Original Article

Evaluation of Melatonin for Prevention of Radiation Myelopathy in Irradiated Cervical Spinal Cord

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Abstract

Objective: Radiation myelopathy (RM) is known as a serious complication of head and neck radiation therapy. Furthermore, the radioprotective roles of melatonin have been investigated on different tissues. The aim of this study was to assess the radio protective effects of melatonin on biochemical, histopathological and clinical manifestations of RM in the rat cervical spinal cord.

Materials and Methods: Four groups of rats were investigated as follows: The control group was treated with vehicle. The second group (melatonin only) was intraperitoneally injected with 100 mg/kg melatonin. The third group's (radiation) cervical spinal cord area was irradiated with 22 Gy cobalt-60 gamma-rays. The fourth group (melatonin plus irradiation) received 100 mg/kg melatonin intraperitoneally, and after 30 minutes their spinal cord area was irradiated with 22 Gy gamma radiation. Five animals from each group were randomly selected. 72 hours, 8 and 22 weeks after irradiation for analysis of malondialdehyde (MDA) and glutathione (GSH) levels, and underwent histopathological studies.

Results: The MDA levels in the irradiation group were significantly higher than in the control group (p<0.001). Furthermore, the GSH levels in this group were significantly lower than that of those in the control group (p<0.001). Administration of melatonin markedly reduced MDA (p<0.001) and increased GSH (p<0.05) levels in this group. Demyelination and clinical signs of myelopathy were decreased in the melatonin plus irradiation group in comparison to the irradiated group.

Conclusion: Our study confirms the radioprotective effects of melatonin at early stages of biochemical, as well as late histological and clinical changes in the spinal cord.

Keywords: Melatonin, Myelopathy, Radiation, Spinal Cord

Introduction

It has been accepted that radiation myelopathy is a serious complication following radiotherapy of extraneural neoplasms and the central nervous system (CNS) tumors. Relatively high radiation doses are required to yield long-term local control of tumors with moderate radiosensitivity, but radiation tolerance of the CNS is rather limited. Today, radiation oncologists, are particularly afraid of late CNS complications because they often greatly impair the quality of life in affected patients (1). The most threatening damage in the spinal cord region is radiation myelopathy that is commonly his-topathologically manifested in intramedullary vascular damage which progresses to hemorrhagic necrosis or infarction, as well as in demyelination progressing to white-matter necrosis and glial reactions (2-4). Free radicals generated as a consequence of irradiation may induce cell damage and lead to biochemical and pathological changes.

Development of strategies of response modifications has thus far remained unsatisfactory. Different chemical agents are routinely used in clinical settings to overcome radioresistivity of tumors and radiosensitivity of normal tissues (5). If new approaches could be devel-

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oped, many patients would be able to receive more effective treatment (6). Melatonin has been shown to play a role in many physiological systems (7). It was recently found that melatonin potentially can act as a direct free radical scavenger as well as an indirect antioxidant (8). The pineal gland releases melatonin into blood and cerebrospinal fluid circulations primarily during darkness. It is reported that the ability of melatonin to scavenge free radicals and reactants is the key mechanism of its neuroprotection (9).

Melatonin, in addition to its scavenging action, also stimulates the production of several antioxidative enzymes including superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione reductase (GSR); this action chemically reduces the oxidation of cells and inhibits the prooxidative enzyme nitric oxide synthase (NOS). The protective action of melatonin is typically associated with the preservation of cell viability (10). Although neuroprotective effects of melatonin have been demonstrated by some researchers (11, 12), there are no reports available suggesting any role played by melatonin against the late effects of localized high dose irradiation to the rat cervical spinal cord. Therefore in the present study, we examined the late effect of pre-treatment with melatonin on high-dose locally-irradiated rat cervical spinal cord.

Materials and Methods
Chemicals
Melatonin (N-acetyl-5-methyoxytryptamine) was obtained from Sigma-Aldrich. It was dissolved in a minimum volume of ethanol (8mg/ml) and diluted with saline. All other reagents were obtained from Sigma (St. Louis, MO) and Merck (Germany) pharmaceutical companies.

Animals
Adult male Wistar rats, each weighing 180-220 g, were selected and housed in conventional rodent facilities. They were fed with a standard rodent chow diet and water, and were kept at a constant temperature on 12 hour intervals of light and dark cycles. All procedures in this study are in accordance with the guidelines for care and use of laboratory animals as adopted by the Ethics Committee of the School of Medicine at Tehran University of Medical Sciences.

