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ORIGINAL PAPER

Neuroprotective effects of high-dose vs low-dose melatonin after blunt sciatic nerve injury

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Abstract
Introduction Melatonin, the secretory product of the pineal gland, has potent antioxidant properties. The aim of this study was to compare the effects of low-dose (10 mg/kg) vs high-dose (50 mg/kg) melatonin on early lipid peroxidation levels and ultrastructural changes in experimental blunt sciatic nerve injury (SNI). We believe this to be the first study to assess the dose-dependent neuroprotective effects of melatonin after a blunt peripheral nerve injury.
Materials and methods Rats were randomly allocated into 5 groups of 10 animals each. The SNI only rats underwent a nerve injury procedure. The SNI plus vehicle group received SNI and intraperitoneal injection of vehicle (diluted ethanol) as a placebo. The SNI plus low-dose or high-dose melatonin groups received intraperitoneal melatonin at doses of 10 mg/kg or 50 mg/kg, respectively. Controls had no operation, melatonin or vehicle injection. SNI was induced by clamping the sciatic nerve at the upper border of the quadratus femoris for 2 min.
Results Sciatic nerve samples were harvested 6 h after nerve injury and processed for biochemical and ultrastructural analysis. Trauma increased the lipid peroxidation of the sciatic nerve by 3.6-fold (153.85±18.73 in SNI only vs 41.73±2.23 in control rats, $P<0.01$). Low ($P=0.02$) and high ($P<0.01$) doses of melatonin attenuated the nerve lipid peroxidation by 25% and 57.25%, respectively (65.76± 2.47 in high-dose vs 115.08±7.03 in low-dose melatonin groups).
Discussion Although low-dose melatonin reduced trauma-induced myelin breakdown and axonal changes in the sciatic nerve, high-dose melatonin almost entirely neutralized any ultrastructural changes.
Conclusion Our results suggest that melatonin, especially at a dose of 50 mg/kg, has a potent neuroprotective effect and can preserve peripheral neural fibers from lipid peroxidative damage after blunt trauma. With further investigations, we hope that these data may prove useful to clinicians who treat patients with nerve injuries.

Keywords Lipid peroxidation · Melatonin · Oxidative stress · Peripheral nerve injury

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Introduction

Peripheral nerve trauma remains a major cause of morbidity and economic and social disruption [7]. Even with

optimal surgical repair, sensation and motor function may remain impaired [7, 12]. It may also exert adverse effects upon skilled motor function, particularly fine manipulative work, because adequate sensory feedback is a vital component of normal proprioception and, therefore, motor control [39].

Although the mechanisms of posttraumatic repair of the peripheral and central nervous systems are quite different, the primary success of antioxidant agents in reversing injuries of the central nervous system may also elucidate pharmacologic modulation for the repair of the peripheral nervous system. The primary pathway for peripheral nerve injury after a traumatic incident is now believed to be free radical-induced lipid peroxidation along neural fibers [1, 19, 26]. As the peripheral nervous system is composed primarily of lipids, they are potentially vulnerable to lipid peroxidation. Moreover, neural tissue does not contain highly active oxidative defense mechanisms. Hence, lipid peroxidation is potentially damaging because it may alter the fluidity and permeability of neuronal membranes and therefore, affect the functional and structural integrity of membrane-bound receptors and enzymes.

Melatonin (*N*-acetyl-5-methoxytryptamine), the chief neurosecretory product of the pineal gland, is a potent antioxidant in biological systems [15]. Numerous studies have demonstrated that under many different types of oxidizing conditions, melatonin forms a first line of antioxidant defense that effectively prevents peroxidative injuries [16, 29]. Melatonin is known to reduce the harmful effects of free radicals in the central nervous system either by free radical scavenging or decreasing nitric oxide synthase activity [2, 9, 27, 30, 31, 33]. Melatonin also inhibits posttraumatic polymorphonuclear infiltration [8] and stimulates superoxide dismutase, glutathione peroxidase, and glutathione reductase [34]. All of these effects suggests that melatonin could be a potential therapeutic option in the prevention of trauma-induced peripheral nerve injuries. It is interesting to note that melatonin has been shown to protect the sciatic nerve from ischemia–reperfusion injury by attenuating neural lipid peroxidation [34].

