

## ORIGINAL ARTICLE

## The effect of melatonin on spinal cord after ischemia in rats

S Aydemir<sup>1</sup>, D Dogan<sup>1</sup>, A Kocak<sup>2</sup> and N Dilsiz<sup>3</sup>**Study design:** Experimental animal model to assess ischemic spinal cord injury (SCI) following occlusion of the thoraco-abdominal aorta.**Objectives:** In the present study, we aimed to investigate the role of melatonin on SCI induced by ischemia and following reperfusion.**Setting:** Animal Research Laboratory, Inonu University, Malatya, Turkey.**Methods:** We evaluated oxidative damage and caspase-3 activity. In total, 32 adult Wistar albino rats were divided into four groups: Group 1, control ( $n=8$ ); Group 2 ( $n=8$ ), those subjected to ischemia/reperfusion (IR) by clamping the thoraco-abdominal aorta; Group 3 ( $n=8$ ), melatonin ( $50\text{ mg kg}^{-1}$ ) treated; and Group 4 ( $n=8$ ), melatonin ( $50\text{ mg kg}^{-1}$ ) followed by ischemia. All animals were kept alive for 48 h, and then spinal cord samples were removed. We assayed oxidative damage by measuring malondialdehyde (MDA), apoptosis by measuring activated caspase-3 (using immunoblots) and intrinsic antioxidative capacity by measuring reduced glutathione (GSH) levels in the spinal cord.**Results:** The results indicated a significant decrease in activity of caspase-3 in SCI animals after treatment with melatonin, as it significantly decreased the formation of MDA and decelerated the loss of GSH.**Conclusion:** This study suggested that melatonin could be an effective neuroprotective agent for treatment of SCI.*Spinal Cord* (2016) 54, 360–363; doi:10.1038/sc.2015.204; published online 1 December 2015

## INTRODUCTION

Spinal cord injury (SCI) can result in severe disability, sensory disorder, paralysis, other neurologic deficits and even death. After SCI, many cells are lost because of the injury.<sup>1</sup> Perturbation of the  $\text{Ca}^{2+}$  homeostasis after injury causes defects in mitochondrial respiratory electron transport. Free radical intermediated products are continuously produced and released. These reactive oxygen species rapidly react with unsaturated and polyunsaturated fatty acid side chains of membrane lipids or with other components of the cells, thus harming the cell and generating more free radicals. The saturation of plasma membrane components by lipid peroxidation induces adverse changes of membrane properties like decreased fluidity and permeability. Malondialdehyde (MDA) is a major peroxidation product.<sup>2</sup> An elevated concentration of MDA is one of the markers of tissue damage.<sup>3</sup> Subsequent cell death is believed to be primarily necrotic.

Melatonin is an indoleamine, naturally produced in the pineal gland and other tissue, and it has been shown to possess several properties that may be responsible for its neuroprotective effects.<sup>4</sup> In experimental animal models of SCI, melatonin has been found highly effective in reducing the level of lipid peroxidation following injury.<sup>5</sup> This effect may be due to the facts that melatonin is a powerful antioxidant, and it stimulates several antioxidants including: catalase, glutathione reductase, peroxidase and superoxide dismutase. Melatonin has been shown to be highly effective in reducing oxidative damage in the central nervous system; its efficacy derives from its

ability to directly scavenge a number of free radicals and to function as an indirect antioxidant.<sup>6</sup>

Melatonin is a free radical scavenger, an antioxidant and immunomodulatory agent. Antioxidant properties of melatonin are connected with its neuroprotective activity in several degenerative disorders. One of the causes of neurodegenerative damage in the nervous system in oxidative injury result from an imbalance between free radical formation and antioxidative mechanisms.<sup>7</sup>

Caspases have a pivotal role in the progression of a variety of neurological disorders. The major executioners in the apoptotic program are proteases known as caspases (cysteine-dependent aspartate-specific proteases). Caspases directly and indirectly orchestrate the morphologic changes of the cell during apoptosis. They exist as latent precursors, which, when activated, initiate the death program by destroying key components of cellular infrastructure and activating factors that mediate damage to the cells. Ischemic stroke was the first neurologic disease in which the activation of a caspase was documented.<sup>8,9</sup> Moreover, inhibition of caspases reduces tissue damage and allows remarkable neurologic improvement. Activation of caspases 1,3,8,9 and 11 and release of cytochrome *c* have been demonstrated in cerebral ischemia.<sup>10–12</sup> Melatonin is known to stabilize membranes and thus prevents cell damage and death. As both calpain and caspase-3 activities are known to participate in cell death, the implication is that reduction of their activities by melatonin may have protected neurons in the SCI following injury.<sup>1</sup>

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Received 2 April 2015; revised 1 October 2015; accepted 16 October 2015; published online 1 December 2015

On the basis of these findings, in the current study, we investigated the effects of melatonin on experimental SCI-induced tissue damage in spinal cord by measuring MDA, glutathione and caspase-3 activity.

