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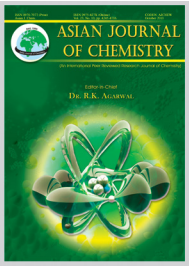

Radioprotective Effects of Melatonin on Bone Marrow Cells in Total Body Irradiated Mice

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This study was undertaken to evaluate the radioprotective effects of melatonin (*N*-acetyl-5-methyloxy tryptamine) in total body irradiated mice. Albino mice were intraperitoneally (i.p.) treated with 5 or 25 mg/kg melatonin, either 0.5 h before or 0.5 h after exposure to 200 cGy whole body irradiation using a ^{60}Co γ-irradiation machine. The animals were divided into six groups. Each group was subdivided equally, half processed for micronuclei examination and the other subgroup was processed for mitotic index assay. All mice were sacrificed 24 h following treatment. The incidence of micronuclei formation and mitotic index in these bone marrow cells (BMCs) were determined for all groups. The results showed that melatonin caused a significant reduction in micronuclei formation and mitotic index of bone marrow cells depending of the melatonin dose and administration time. Administration of one single 25 mg/kg melatonin dose given before irradiation, reduced bone marrow cell mitotic index and micronuclei formation significantly ($p < 0.05$). However, no significant effect was observed when melatonin was given after irradiation. The data obtained from this study indicated that melatonin administration may have an active role in protection against damage inflicted by radiation when given prior to exposure to irradiation and not after, depending on it's dosage.

Key Words: Melatonin, Mitotic index, Micronuclei, Radioprotection, γ-Irradiation.

INTRODUCTION

Radiotherapy is one of the most effective treatments for cancer. Seventy percent of cancer patients may require radiation treatment at some time or other, either for curative or palliative purpose of their disease. Bone marrow is well known to be susceptible to ionizing radiation and during the radiotherapy course if covered substantial amounts of the bone marrow (*e.g.* mantle or inverted Y for Hodgkin's disease), irradiation may lead to bone marrow failure[1].

Melatonin, *N*-acetyl-5-methyloxy tryptamine is the principal secretory product of the pineal gland. The molecule has wide-ranging functions in the body; it not only has endocrine actions on other glands, but also has been shown to have an efficient free radical scavenger, antioxidant and immunomodulator function[2-5]. Melatonin is highly lipid soluble and enters cells and subcellular compartments easily[6]. Melatonin has not been shown to be toxic[7].

Although compelling evidence suggest that melatonin has significant radioprotector effect, the optimum dose and administration time of melatonin for the most efficient radioprotection is yet to be determined. In this study, we aimed to investigate possible radioprotective effect of two different dose of melatonin *in vivo* given before and after irradiation of albino mice. The frequency of micronuclei formation (MN) and mitotic index (MI) in bone marrow cells (BMCs) was used to asses the effect of melatonin.

EXPERIMENTAL

This study was performed with the review approval of The Animal Experimental Ethics Committee of Erciyes University; Kayseri, Turkey. Sixty adult Albino Swiss male mice with body weight (BW) ranging from 20 to 35 g, were obtained from Erciyes University Hakan Çetinsaya Research Center, Erciyes University; Kayseri, Turkey and used in this study. The animals were housed in plastic cages (five per cage) in a 23 ± 2 ºC-controlled animal care room with a light/dark cycle of 14/10 h and had free access to tap water and commercial chow (Aytekinler Yem Sanayi, Konya, Turkey) during the experiment. A pure liquid of melatonin [Sigma, St. Louis, MO] was purchased from Farma Pharmaceticals, Ankara, Turkey. Melatonin solution (freshly prepared melatonin in 100 µL of 5 % ethanol in phosphate buffered saline or the same volume of solvent alone) was given an intraperitoneal (i.p.) injection. All procedures were performed at the Experimental Animals Breeding and Research Center Medical Faculty, Erciyes University.

TABLE-1
RESULTS OF MICRONUCLEUS EXAMINATION FOR EACH TREATMENT GROUPS

Groups	I (n=10)	II (n=10)	III (n=10)	IV (n=10)	V (n=10)	VI (n=10)		
Micronucleus frequency	2	2	2	3	4	0		
	2	2	3	1	0	4		
	2	3	2	2	3	2		
	1	4	1	1	2	3		
	1	2	2	3	3	3		
	1	3	2	1	3	0		
	2	2	1	3	3	2		
	2	2	3	2	2	4		
	2	4	2	2	1	3		
	1	2	3	1	4	3	F	*P*
	1.6±1.0	2.6±1.3	2.1±0.7	1.9±0.9	2.5±1.5	2.4±0.8	1.1	*>0.05*

Group I: No melatonin or irradiation, Group II: Irradiation only, Group III: 5 mg melatonin / kgBW 30 min before irradiation, Group IV: 25 mg melatonin / kg BW 30 min before irradiation, Group V: 5 mg melatonin / kg BW 30 min after irradiation, Group VI: 25 mg melatonin / kg BW 30 min after irradiation

Administration of melatonin and γ-irradiation: After a 14-day acclimatization period, a randomized block design based on animal body mass was used to divide mice into 6 different groups each of 10 mice. Each group was subdivided equally, half processed for micronuclei examination and the other subgroup was processed for mitotic index assay.