The objective of this study was to assess and compare the neuroprotective effects of high (50 mg/kg) and low doses (10 mg/kg) of melatonin on neural fiber (axon and myelin) damage and lipid peroxidation after a blunt sciatic nerve trauma. These pharmacologic doses of melatonin are well-tolerated. Administration of either low-dose or high-dose melatonin early after a blunt peripheral nerve trauma provides a clinically favorable experimental model. In addition, it is now believed that processes that take place immediately after an injury largely determine the success of regeneration after a peripheral nerve lesion [10].

Materials and methods

Animals and maintenance

A total of 50 young male Wistar rats weighing 225 to 280 g were used in this study according to the Guide for Care and Use of Laboratory Animals (DHEW Publication No. 78-23, NIH revised 1978) and local guidelines for the humane use of animals in research. The animals were housed three per cage and provided with free access to compact food and water. This food consisted of all essential ingredients including vitamins and minerals. Animals were kept under constant laboratory conditions (e.g., 18°C to 21°C room temperature, humidity, and illumination of 12-h cycles of light and darkness with the latter beginning at 7:00 P.M.).

Surgical technique

Under general anesthesia, groups of rats underwent unilateral exposure of the right sciatic nerve at the upper border of the quadratus femoris. An acute blunt (constriction) injury was initiated by clamping the sciatic nerves with a Geister Yasargil standard (titanium) temporary aneurysmal clip (41-5112.T_1) for 2 min. This delivered a force of approximately 70 g. Next, the clip was removed, the skin incision was sutured, and animals were returned to their cages. This technique, which is similar to the clip compression model introduced by Rivlin and Tator [11] for the production of spinal cord trauma, ensures a sufficient degree of sciatic nerve injury (SNI).

Drug preparation and administration

Melatonin (ACROS, 12536-0010) was dissolved in absolute ethanol and then diluted to either 5 or 25 mg/ml of normal saline. The ethanol concentration in the final solutions was 5%. A vehicle of 5% ethanol in normal saline was also produced to be used as a placebo. Melatonin solutions or vehicle of 2 ml/kg was injected intraperitoneally immediately after the induction of SNI to obtain a total injection of 10 and 50 mg of melatonin and a comparable volume of the vehicle in the designed groups. It is interesting to note that melatonin has been shown to be rapidly absorbed irrespective of its administration route and readily crosses the blood–brain (or nerve) barrier because of its lipophilic nature [23, 32, 40].

Group design

Animals were randomly allocated into 5 groups: *control animals* (n=10) had no operation, melatonin or vehicle

injection. The *SNI only animals* (n=10) underwent sciatic nerve exposure and induction of SNI. The *SNI plus vehicle group* (n=10) received intraperitoneal injection of vehicle as a placebo after induction of the nerve injury. The *SNI plus melatonin animals* were divided into 2 groups of 10 animals each receiving either low-dose or high-dose melatonin intraperitoneally as described before (10 vs 50 mg/kg of melatonin).

Sample preparation and determination of lipid peroxides

Under anesthesia, a 1-cm segment of the injured sciatic nerve from the site of clip compression was harvested 6 h after the induction of SNI and at a corresponding time in control rats. Lipid peroxidation in the sciatic nerve of the rat was measured as thiobarbituric acid reactive substance. Tissue homogenates (10% *w*/*v*) were prepared by homogenizing sciatic nerve tissue in 50 mM cold potassium phosphate buffer (pH 7.4) using a glass/glass homogenizer. In a test tube, 0.5 ml of tissue homogenates was mixed with 3 ml of 1% orthophosphoric acid. After the addition of 1 ml of 0.67% thiobarbituric acid, the mixture was heated in boiling water for 45 min. The color produced was extracted into 4 ml of *n*-butanol, and the spectrophotometric absorbance was measured at 532 nm using tetramethoxypropan as the standard. Tissue malondialdehyde (MDA) levels were calculated as nanomole per gram (nmol/g) of wet tissue [17, 25]. Animals were given lethal doses of anesthesia after harvesting of their sciatic nerve.

