Original Article

Protective effect of melatonin on experimental spinal cord ischemia

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Study design: Experimental animal model to assess ischemic spinal cord injury following occlusion of the thoraco-abdominal aorta.

Objectives: To measure whether melatonin administered to rabbits before and after occlusion exerts an effect on the repair of ischemia–reperfusion (IR) injury.

Setting: Medical Biology Laboratory, Inonu University, Malatya, Turkey

Methods: Rabbits were divided into three IR treatment groups and one sham-operated (ShOp) control group. The three treatment groups had their infrarenal aorta temporarily occluded for 25 min, while the ShOp group had laparotomy without aortic occlusion. Melatonin was administered either 10 min before aortic occlusion or 10 min after the clamp was removed. Physiologic saline was administered to the control animals. After treatment, the animals were euthanized and lumbosacral spinal cord tissue was removed for the determination of relevant enzyme activities.

Results: Malondialdehyde levels, indicating the extent of lipid peroxidation, were found to be significantly increased in the nonmelatonin treated (IR) group when compared to the ShOp group. Melatonin, whether given to pre- or post occlusion groups, suppressed malondialdehyde levels below that of the ShOp group. Catalase (CAT) and glutathione peroxidase (GSH-Px) enzyme activities were increased in the IR group compared to the ShOp group. Melatonin given preocclusion resulted in a significant decrease in both CAT and GSH-Px enzyme levels. The superoxide dismutase (SOD) enzyme activity was decreased in the ischemia–reperfusion treatment group. However, the melatonin treatment increased SOD enzyme activity to levels approximating that of the ShOp group.

Conclusion: To our knowledge, this is the first study that shows the effects of melatonin administered both pre- and postischemia on induced oxidative damage to injured spinal cords. Our data also expands on reports that melatonin administration may significantly reduce the incidence of spinal cord injury following temporary aortic occlusion.

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Keywords: catalase; glutathione peroxidase; lipid peroxidation; melatonin; superoxide dismutase; spinal cord ischemia

Introduction

Experimental models and clinical observations of acute spinal cord injury support the concepts of primary and secondary injury in which the initial mechanical insult is followed by a series of deleterious events that promote progressive tissue damage and ischemia. Whereas the fate of the primary injury depends upon the circum-

stances of the trauma, the outcome of the secondary injury may be amenable to therapeutic modulation. The extent of lipid peroxidation is a useful parameter for evaluating the cellular disturbances caused by spinal cord injury caused by ischemia/reperfusion (IR) in experimental conditions.^{1–10}

Free oxygen radicals have been implicated in post-ischemic cell injury and cellular death, while free radical scavengers such as superoxide dismutase (SOD) and catalase (CAT) are associated with an amelioration of ischemic injury.¹¹ To prevent such damage, a variety of

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biological defense systems are activated in normal cells. Among these, the enzymes superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), and glutathione peroxidase (GSH-Px, EC 1.11.1.9) are the best-known components of the biological protective system.^{12–15} It is recognized that these enzymes may be important protectors against lipid peroxidation and damage due to free radicals after the onset of neuronal ischemia.

Melatonin, which is considered to be an antioxidant agent for free oxygen radicals, has been reported to have a role in the treatment of a variety of cerebral pathologies including ischemia, vasospasm and brain edema in experimental settings.^{10,16–18}

However, there are conflicting reports concerning melatonin's scavenging activity on superoxide anions.¹¹ The results presented here indicate that melatonin is effective in preventing secondary damage due to peroxidation of lipid membranes in the ischemic spinal cord. Melatonin treatment promotes a significant increase in protective enzyme levels and may have a potential role in an alternative treatment for spinal cord ischemia.

Materials and methods

General animal care and surgery

This study was performed in the Medical Biology Research laboratory of Inonu University, Faculty of Medicine. The experimental protocol was evaluated and approved by the Ethics Review Committee of Inonu University, Faculty of Medicine, prior to the study. In total, 32 adult male New Zealand rabbits weighing 2.8–3.5 kg were used. The animals were kept at optimal (18–21°C) room temperature and fed a standard diet (Oguzlar Yem Sanayi Eskisehir, Turkey). A 12 h light–dark cycle (8:00 am–8:00 pm light/ 8:01 pm–7:59 am dark) was implemented. Free access to food and water was allowed.