## MATERIALS AND METHODS

### Animal handling and treating with melatonin

This study was performed in the Medical Biology Research Laboratory of Inonu University, Faculty of Sciences and Arts. The experimental protocol was evaluated and approved by the Ethics Review Committee of Inonu University, Faculty of Medicine. The animals were kept at optimal (18–21 °C) room temperature and fed a standard diet. A 12-h light–dark cycle (0800–2000 hours light/2001–0759 hours 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<sup>-1</sup>) (Ketalar, Parke-Davis, Eczacıbaşı, Istanbul, Turkey) and xylazine (5 mg kg<sup>-1</sup>) (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.

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.

The spinal cord ischemia model described by Zivin and De Girolami was used in this study.<sup>13</sup> In total, 32 rats were divided into four groups. The control group contained eight rats (C). The control group was not injured in order to compare the effects of melatonin treatment intact animals and injured animals. The second group of eight animals was subjected to IR by clamping the thoraco-abdominal aorta (IR). Another eight rats in the pre-IR group were given 50 mg kg<sup>-1</sup> melatonin intraperitoneally (i.p.) 10 min. before the aorta was clamped for ischemia and reperfusion (Mel+IR). The remaining eight rats were given 50 mg kg<sup>-1</sup> melatonin (Mel) but were not injured.

### Ischemia–reperfusion procedure

The spinal cord ischemia model described by Zivin and DeGirolami<sup>13</sup> 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. At the end of the occlusion period, the clamp was removed, and restoration of blood flow was visually verified. For the control study, the same group was subjected to only laparotomy without aortic compression.

Rats were killed by ketamine overdose after reperfusion. Intra-arterial perfusion was performed with a left ventricular cannula, whereas the right atrium was opened widely. During the perfusion, circulating blood was washed out with 500 ml physiological saline under 110 cm H<sub>2</sub>O pressure. The lumbosacral cords between L4 and S1 were immediately removed. The tissues were collected under a surgical microscope at ×400 magnification using a ×16 ocular and a ×25 objective (OPMI-99 microscope, Carl Zeiss Inc., Oberkochen, Germany). Spinal cord samples were labeled and placed in liquid nitrogen

immediately and then transported to the laboratory for measurement of caspase 3 activity and lipid peroxidation levels (nmol per gwt).

### Measurement of MDA

To determine the amount of oxidative damage in the spinal cord, levels of MDA were measured spectrophotometrically by the method of Uchiyama and Mihara,<sup>14</sup> which follows changes in malondialdehyde precursors in tissue through the use of the thiobarbituric acid test. To 0.5 ml of tissue homogenate, 3 ml of 1% H<sub>3</sub>PO<sub>4</sub> and 1 ml 0.6 thiobarbituric acid aqueous solutions were added, then 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 thiobarbituric acid value. A calibration curve was drawn by using 1,1,3,3-tetramethoxypropane, and results were presented as nanomoles of MDA per gram of wet tissue (g.w.t).

### Measurement of GSH

GSH was assayed by reacting with O-phthalaldehyde (10 mg/10 ml methanol) according to the modified method of Lee and Chung.<sup>15</sup> Briefly, tissue was homogenized in ice-cold 6% perchloric acid at 4 °C. Supernatant was collected after centrifugation at 4000 × g for 10 min at 4 °C.

An aliquot of the supernatant was allowed to react with O-phthalaldehyde for 5 min. Pure reduced GSH was used as standard for calibration. GSH samples were measured by using spectrofluorimetry (excitation at 345 nm and emission at 425 nm).