Ultrastructural studies

For transmission electron microscopy, the sciatic nerve samples obtained from 2 rats in each group were cut into 2×2 mm segments and fixed in 2.5% glutaraldehyde and 1% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Semi-thin sections (1 μm) and ultrathin sections (60–70 nm) were cut and stained with toluidine blue, uranyl acetate, and alkaline lead citrate, respectively. They were then observed under a transmission electron microscope (Zeiss LEO 906, Germany).

Data analysis

All data were expressed as the mean±SEM. One-way analysis of variance (ANOVA) was done to compare the sciatic nerve MDA levels between groups using LSD for the post hoc. Analysis was performed using the SPSS version 11.0 software. P values less than 0.05 were considered to indicate statistical significance.

Results

Figure 1 compares the MDA levels of the sciatic nerve samples of all groups. Trauma was found to produce a 3.6-fold increase in sciatic nerve MDA levels (153.85±18.73 in SNI only group vs 41.73±2.23 in control rats, P<0.01). Intraperitoneal administration of vehicle had no effect on nerve MDA levels (153.85±18.73 in SNI only group vs 150.63±13.75 in SNI plus vehicle group, P=0.83). Administration of low-dose melatonin decreased MDA levels by 25% in the injured sciatic nerve (115.08±7.03 in low-dose melatonin group vs 150.63±13.75 in SNI plus vehicle group, P=0.02). High-dose melatonin also decreased MDA levels of the injured nerves by 57.25% (65.76±2.47 in high-dose melatonin group vs 150.63±13.75 in SNI plus vehicle group, P<0.01). The observed effect of high-dose melatonin in the reduction of MDA levels exceeded that of low-dose melatonin by 32% (65.76±2.47 in high-dose melatonin group vs 115.08±7.03 in low-dose melatonin group, P<0.01). It is interesting to note that no significant difference was found between sciatic nerve MDA levels of SNI plus high-dose melatonin and control rats (P=0.13).

In the controls, axons, myelinated and unmyelinated fibers, and Schwann cells all showed normal ultrastructural features (Fig. 2a). However, trauma caused axonal damage in most of the myelinated fibers. Axonal shrinkage and swollen axons were common ultrastructural features of the traumatized sciatic nerves. Vacuolization and lamellar separation of the myelin (myelin breakdown) were also the most striking ultrastructural alterations seen in the traumatized myelin (Fig. 2b). However, myelin breakdown was considerably attenuated with melatonin-treated animals. Vacuolization and lamellar separation of the axonal myelin was less obvious in animals given melatonin than in SNI or controls. Furthermore, improvement of these