The animals were anesthetized by intramuscular (i.m.) injection of ketamine (70 mg/kg) (Ketalar, Parke-Davis, Eczacibasi, Istanbul, Turkey) and xylazine (5 mg/kg) (Rompun, Bayer, Istanbul, Turkey) and allowed to breathe spontaneously. Additional doses were occasionally required during the surgery. Body temperatures were measured by rectal thermometry and maintained at 37°C with a pad and heating lamp. The arterial pressure, heart rate and arterial blood gases were monitored continuously. pO_2 , pCO_2 and pH were determined to be normal during surgery.

Melatonin preparation

Melatonin (Sigma, St Louis, MO, USA) was dissolved in a minimum volume of ethanol (0.5 ml) and diluted to the desired concentration with physiological saline.

Ischemia–reperfusion procedure

The spinal cord ischemia model described by Zivin and DeGirolami¹⁹ was used in this study. Briefly, the

thoraco-abdominal aorta was clamped by a 'Bulldog' atraumatic infantile vascular compression clamp approximately 1 cm below the renal artery. The location of the clamping corresponds to the L3 vertebrae of the animal. Experimental animals were subjected to 25 min of cross clamp time. At the end of the occlusion period, the clamp was removed and restoration of blood flow was visually verified. For the control study, the ShOp group was subjected to only laparotomy without aortic compression.

Setting of groups and melatonin administration

In total, 32 rabbits were divided into three treatment groups and one control group. The control group containing eight rabbits was ShOp. The second group of eight animals was subjected to IR by clamping the thoraco-abdominal aorta for 25 min. Each rabbit in the IR group received an equivalent dose of aqueous-buffered saline administered intraperitoneally (i.p.) 10 min after the clamp was removed. Another eight rabbits in the Pre-IR group were given 10.0 mg/kg melatonin i.p. 10 min before the aorta was clamped for ischemia and reperfusion.^{7,20} The remaining eight rabbits (Post-IR group) were given 10.0 mg/kg melatonin IP 10 min after the aortic clamp was removed.

Sacrificing of animals and sample preparation

Rabbits were killed by ketamine overdose after reperfusion. Intra-arterial perfusion was performed with a left ventricular cannula while the right atrium was opened widely. During the perfusion, circulating blood was washed out with 500 ml physiological saline under 110 cmH₂O pressure. The lumbosacral cords between L4-S1 were immediately removed. The tissues were collected under a surgical microscope at $\times 400$ magnification using a $\times 16$ ocular and a $\times 25$ objective (OPMI-99 microscope, Carl Zeiss Inc. Germany). Spinal cord samples were, labeled as group 1A, 1B, etc to 4A, 4B, etc for coded blind biochemical studies, placed in liquid nitrogen immediately and then transported to the laboratory for measurement of antioxidant enzyme activities and lipid peroxidation levels. Spinal cord tissue samples were divided into four equal parts for homogenization. An Ultra-Turrax homogenizer (model T25, Janke and Kunkel, Germany) 9500 rpm (4×10 s at 4°C) was used. The homogenates were centrifuged at 1000 g for 10 min and supernatants were collected. The first part was homogenized in a ratio of one part wet tissue to 9 ml of cold 1.15% KCl. From this 1:9 homogenate, 0.5 ml was used for the MDA assay. The remaining three parts were homogenized as above (1:9) but with the addition of 0.02 M potassium phosphate (pH 7.0) as a buffer. The buffered homogenate was equally divided for CAT, SOD and GSH-Px activity determination. Chemicals were purchased from Sigma Chemical Co. (St Louis, MO, USA) unless otherwise stated.