Experimental design
The rats were divided into four groups; the first group was treated with vehicle and considered as control (n=35); the second group was given an intraperitoneal (i.p.) injection of 100 mg/kg body weight melatonin and was considered to be the melatonin only group (n=15). In the third group, considered as radiation only group (n=35), rats were administered an i.p. injection of vehicle, and their cervical spinal cords were after thirty minute irradiated by 22 Gy of gamma rays. Rats in the fourth group, considered as radiation + melatonin group

(n=35), were administered melatonin in the same manner as the second group, and after thirty minutes their cervical spinal cords were exposed to 22 Gy of gamma radiation in the same manner as those in the third group. Melatonin dose was selected on the basis found in Gul and Serin studies (12, 13).

Irradiation
The animals were anesthetized with an i.p. injection of ketamin (60 mg/kg) and xylazin (20 mg/kg), then placed in prone position. The rats of Groups 3 and 4 were treated with Cobalt 60-gamma irradiation (Theratron 760-C) to the cervical segment of the spinal cord (C1-T2). A single dose of 22 Gy at dose rate of 1.8 Gy/min and source skin distance of 79.5 cm was delivered to the depth of 0.5 cm based on lateral simulation radiographs. This dose is proposed as the "Effective Dose" for white matter necrosis and limb paralysis (14). Sham irradiation was performed on groups 1 and 2; they were anesthetized, but not irradiated.

Samples preparation
For the purpose of obtaining post radiation-therapy biochemical and histopathological assays, at 72 hours, and at 8 and 22 weeks after exposure, five rats from each group were injected with ketamine and xylazine; sample tissues of the cervical spinal cord through posterior approach were then taken for chronological assessments of early, intermediate and late responses of spinal cord tissue.

Clinical responses survey
From each of the control, irradiated, and irradiated plus melatonin groups, twenty rats were kept for late evaluation of melatonin effect on survival and clinical symptoms of radiation myelopathy. The rats were observed and examined every other day for any development of clinical signs of spinal cord myelopathy including paralysis of hind and fore-limbs for a duration of 50 weeks (clinical endpoint).

Histopathologic study
The tissue samples for light microscopy were fixed in 10% neutral formalin. After routine processing, the spinal cord tissues were embedded in paraffin wax. Slices of 5 thickness were prepared and stained with hematoxylin and eosin (H&E), as well as Luxol fast blue, and counterstained with cresyl violet. With Luxol fast blue staining, myelin damage was defined as presence of areas with less Luxol fast blue color-intesity compared to the color intensity of the control group. White matter disarrangement, demyelination and cavitation due to histopathological changes were blindly evaluated in the samples. The semi-quantitative scoring of each variable was done by a histopathologist using the following scale: 0 for no change, 1 for mild, 2 for moderate, and 3 for severe injury.

Biochemical survey

Malondialdehyde (MDA) levels (in µg/mg of protein) were measured in each of the tissue samples (15); therefore, the samples were briefly homogenized in 1 ml of 0.9% cold saline,. After the addition of 200 µl TCA (25%) they were then centrifuged at 6000 rpm for 15 minutes and their absorbance of supernatant at 535 nm was measured. In order to determine GSH concentrations in the samples, an aliquot of deproteinized supernatant fraction was added to 2 ml of 0.3 M Na_2HPO_4 solution followed by the addition of 0.5 ml of 0.04% 5,5'-dithiobis (2-nitrobenzoic acid) dissolved in 10% sodium citrate. The absorbance of the mixture was measured at 412 nm. The GSH values were determined by extrapolations from a standard curve (nmol/mg protein). Total protein concentration was determined using the method developed by Bradford (16).

Biochemical measurements were carried out at room temperature using a spectrophotometer (Pharmacia Biotech, Cambridge, UK).

Statistical analysis

The biochemical data are presented as mean±SEM, and the difference between the groups was analyzed by using a two-way variance analysis (ANOVA) followed by Tukey's multiple comparison test. For nonparametric histopathological comparisons at each time point, the Kruskal-Wallis test was used. Survival data were analyzed in an actuarial fashion by means of Kaplan-Meier analysis and compared with the log-rank test. Significance was accepted at p<0.05.

## Results

Tissue MDA levels

72 hours post irradiation, tissue MDA levels in the spinal cord samples were found to be significantly higher in irradiation group than in the control group (p<0.001). Pretreatment with intraperitoneal melatonin (100 mg/kg) significantly reduced MDA levels in the spinal cords of rats subjected to irradiation. No significant differences between the levels of MDA in the spinal cord tissues of control and melatonin only groups were observed (Fig 1). However, there was no significant difference in spinal cord lipid peroxidation product between control and other groups at 8 and 22 weeks post irradiation.

