50, 51]. The results obtained in birds and man indicate the presence of one single class of binding sites in the gut, which appears to be of very high affinity [49]. In our study melatonin was injected by i.p. route, on the basis of the reported data, it is possible that the hormone could act via a direct peripheral mechanisms on gastric target cells or indirectly via the central nervous system.

In conclusion, the results of the present study, confirm that not only the free radical scavenger activity of melatonin mainly of the superoxide anion, probably derived via the XO pathway, but also the increase of antioxidative enzymes (SOD and GSSG-rd) significantly contribute to mediating the protection by the hormone against I-R gastric injury. Nevertheless, further studies are needed to elucidate the molecular mechanisms of the antiulcer action of melatonin and study in-depth the pharmacological possibilities of the hormone in digestive disorders.

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