### Assessment of caspase-3 activity

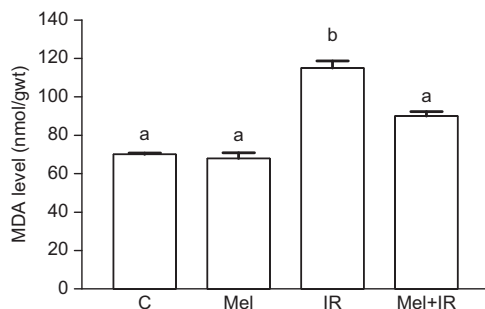
All spinal cord samples were homogenized in lysis buffer (Tris-NaCl-EDTA-protease inhibitors) by using glass-dounce homogenizer. The homogenate was centrifuged to pellet protein at 13 000 r.p.m. at 4 °C for 15 min. Supernatants were run on SDS-PAGE after heating at 65 °C for 3 min by standard procedures using the Mini Protean II system (Bio-Rad, Hercules, CA, USA). After electrophoresis, proteins were transferred to a PVDF membrane (Bio-Rad) using the Trans-Blot Semi-Dry system (Bio-Rad) at 20 V for 30 min. The membranes were blocked in Roti-Block reagent (Roth, Germany) with gentle shaking at RT for 1 h. After washing with tris-buffered saline (TBS) (20 mM Tris, pH 7.5, 150 mM NaCl), blots were incubated with anti active product of caspase-3 (that react only with 17 kDa subunit, Chemicon, AB3623) antibody (1:200) for 2 h. Blots were washed three times with tris-buffered saline and tween 20 (TBS-T) (20 mM Tris, pH 7.5, 150 mM NaCl, 0.05% Tween 20) and incubated with secondary goat anti-rabbit antibody conjugated with alkaline phosphatase (1:30 000) for 1 h. After washing with TBS-T(3×) and with TBS only (1×), a color reaction was performed in the dark with NBT/BCIP alkaline phosphatase substrate tablets (Sigma fast tablet) under agitation for 10–30 min, until development of a black–purple color. The membranes were then scanned, and the integrated optical density of the resulting bands was determined by Fluor-S-Multi-Imager (Bio-Rad, München, Germany) and given as arbitrary units.

### Statistical analysis

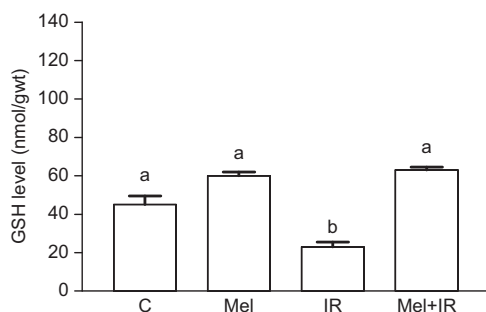
Statistical calculations were performed by using Excel (Microsoft, Redmond, WA, USA) and SPSS (SPSS, Inc., Chicago, IL, USA). The normality of distributions of data was evaluated with the Shapiro–Wilk test and was found abnormal ( $P < 0.05$ ). The results are expressed as mean ± s.e.m. Differences between the group means were analyzed for significance using the Mann–Whitney *U*-tests. In all cases, a *P*-value of  $< 0.05$  was considered to be statistically significant.

## RESULTS

As expected, IR caused spinal cord damage as indicated by increased levels of MDA, decreased GSH contents and the occurrence of measurable caspase-3 activity. MDA levels were found to be significantly increased in the IR group compared with the control group, IR and mel+IR groups (Figure 1). Control group GSH levels were found



**Figure 1** Spinal cord tissue (L3–L4) malondialdehyde (MDA) levels in the experimental groups. Control (C), Melatonin (Mel), Ischemia/reperfusion (IR), Melatonin+Ischemia/reperfusion (Mel+IR), gram wet tissue (gwt). The values are shown as mean ± s.e.m. Different letters indicate significant difference between the bars ( $P < 0.05$ ).



**Figure 2** Spinal cord tissue (L3–L4) reduced glutathione (GSH) levels in the experimental groups. Control (C), Melatonin (Mel), Ischemia/reperfusion (IR), Melatonin+Ischemia/reperfusion (Mel+IR), gram wet tissue (gwt). The values are shown as mean  $\pm$  s.e.m. Different letters indicate significant difference between the bars ( $P < 0.05$ ).

to be significantly decreased ( $P < 0.05$ ). GSH levels were found to be significantly increased in the mel+IR group (Figure 2).

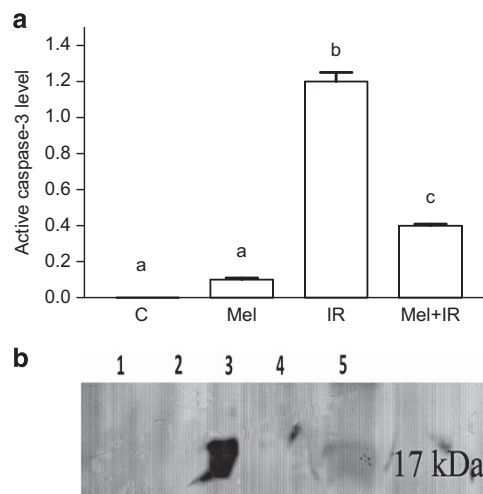
In the normal control of spinal cord tissue, we failed to find any measurable active p17 fragment of caspase-3 (Figures 3a and b). However, in the spinal cord tissue of the IR group, a marked activation of caspase-3 occurred (to 1.2 AU;  $P < 0.05$ ).