GSH activity

As shown in Fig 2, GSH levels of the irradiated group were found to be significantly lower than that of the control, melatonin, and radiation plus melatonin groups at 72 hours after irradiation (p<0.001). There was no significant difference between control and melatonin only groups. There was no significant difference in the spinal cord glutathione content between the control and other groups at 8 and 22 weeks after irradiation.
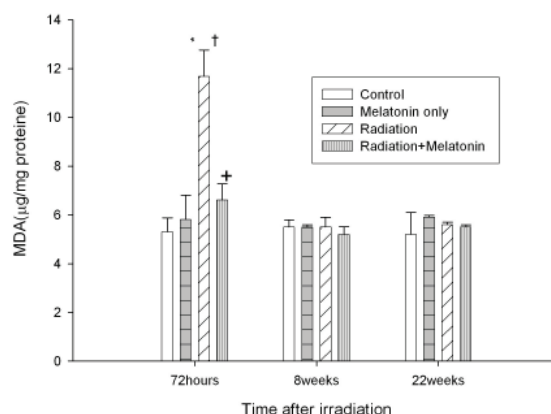
*Fig 1: Effect of pre-treatment with melatonin on MDA levels (microgram per milligram protein) after 72 hours, and 8 and 22 weeks. 72 hours after irradiation, MDA levels of the irradiated group are significantly higher than that at 8 and 22 weeks after irradiation. 72 hours after irradiation, Melatonin significantly reduced MDA levels in the spinal cords of rats subjected to irradiation. Data are given as Mean ± SEM of 5 rats. *p<0.001 compared to control at 72 hours after irradiation. + p<0.001 compared to radiation group at 72 hours after irradiation. † p<0.001 compared to irradiated group at 8 and 22 weeks after irradiation*
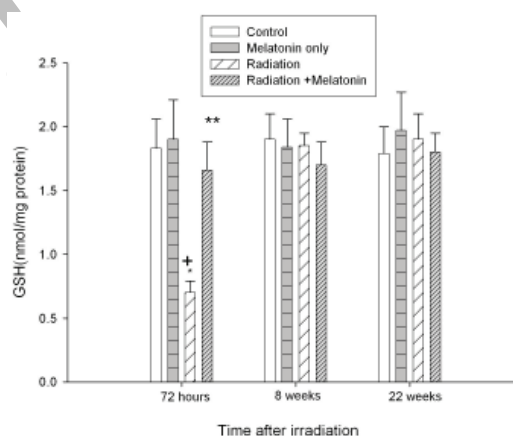

Fig 2: Effect of pre-treatment with melatonin on radiation-induced changes in glutathione (nanomol per milligram protein) after 72 hour, and 8 and 22 weeks.
*72 hours after irradiation, melatonin significantly compensate GSH levels in the spinal cord of rats subjected to irradiation (** p<0.001).*
Data are given as Mean ± SEM of 5 rats. p<0.001 compared to control at 72 hours after radiation. + p<0.001 compared to radiation groups at 8 and 22 weeks.*

Table 1: The histopathological evaluation of the spinal cord by groups 22 weeks after irradiation.

item scores	Control	Melatonin	Radiation	Radiation +Melatonin
Disarrengment	0	0	3*	1
Demyelination	0	0	3*	1
Cavitation	0	0	2*	0