Measurement of MDA levels and antioxidant enzyme activities

Malonaldehyde Malonaldehyde levels were measured spectrophotometrically by the method of Uchiyama and Mihara,²¹ which follows changes in malonaldehyde precursors in tissue through use of the thiobarbituric acid (TBA) test. To 0.5 ml of tissue homogenate, 3 ml of 1% H_3PO_4 and 1 ml 0.6% TBA aqueous solution was added, stirred and heated in a boiling water bath for 45 min. After cooling, 4 ml of *n*-butanol was added and the mixture was shaken. The butanol layer was separated by centrifugation and the absorbance of the butanol layer was measured spectrophotometrically at 535 and 520 nm. The difference between the two optical densities was a measure of the TBA value. Results are expressed as nmol per gram wet weight (nmol/gww).

Superoxide dismutase SOD enzyme activity was measured by the indirect spectrophotometric assay described by McCord and Fridovich.²³ SOD catalyzes the dismutation of superoxide radicals. SOD activity was determined in the supernatant using its inhibitory action on the superoxide-dependent reduction of cytochrome *c* by a xanthine/xanthine oxidase mixture. In a total volume of 1.1 ml, the reaction mixture contained phosphate buffer (20 mM, pH 7.0), 0.1 M EDTA, 50 μM xanthine and 10 μM cytochrome *c*. The reaction was initiated by adding xanthine oxidase, and the rate of cytochrome *c* reduction was monitored spectrophotometrically at 550 nm.

Catalase CAT activity was measured spectrophotometrically by the method of Luck.²⁴ Samples were diluted 1:10 in cold potassium dihydrogen phosphate buffer (pH 7.0) incubated with occasional shaking for 10 min at 4°C and centrifuged, followed by a repeated extraction. Supernatants were combined, and 0.04 ml was added into 3 ml 0.01 M of H_2O_2 -phosphate buffer, mixed well, and read on a spectrophotometer containing a constant temperature unit attachment. Blank readings were performed in a control cuvette that included enzyme solution as in the experimental sample but with H_2O_2 -free phosphate buffer. Decreases in absorbance measurements at 240 nm are followed until the optical density reaches 0.450. The time (Δt) required for the optical density to reach 0.400 was recorded and used to obtain the value *k* from the formula $0.1175/\Delta t$. The value *k* is then converted to CAT units.

Glutathione peroxidase GSH-Px activity was measured spectrophotometrically by the method of Lawrance and Burk.²⁵ The assay measures the oxidized glutathione formed in the GSH-Px reaction by coupling it to the oxidation of NADPH via glutathione reductase. The reaction mixture contained 50 mM potassium phosphate buffer (pH 7), 1 mM EDTA, 1 mM NaN_3 , 0.01 EU/ml

GSSG-reductase, 1 mM GSH, 1.5 mM cumene-hydroperoxide and 0.25 mM H_2O_2 . All ingredients were prepared fresh each time except for the enzyme. For each assay, the enzyme was added to 0.8 ml of the above mixture and allowed to incubate at room temperature before initiation of the reaction with the peroxide solution. Absorbance at 340 nm was recorded for 5 min. Enzyme activity was calculated as micromoles per minute. In blank reactions, the enzyme was replaced by water and readings were subtracted from each assay. Protein was measured by the Lowry procedure,²² using bovine serum albumin as a standard.

Statistical evaluation

The Mann–Whitney *U*-test was used for determining the significance of the results. Treatment differences with *P*-values less than 0.05 are considered significant and are represented by a single star (*) and *P*-values less than 0.01 are considered very significant and are represented with two stars (**). The Kruskal–Wallis nonparametric analysis of variance test (ANOVA) was used to compare inter- and intragroup variations of antioxidant enzyme activities as well as lipid peroxidation levels of the lumbosacral cord tissue. The values are given as the mean of eight duplicate measurements (16 samples per group) as well as the standard error of the mean.

Results

The clinical and physiological status of the animals did not change during the course of the study. Two deaths occurred during the ischemia procedure, and the dead rabbits were replaced with new ones to keep the numbers in each group equal.

Lipid peroxidation

MDA levels were found to be significantly increased in the IR group compared to the ShOp, Pre-IR and Post-IR groups. However, in the Pre-IR and Post-IR groups, MDA levels did not show significant differences when compared to the ShOp group (Table 1).