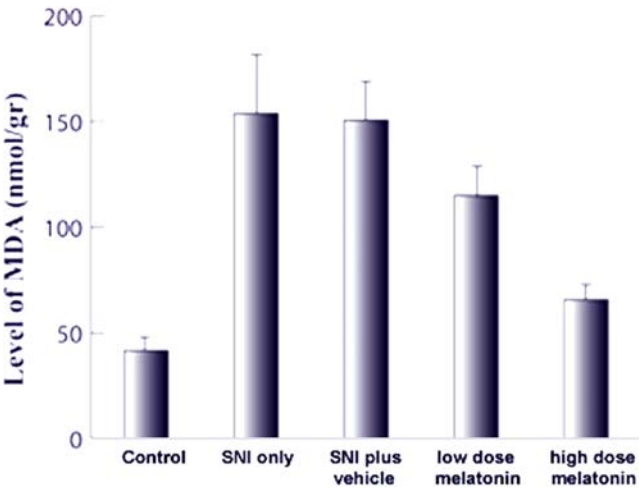

Fig. 1 Comparison of sciatic nerve MDA levels among animal groups

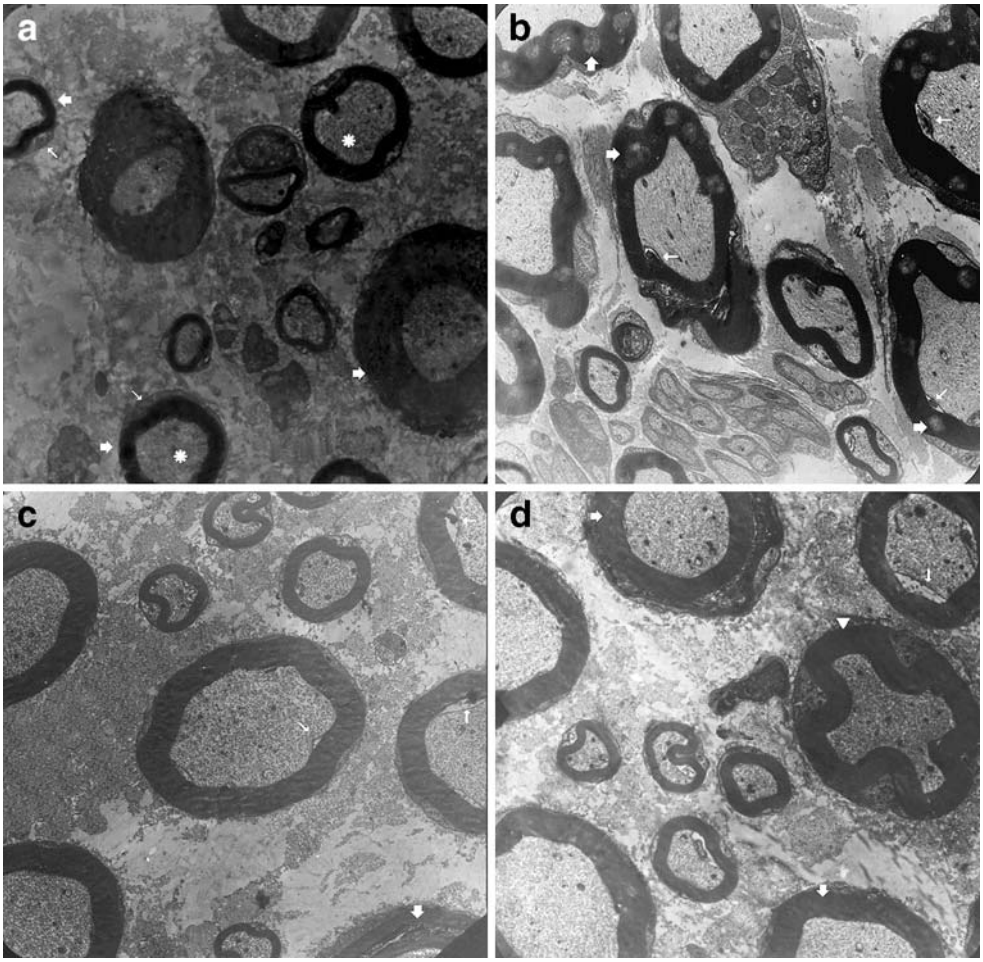

Fig. 2 Electron micrographs of animal groups (original magnification, 3,750). **a** Control group: myelinated fibers (*thick arrows*), Schwann cells, and axoplasm (*asterisk*) are seen. **b** SNI only rats: there are many vacuoles within the myelin sheath (*thick arrows*). Axonal shrinkage (*thin arrows*) is seen. **c** Low-dose melatonin group: there are limited vacuolizations of the myelin sheath (*thick arrows*) and minimal features of axonal shrinkage (*thin arrows*). **d** High-dose melatonin group: myelinated fibers show normal ultrastructure (*thick arrows*). Axonal shrinkage is rare (*thin arrows*). A cruciate myelinated fiber is seen (*arrowhead*). Myelin vacuolizations are not present

ultrastructural features was more prominent in the high-dose melatonin group than the low-dose melatonin group (Figs. 2c,d and Fig. 3).