*p<0.05 vs control group

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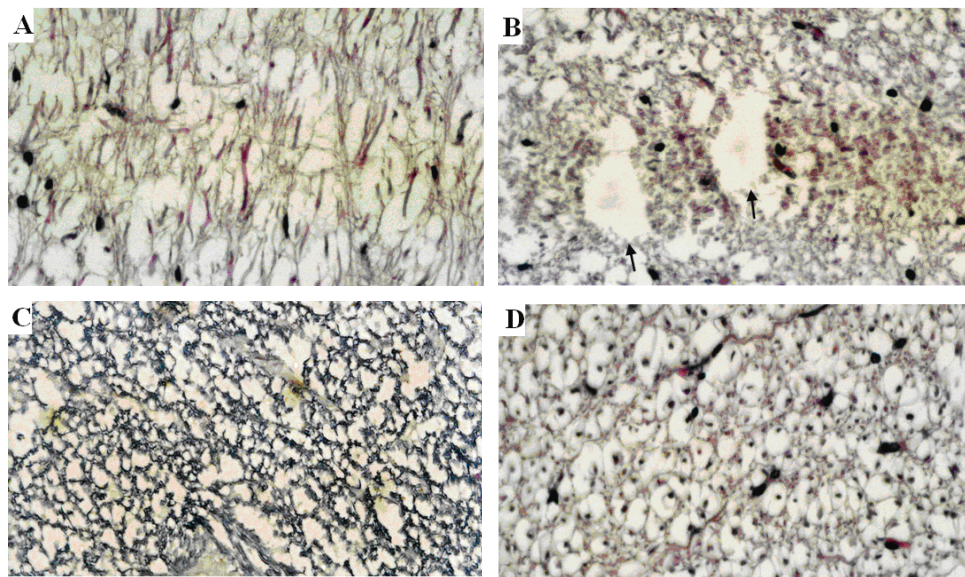
Fig 3: Histopathological effect of 22 Gy gamma radiation and protection by melatonin 22 weeks post irradiation: (A) Disarrangement of white matter is noticeable. (B) Arrows indicate patchy cavitations in the white matter. (40× magnification, H&E staining). (C) Patchy areas of loss of Luxol fast blue staining are seen in the white matter (20× magnification, Luxol fast blue staining). (D) Prominent reduction in disarrangement, demyelination, and cavitation in the 100 mg/kg intraperitoneally melatonin pre-treated group (×40 magnification, H&E staining).

Histopathological study

No histopathologic differences were observed in terms of disarrangement, demyelination and cavitation between the examined groups at 72 hours and eight weeks post irradiation (p>0.05). Histopathological examinations 22 weeks post irradiation is presented in table 1.

The spinal cord of rats in the control group and melatonin only group showed normal light-microscopic structures and no lesions were evident. There were statistically significant differences between the control and radiation groups in terms of disarrangement, demyelination, and cavitation (p<0.05). Differences between the control and radiation plus melatonin groups were insignificant for disarrangement, demyelination, and cavitation (p>0.05). Briefly, irradiation significantly caused disarrangement, cavitation (Fig 3A, B), and demyelination (Fig 3C). As shown in Fig 3D, melatonin was found to be efficacious in preventing all of these variables.

Frequency and onset of myelopathy

None of the irradiated animals developed typical manifestation of RM after short-term study, but clearly showed weight loss compared to the control, melatonin, and irradiated plus melatonin groups. Onset of paresis occurred 22 weeks after irradiation in the irradiated group. Sixteen of the twenty irradiated animals showed hind-limb and fore-limb paralysis and finally death, 28 to 50 weeks after irradiation. Incidence of myelopathy was calculated to be 80% in this group. Clinical signs of myelopathy began in the melatonin

pretreatment group at 28 weeks post irradiation. Also, paresis occurred 6 weeks later in the melatonin plus radiation group than in the irradiated group. The radiation myelopathy incidence in melatonin pretreatment group was calculated to be 50% at the 50th week. There were significant differences in the incidence of RM between irradiated and irradiated plus melatonin groups (p<0.05) (Fig 4).
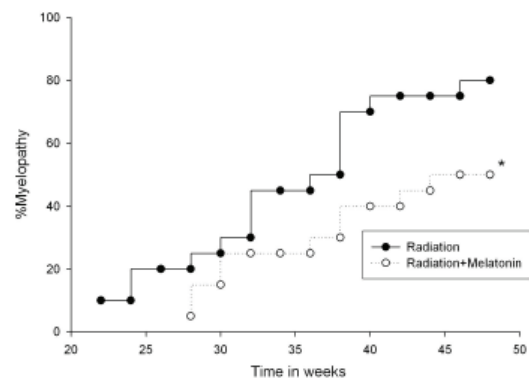
Fig 4: Changes in occurrence of paresis after exposure to a single dose of 22 Gy radiation (solid line), and pre-treatment with melatonin plus irradiation (dashed line) of spinal cord. A significant decrease in myelopathy rate was seen (* p<0.05 compared to radiation group)

Discussion

Despite recent advances in radiotherapy, the spinal cord still represents a major dose-limiting organ. RM is a serious, complex condition that usually develops in a time- and dose-dependent manner after the thresh-

old dose has been exceeded (14). Free radicals not only damage the cellular components of neural membranes, but also myelin lipoproteins of axonal structures (17). Thus, the antioxidant defence system needs to be activated for protection against the damaging effects of free radicals (18).