Antioxidant enzyme activities

Cu–Zn SOD activity in the IR group was significantly reduced when compared to the ShOp group. Between the Post-IR and IR groups, Cu–Zn SOD enzyme activity showed significant increase. Between the Post-IR and Pre-IR groups, Cu–Zn SOD activity also had a significant decrease. However, when both groups were compared with the ShOp group, the changes were not statistically significant (Table 1).

CAT enzyme activity in the IR group showed significant increases compared with ShOp, Post-IR and the Pre-IR group. CAT enzyme activities in the Pre-IR and Post-IR group were reduced in comparison with the ShOp group. CAT enzyme activity was significantly higher in the Post-IR group than in the Pre-IR group as seen in Table 1.

Table 1 MDA and antioxidant enzyme levels in each study group

Experimental conditions	MDA (nmol/gww)	SOD (μ mol/mg total protein)	CAT (μ mol/mg total protein)	GSH-Px (μ mol/mg total protein)
ShOp	173.39 $\pm$ 11.29*	10.72 $\pm$ 1.52	76.78 $\pm$ 9.10**	1.45 $\pm$ 0.21**
Pre-IR	157.72 $\pm$ 15.44	8.97 $\pm$ 0.05*	34.16 $\pm$ 4.07**	1.89 $\pm$ 0.50
Post-IR	160.46 $\pm$ 8.85	11.92 $\pm$ 1.33*	54.37 $\pm$ 6.54*	3.38 $\pm$ 0.26**
IR	213.50 $\pm$ 10.80*	7.21 $\pm$ 1.23*	103.13 $\pm$ 6.15**	2.70 $\pm$ 0.26**

Values represent the mean $\pm$ SEM; * P < 0.05; ** P < 0.01

GSH-Px enzyme activity in the IR group and the Post-IR group showed significant increases when compared to the ShOp group. Also, GSH-Px enzyme activity in the IR group and in the Post-IR group showed significant increases when compared to the Pre-IR group. Observed GSH-Px enzyme activity increased in the IR group when compared to the ShOp group. This is most likely due to an increase in peroxide levels in ischemic tissues. When melatonin was exogenously administered to the Pre-IR group, its protective effect on free radical production minimized the need for GSH-Px increased enzyme activity (Table 1). However, exogenous melatonin administration to the Post-IR group was not as effective as in the Pre-IR group in spite of melatonin's protective effect, and GSH-Px enzyme activity increased two and one-half-fold.

Discussion

Ischemic injury to the spinal cord, that is, vascular occlusion, global ischemia, surgical repair of aortic pathologies, coagulopathy and spinal trauma, results in cell and tissue damage.^{11,18,26–30} Free oxygen radicals can impair the function of the cellular components.^{14,15,31} Free oxygen radical-induced damage has been implemented in postischemic cell injury and cell death, while free radical scavengers such as SOD, CAT and GSH-Px are associated with partial amelioration of ischemic injury.^{4,5}

The extent of lipid peroxidation is a useful parameter for evaluating cellular disturbances caused by spinal cord trauma under experimental conditions.^{32–34} The literature mentioned above outlines the biochemical basis and evidence for the occurrence of oxygen radical generation and lipid peroxidation during the acute phase of central nervous system (CNS) trauma or stroke (ischemic and hemorrhagic).

Identification of oxygen radicals and lipid peroxidation, important pathophysiological mediators of trauma or stroke-induced neural degeneration rather than simple epiphenomena, depends upon the successful demonstration of their association with actual secondary physiological and structural degenerative events. Moreover, their significance in the pathophysiology of CNS trauma or stroke must be supported by experimental observations that pharmacological antagonism

of either oxygen radical generation and/or lipid peroxidation results in therapeutic effects.^{1–9,11,17,28,35,36}

Work by Fujimoto *et al*⁵ showed that potent protective effects of melatonin on experimental trauma induced on rat spinal cords at T12 showed increased MDA levels after the injury. This work showed that melatonin facilitated recovery of the damaged spinal cord. As a measure of melatonin protection, Fujimoto *et al* determined the reduction of TBA reactive substance content. The studies presented in this paper also show the protective effects of melatonin when administered both before and after IR injury to the spinal cord as opposed to Fujimoto's use of direct physical trauma to the spinal cord.