Discussion

The results of this study demonstrate that high-dose melatonin (50 mg/kg) significantly reduces sciatic nerve lipid peroxidation, axonal injury, and myelin breakdown after a clamping injury; effects that were more pronounced than those afforded by low-dose melatonin (10 mg/kg). To our knowledge, this study is the first to compare the neuroprotective and antioxidant effects of high and low doses of melatonin in an experimental blunt peripheral nerve injury. Gul et al. [13] found no dose-dependent effects for melatonin in the reduction of early lipid peroxidation after spinal cord clamping in rats. Rogerio et al. [33] revealed that melatonin at doses of 1, 5, 10, and 50 mg/kg administered before and at 7 time intervals after sciatic nerve transection in neonatal rats significantly decreased motor neuron death. However, in their study, the neural survival rate was higher in those animals treated with lower doses of melatonin rather than higher doses. A possible explanation is that Rogerio et al. [33] had intoxicated their animals with high doses of melatonin (a total of 400 mg/kg). Likewise, their lower-dose groups received a total of 8, 40, and 80 mg/kg of melatonin that were rather comparable to the low-dose and high-dose melatonin (10 vs 50 mg/kg) groups of our study. It is interesting to note that animals treated with 8 mg/kg of melatonin had a relatively higher spinal cord neural survival ratio compared to those treated with 40 mg/kg. [33] Chang et al. [3] also showed that melatonin dose-dependently reduces neural nitric oxide synthase expression in the rat hypoglossal nucleus after hypoglossal nerve transection and thus, they suggested that dose-dependent neuroprotection of melatonin in their model was due to its antioxidant properties.

We found that blunt trauma increases sciatic nerve lipid peroxidation by 3.6-fold. These figures were reduced by 25% and 57.25% with low and high doses of melatonin, respectively. Oxygen free radical-induced lipid peroxidation has been suggested to be an important factor in posttraumatic neural tissue degeneration [14]. Oxygen free radicals