Some agents have been used for protection against the deleterious effects of ionizing radiation to the spinal cord. Nider and colleagues (2004, 2005) found a delay in the onset of paralysis after spinal cord irradiation associated with cisterna magna or subcutaneous injection of insulin-like growth factor-1 (IGF-1) and platelet-derived growth factor. These compounds have increased the tolerance of spinal cord (6). The combination of IGF-1 and amifostine has shown better efficiency. While IGF-1 may help tumor development via proliferation or angiogenesis (19), amifostine displays the adverse effects such as vomiting, hypotension, heat intolerance, somnolence, and hypocalcaemia. In addition, it can not cross the blood-brain barrier (20). The ideal radioprotector must be safe, easily administered, and rapidly effective.

Melatonin acts as a scavenger of free-radicals and may serve as a protectant against the damaging effects of radiation through activation of the antioxidant defence system and inhibition of pro-oxidative enzymes. Furthermore, melatonin crosses through all biological membranes, e.g the blood-brain barrier and does not require receptor interaction (21-25).

The *in vitro* and *in vivo* studies have revealed that melatonin decreases CNS injury due to hypoxia, ischemia, and trauma (12, 26, 27). It was recently shown that melatonin mitigates oxidative damage and has a protective role against high LET 56-Fe particle irradiation on the mouse cerebellum (28).

In this study, melatonin significantly prevented the increase of MDA as an index of lipid peroxidation at the early stages; in fact, melatonin reduced lipid peroxidation by about 45%. The ameliorating action of melatonin against radiation-induced lipid peroxidation might be due to its free-radical scavenging property. Sener et al reported that tissue levels of MDA were elevated at 12 and 72 hours after 8Gy proton irradiation of the ileum and colon in rats while GSH levels were reduced. They also demonstrated that melatonin decreased the oxidative organ damage induced by ionizing radiation (29).

GSH, with its sulfhydryl group, plays a role in the maintenance of the sulfhydryl group of other molecules. It also acts as a catalyst for disulfide exchange reactions and in the detoxification of foreign compounds like hydrogen peroxide and free radicals. Decreased brain GSH levels have been reported in neurodegenerative diseases such as Parkinson's and Alzheimer's diseases in which oxidative processes contribute to the pathology (30). In our experiment, early decrease of GSH concentration in the irradiated spinal cord can be due to interaction of this enzyme with free radicals induced by radiation. However, the melatonin pre-treated group showed significantly higher levels of GSH in comparison to the untreated irradiated group. This data demonstrates that treatment with melatonin, compensates for the decreased GSH due to irradiation. In the long run, it seems when free radicals are removed from cells, the GSH level will return to its normal state.

Histopathologically, we found that melatonin can modulate demyelination and cavitation 22 weeks post irradiation. Results indicate that melatonin has the ability to modulate the delayed effects of radiation on spinal cord. Our results also indicate that the accumulation of peroxidation by-products and the consumption of antioxidant agents might be transient early reactions and pre-histopathological events, but their effects in the form of pathological processes last several months after irradiation when biochemical changes return to their normal states.

Our study showed that pre-treatment melatonin significantly increased the latency period by delaying the onset of paralysis and reducing RM incidences in irradiated animals. This is in agreement with the Blickenstaff et al study which reported when pre-treated with melatonin, 43% of all irradiated mice survived at least 30 days after being exposed to a lethal dose of ionizing radiation (31). Also, Vijayalaxami et al observed that pre-treatment with melatonin at a dose of 125 mg/kg body weight increased survival to 60% (25).

The present data support the hypothesis that modulation of early radiation-induced spinal cord toxicity by melatonin administration is able to prevent long-stage radiation myelopathy.

However, a radiation dose of 22 Gy can be fatal for rats suffering from myelopathy symptoms, but it may not have direct clinical application, because the radiation dose necessary to induce RM in humans is different from animals, although the underlying mechanisms may be similar.

Conclusion

To our knowledge, this is the first *in vivo* study related to the effects of melatonin administration on delayed radiation injury in spinal cord. This study reveals that melatonin may have radioprotective effects on radiation–induced injury to the spinal cord due to its antioxidative and free radical scavenging properties. Melatonin has the potential to reduce MDA levels as well as increase GSH concentrations shortly after irradiation. It seems that these short-term antioxidative effects can lead to a long-term protective effects on radiation induced injuries to the spinal cord. We believe that melatonin supplementation may be of significant benefit for patients scheduled to receive radiotherapy for spinal cord lesions or targets close to the cord. The protection offered by melatonin might even allow dose limits to be increased in some cases, leading to more effective treatment of the lesions. However, radiobiologically, more experimental studies are needed to determine the selectivity of the effects of melatonin on malignant tissues.

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