Group I, control group (C): Animals received no melatonin or irradiation (sham control). Group II, radiation group (R): animals were exposed to 200 cGy total body irradiation only delivered from a ^{60}Co γ-source [Theratronics-780C, Canada], at a dose rate of 0.52 Gy/min, administered at 1.5 cm depth below the skin, the source-skin distance being 80 cm and sacrificed 24 h later. Group III, 5 mg/kg melatonin before radiation group (MBR-5): animals received a single i.p. injection of 5 mg/kg body weight melatonin, 0.5 h before to their exposure to 200 cGy total body γ-irradiation, then sacrificed 24 h after irradiation. Group IV, 25 mg/kg melatonin before radiation group (MBR-25): Similar to group III except that melatonin was administered at a dose rate of 25 mg/kg body weight. Group V, 5 mg/kg melatonin after radiation group (MAR-5): Similar to group III except that melatonin was administered 0.5 h after radiation. Group VI, 25 mg/kg melatonin after radiation group (MAR-25): Similar to group V except that melatonin was administered at a dose rate of 25 mg/kg body weight.

To obtain bone marrow, all irradiated animals were decapitated at 24 h after irradiation. One femur was removed from each animal. After removal of the epiphyses, the core of the long bone was flushed with cool fetal bovine serum to obtain a bone marrow sample. Femoral marrow cell suspension were smeared onto two clean glass slides and fixed with methanol for micronuclei and mitotic index determination.

Stain method of micronuclei: Slides were immersed in 5 N HCl for 20 min at room temperature. After washing with tap water slides were left in Schift solution 250 mg basic fuxin dissolved in 50 mL boiling of water and cooled to 50 ºC. After adding 250 mg sodium bisulphate, the colour changed to a red wine colour and the slides were left for 1.5 h in the dark. The slides were passed through sodium bisulphite for 2 min three times and then allowed to cook in tap water 3 times, 30 min each time, stained for 40 min in 5 % fast green solution; 250 mg fast green dissolved in 50 mL 95 % ethanol and rinsed in tap water to remove excess stain.

Micronucleus examination: Slides were randomized and scored by a single observer. From each slide, approximately 1500 red blood cells were examined under 600 magnification and when cells with micronuclei were located, they were examined under 1000 magnification. The criteria suggested by Sarto *et al.*[8] for recognizing micronuclei were followed. Dead or degenerating cells were excluded from evaluation. Nuclear blebbings micronuclei-like structures connected to the nucleus via a bridge, were also excluded. Only micronuclei equal to or smaller than one-fifth of the nucleuses were assumed to have resulted from chromosome breakage and were considered. Multi micronucleated cells were also scored but not included in the evaluation of micronuclei frequency[9].

Mitotic index examination: The femur was dislocated from each mouse and bone marrow preparations were performed by the method as described by Schmid[9], with some modifications. Bone morrow cells were incubated in 0.07 M KCl for 20 min at 37 ºC and centrifuged at 200 g for 5 min. Then the pellets were resuspended in 6 mL of 3:1 methanol: acetic acid fixative 3 times. They were prepared and stained with 5 % Giemsa in Gurr's buffer for 6 min at room temperature. One thousand and five hundred cells were counted for each mouse to determine the mitotic index and metaphase cells were included only.

Statistical evaluation: One way Anova variance analyses were used to test for statistical significance of *p*-values obtained for each treatment group compared with the control group. *p*-value < 0.05 was considered significant.

RESULTS AND DISCUSSION

In this study, the effect of melatonin as a radioprotector agent was assessed in vivo using two different test systems: micronuclei formation (MN) and the mitotic index frequency (MI) of bone morrow cells (BMCs). Five or 25 mg/ kg body weight melatonin were used as the radioprotector agent.

Micronuclei assay: The frequency of micronuclei was determined in bone morrow cells in each of the different groups (Table-1). The results showed remarkable decrease in the frequency of micronuclei in bone morrow cells in the melatonin groups (Group III and IV) as compared to the control group (Group I). Also the protective effect of melatonin against radiation when given prior to irradiation was statistically

TABLE-2
RESULTS OF MITOTIC INDEX EXAMINATION FOR EACH TREATMENT GROUPS

Groups	I (n=10)	II (n=10)	III (n=10)	IV (n=10)	V (n=10)	VI (n=10)		
Mitotic index frequency	10	9	9	5	9	6		
	11	8	8	6	8	7		
	10	7	5	4	6	6		
	12	10	7	6	6	9		
	10	9	9	5	6	7		
	11	8	8	8	7	8		
	10	7	5	4	8	9		
	12	10	7	6	6	7		
	10	8	6	8	6	9		
	11	10	7	6	9	7	F	P
	10.7±0.8	8.6±1.8	7.3±1.0	5.6±1.0	7.1±1.3	7.5±1.1	18.4	<0.001

Group I: No melatonin or irradiation, Group II: Irradiation only, Group III: 5 mg melatonin / kgBW 30 min before irradiation, Group IV: 25 mg melatonin / kg BW 30 min before irradiation, Group V: 5 mg melatonin / kg BW 30 min after irradiation, Group VI: 25 mg melatonin / kg BW 30 min after irradiation

significant in Group IV, MBR-25. And also, there is a several-fold increase in the frequency of micronuclei in radiation alone group (Group II). Five mg/kg body weight melatonin before irradiation showed statistically unsignificant decrease, whereas 25 mg/kg melatonin body weight before irradiation resulted in maximum and also statistically significant decrease of the micronuclei formation in bone morrow cells ($p < 0.05$). This result suggest that melatonin dosage and administration time are important factors.