Our results are in good agreement with those in the literature,^{35–37} indicating that increases in MDA levels are indicators of the extensiveness of the IR injury and most likely due to tissue membrane damage after IR injury.

CAT is an important scavenging enzyme against free oxygen radicals since it removes H₂O₂ produced during metabolic processes.^{10,31,38,39} In our study, observed changes in CAT activity indicate that IR-induced tissue damage could be treated with melatonin. The administration of melatonin brings CAT activity to levels even below that of the ShOp group. The amount of CAT may be critical for the protective effect against the oxidative stress under pathological conditions such as IR injury.

Protective effects of melatonin were shown by decreased SOD activity in damaged spinal cord tissues but at a smaller scale than the decreases observed in CAT activity.¹⁵ However, Nishibe showed that SOD activity is generally much lower than that of CAT activity in spinal cord tissues.⁴⁰ Therefore, SOD activity changes observed due to melatonin treatment are less pronounced than those seen in CAT.¹⁴ The data reported here are in agreement with Kaynar *et al*,³¹ who found that SOD and glutathione activities were not significantly different, while CAT activity was significantly higher in traumatic spinal cord segments.

In addition to its documented free radical scavenging activity, melatonin may regulate glutathione synthesis through glutathione synthetase activation.²⁵ On the other hand, Barlow-Walden *et al*¹⁶ reported that acute administration of melatonin causes a two-fold rise

in GSH-Px activity in rat brain. GSH-Px is thought to be the principal enzyme eliminating peroxides in the brain. This oxidative enzyme reduces the formation of hydroxyl radicals formed via iron-catalyzed Fenton-type reactions from H_2O_2 . Our results show statistically significant increases in GSH-Px activity in the Post-IR (ie, melatonin administrated) group versus the ShOp group, suggesting that melatonin has a free radical scavenging effect. GSH-Px activity in the cerebral cortex and hippocampus is more stable than that of SOD activity observed in the same locations.³¹ However, GSH-Px shows a significant decrease only at 48 h in rat brains.¹⁶ The administration of pharmacological doses of melatonin increases GSH-Px activity in the brain. Thus, our data show that both CAT and GSH-Px activities were higher in IR injury.

Barlow-Walden *et al*¹⁶ proposed two possible explanations for the induction of the GSH-Px activity: (1) the increase in GSH-Px activity is elicited by the antioxidative activity of melatonin, which protects the enzyme from inactivation by hydroxyl radicals and (2) an enzyme induction is mediated by changes in GSH-Px gene expression. In agreement with these theories, our studies showed that the highest levels of GSH-Px activity were in the post-IR group.

The apparent role of oxygen free radicals in ischemic neuronal damage and other types of injuries has stimulated interest in antioxidants and free radical scavengers as potential therapeutic agents.^{16,41} Among these, melatonin molecule is a potent-free radical scavenger.^{17,37} Melatonin is also a highly soluble and easily diffusible molecule. Owing to its extreme lipophilicity, it crosses morphophysiological barriers such as the blood brain barrier, cell membrane and basal membrane, diffusing into all body fluids.⁴² Thus, exogenous melatonin administration can provide on-site protection against free radical damage.^{18,43}

The present study shows that antioxidant enzyme levels due to the extensive presence of free radicals in damaged tissue were significantly changed in the presence of melatonin. Changes observed in free radical scavenger enzyme concentration indicate that melatonin might have provided significant protection against the effect of the free radical damage. We suggest that the mechanisms of this protection are either (1) melatonin causing an induction of antioxidant enzyme levels or (2) melatonin acting as a free radical scavenger that lowers the need for extensive production of endogenous antioxidant enzymes. Further experiments must be carried out to determine the protective mechanism of melatonin action. In the literature, there are studies that demonstrate the protective effect of melatonin. However, in these studies, the treatment with melatonin was always administered after IR damage.^{17,18,37}

To our knowledge, this is the first study that shows the protective effects of melatonin administration in an attempt to prevent IR induced oxidative damage on spinal cord injury before it has occurred. Our results demonstrate that prior application of melatonin

can reduce damage to spinal cord IR-induced injury. The data show that spinal cord ischemia caused significant changes in the activities of endogenous antioxidants.

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