Melatonin application reduced the IR-induced caspase-3 activation to 0.1 AU ( $P < 0.05$ ).

## DISCUSSION

Although oxygen is critical for life and for maintenance of metabolic processes, reactive metabolites of oxygen may be toxic to cells. In particular, the cellular damage that occurs secondary to ischemia may be exacerbated by the sudden re-oxygenation into tissues during onset of the reperfusion period, triggering free radical cascades that overwhelm endogenous free radical scavengers. Here we show that the IR-induced alterations of rat spinal cord involve an activation of caspase-3, as an indicator of apoptosis, an increase in lipid peroxidation (assayed by measuring MDA) and an exhaustion of the endogenous free radical scavenger, GSH.

Melatonin is a free radical scavenger, an antioxidant and an immunomodulatory agent. In addition to many normal physiological roles of melatonin, the neuroprotective properties of this pineal gland hormone have been investigated in neurodegenerative diseases and injuries. Investigations of the chemical properties of melatonin, both *in vivo* and *in vitro*, indicate that melatonin and its metabolites are potent antioxidants that directly scavenge both reactive oxygen species (ROS) and reactive nitrogen species (RNS) in the cells.<sup>4,6,16</sup> Besides, melatonin stimulates many antioxidant enzymes. The antioxidant properties of melatonin are connected with its neuroprotective activity in several degenerative disorders. One of the causes of neurodegenerative damage in the nervous system is oxidative injury, which results from an imbalance between free radical formation and antioxidative mechanisms.<sup>17</sup> The efficacy of melatonin in the inhibition of the oxidative stress was estimated in various neurodegenerative disorders. The mechanisms involved in melatonin protection against ischemia–reperfusion damage seem to be mainly mediated by its antioxidant properties. A direct relationship between the protection by melatonin and its antioxidant properties was found by Sinha *et al.*<sup>18</sup> who besides the decreased lesion volume and the improvement of neurological deficits, reported prevention by melatonin of lipid peroxidation and glutathione reduction induced by ischemia. In the other experimental model, Kılıç *et al.*<sup>19</sup> confirmed the recovery by melatonin of Akt phosphorylation levels, also reporting the increase in



**Figure 3** (a) Spinal cord tissue (L3–L4) optical density of active caspase-3 protein from western blotting. Control (C), Melatonin (Mel), Ischemia/reperfusion (IR), Melatonin+Ischemia/reperfusion (Mel+IR). The values are shown as mean  $\pm$  s.e.m. Different letters indicate significant difference between the bars ( $P < 0.05$ ). (b) Western blot analysis of rat spinal cord tissue (L3–L4). 1. Control, 2. Melatonin, 3. Ischemia reperfusion, 4. Melatonin+Ischemia reperfusion, 5. Marker. The experiment was repeated three times, with similar results.

the antiapoptotic Bcl-XL protein expression and the decrease in caspase-3 activity.

In the present study, we used a powerful antioxidant agent, melatonin, to examine its effects on the SCI-induced oxidative injury and apoptosis. It is well known that the formation of MDA, an end product of lipid peroxidation, is a major indicator of oxidative injury.<sup>1,5,20</sup> In the current study, SCI caused significant elevations in MDA levels in spinal cord tissues demonstrating oxidative stress, while caspase-3 activity was also increased. It was reported that, in animal models of SCI, melatonin has been found highly effective in reducing the level of lipid peroxidation following injury, and the effects are attributed to its antioxidative actions.<sup>20,21</sup> Furthermore, our results are also in agreement with the study of Samantaray *et al.*<sup>1</sup> who demonstrated that melatonin significantly decreased caspase-3 activity in SCI animals. Melatonin prevents cell damage and death. As both calpain and caspase-3 activities are known to participate in cell death, the implication is that reduction in their activities by melatonin may have protected neurons in the SCI following injury.<sup>1</sup>

Reduced GSH has an important role in cell metabolism, differentiation, proliferation and apoptosis, and as a result disturbances in its homeostasis are implicated in the etiology and/or progression of a number of human diseases, including neurodegenerative diseases and injuries. Several reports indicate that tissue injury induced by various stimuli is coupled with GSH depletion. Melatonin is a well-known direct, free radical scavenger and neutralizer of a number of reactive oxygen and nitrogen species.

In conclusion, because melatonin has almost no toxicity, is available and easily administered, clinical studies of its potential for protecting SCI may be warranted.

## DATA ARCHIVING

There were no data to deposit.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

## ACKNOWLEDGEMENTS

This work was supported by a grant from The Scientific and Research Project Unit of Inonu University (BAP-2009004), Turkey.

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