On the other hand, post-treatment with melatonin after irradiation had no effect on micronuclei formation. Significance of differences in the frequency of micronuclei was tested by One way Anova test and the results are given in Table-1.

Mitotic index assay: Different levels of mitotic index were observed on bone morrow cells (Table-2). There is marked increase in the total amount of mitotic index in the radiation alone group compared to the control and melatonin groups. In the melatonin before radiation group-25 (Group IV) the frequency of total mitotic index decreased significantly ($p < 0.001$) whereas it remained nearly unaffected when melatonin was given after irradiation. Radiation has caused an overall increase in the frequency of mitotic index (Table-2). Treatment with melatonin prior to irradiation has resulted in a marked decrease in all types of the observed structural parts of the bone morrow cells. However, administration of melatonin after irradiation caused either no or insignificant change in the frequency of mitotic index. Table-2 also shows that the statistical comparison of different groups regarding the frequency of mitotic index in bone morrow cells. The elevation in the frequency of different types of mitotic index in the bone morrow cells observed in radiation group were statistically significant as revealed by One way Anova test (Table-2).

It is well estabilished that ionizing radiation causes its harmful effect through generation of free radicals[10], the melatonin principle mechanism of action for radioprotection, therefore, could be through its ability as a scavenger for free radicals[11]. Due to its small size and high lipophilicity, melatonin crosses biological membranes easily and reaches all compartments of the cell[6,12].

The radioprotective effect of melatonin given before irradiation observed *in vitro* was reported by Vijayalaxmi *et al*.[13] and was suggested to occur through free radical scavenger activities. Melatonin may have an active role in protection against genetic damage due to endogenously produced free radicals and it may be of use in reducing damage from physical and chemical mutagens and carcinogens that generate free radicals[14], Vijayalaxmi and Meltz[15] also reported that whole-body irradiated mice pretreated with melatonin exhibited a significant and dose-dependent reduction in the incidence of micronuclei. They suggest that their data indicated that melatonin has the ability to protect cells of mice from the damaging effects of acute whole-body irradiation[13,15].

In this study, we tested the radioprotective effect of two different doses (5 or 25 mg/kg body weight) of melatonin, given before or after radiation, on bone morrow cells of total body irradiated albino mice. And we used two different test systems: micronuclei assessment and mitotic index (MI) count. A dosage of 5 mg/kg melatonin body weight given before irradiation reduced micronuclei formation numerically but not statistically significant (Group III). However, 25 mg/kg body weight melatonin given before irradiation (Group IV) showed statistically significant low micronuclei formation compared to all groups ($P < 0.05$). This result suggests that melatonin dosage and administration time was important. These results are resembling the findings of Vijayalaxmi *et al*.[13] and Badr *et al.*[16].

Similar results have been reported by Sewerynek *et al*.[17]. Here, it has been shown also that administration of melatonin after exposure of animals to radiation had no significant effect on mitotic index and micronuclei. However, the effect of melatonin *per se* doubled the micronuclei frequency in bone morrow cells as compared to control. This suggest a cytotoxic effect of melatonin at a relatively high dose (25 mg/kg body weight). The increase in melatonin from exogenous sources could disturb the delicate balance of mechanisms regulated by endogenous doses of melatonin and might explained the noted enhanced incidence of micronuclei and suggests a more direct action on DNA. The radiation-induced increase in the frequency of micronuclei and mitotic index in bone morrow cells declined significantly when melatonin was administered 1 h before irradiation. This finding suggests a radioprotective role for melatonin and that it plays a vital role in reducing the risk of micronuclei and/or mitotic index frequency in bone morrow cells. On the other hand, administration of melatonin

after irradiation had little or no effect on micronuclei and mitotic index frequency in bone morrow cells as a result of fixation of radiation induced damage. These results strongly suggest that the presence of melatonin during radiation exposure is essential to its radioprotective effect. The finding that melatonin after irradiation provides no radioprotective effect indicates that melatonin should be inside the cell at the time of exposure to radiation in order to confer protection.

Conclusion

In this study we tested two different dose of melatonin, before or after exposure to irradiation. Whether its radioprotective effect is dose-dependent needs further investigations. However, melatonin was reported to diminish radiation-induced DNA damage in a dose-dependent manner[15] and if it has been given at an appropriate dose and at least 1 h before irradiation, probably, it could show the maximal radioprotective effect. But the optimal dose and schedule of melatonin administration for the maximal radioprotection needs further investigations.

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