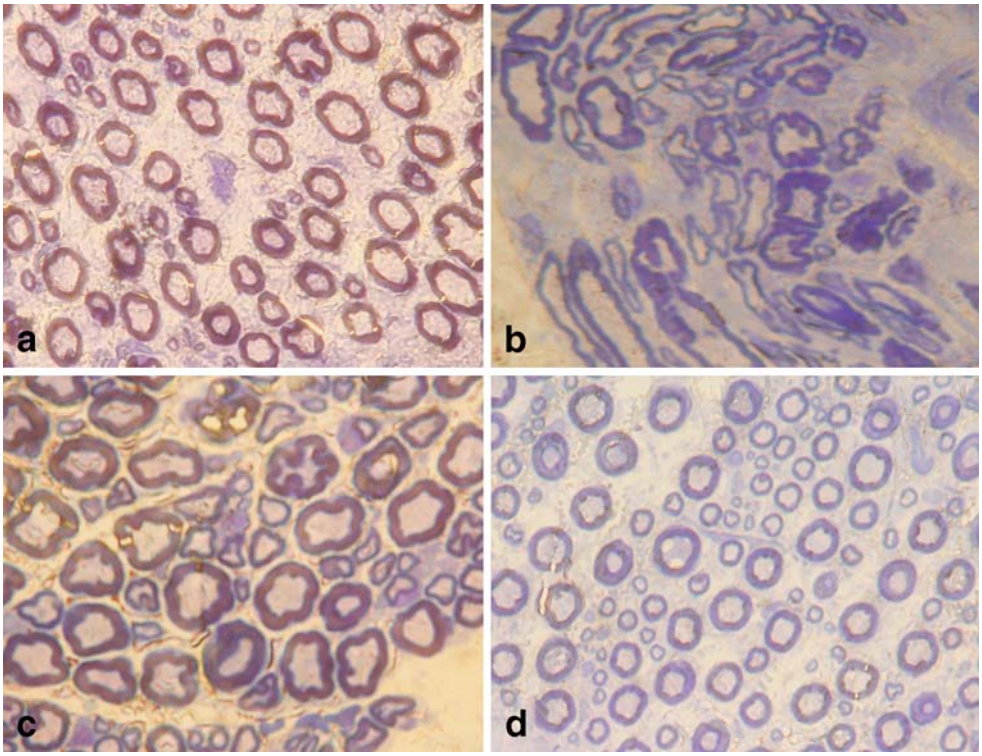

Fig. 3 Semithin sections stained with toluidine blue (original magnification, 200–400). **a** Control group: regular myelinated fibers of different sizes are seen. **b** SNI only rats: the thin myelin sheath, small and crenated axons, and frequent irregular and degenerating nerve fibers are found. **c** Low-dose melatonin group: thick myelinated fibers are interspersed among small and irregular figures characteristic of degenerating fibers. **d** Round and regular myelinated fibers are seen similar to that of the control group

not only damage phospholipids of the neural membranes but have also been proposed to make myelin proteins more susceptible to the attack of reactive oxygen species [20]. Vitamin E, as an antioxidant and potent neuroprotective agent, has also been investigated in peripheral nerve crush injuries [1, 6]. Vitamin C has also been found to attenuate neural injuries in a rat model of diabetic neuropathy and in cold injury of peripheral nerves [5, 18, 28, 36]. Sinha et al. [35] and Kondoh et al. [21] postulated that melatonin is a more potent antioxidant than vitamin E, vitamin C, mannitol, and glutathione with regard to its ability to scavenge hydroxyl radicals. Likewise, melatonin has been shown to enter the nucleus of the cell, thereby, providing protection to DNA [23, 24]. The extreme diffusibility of melatonin makes it a very available molecule for every subcellular component [4, 6, 23].

We found that the most prominent morphologic changes after sciatic nerve trauma occurred in myelinated fibers. Sayan et al. [34] reported a similar finding in a sciatic nerve ischemia–reperfusion injury and suggested that this might be due to the fact that myelin, a rich source of lipids, is the main target of free radical-mediated lipid peroxidation during trauma. They also demonstrated that ultrastructural alterations of the injured neural fibers significantly decreased with melatonin administration. Our observations, at the ultrastructural level, suggest that melatonin is effective in attenuating the trauma-induced sciatic nerve ultrastructural changes including myelin breakdown and axonal shrinkage or swelling. Gul et al. [13] reported a dose-independent neuroprotective effect of melatonin on white matter (axons and myelin sheaths) of traumatized rat spinal cord. We believe that our study is the first to indicate the neuroprotective effects of melatonin on peripheral nerve injury is dose-dependent, as according to our results, high-dose melatonin (50 mg/dl) significantly reduced trauma-induced ultrastructural changes in the sciatic nerve.

Mallo et al. [22] found that after melatonin infusion, plasma hormone levels reached a steady-state after 60 and 120 min. In another study, after a single subcutaneous injection of melatonin in hamsters, the maximal mean serum value of melatonin reached 20 min after injection (50 ng/ml), was more than 1,000 times the normal nocturnal melatonin concentration [37]. Moreover, with the administration of crystalline melatonin to 5 young male volunteers, peak serum melatonin levels were observed after 60–150 min and remained stable for approximately 1.5 h [38]. We injected melatonin preparations immediately after the induction of nerve trauma and obtained sciatic nerve samples 6 h thereafter. As the peak melatonin concentration was between 20 min and 1.5 h after its administration, we believe that the antioxidant effect of melatonin could be achieved with this timing.

Conclusion

We conclude that the early administration of high (50 mg/kg) and low (l0 mg/kg) doses of melatonin could attenuate lipid peroxidation, axonal injury, and myelin breakdown of traumatically injured sciatic nerve after an acute blunt injury. The neuroprotective effects of high-dose melatonin were more pronounced than that of the low-dose regimen. The high-dose regimen of melatonin more or less neutralized all trauma-induced ultrastructural changes of the sciatic nerve.

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