

The potential ameliorative effects of melatonin against cyclophosphamide-induced DNA damage in murine bone marrow cells

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Abstract. – OBJECTIVES: The protection afforded by melatonin, a pineal secretory product, against cyclophosphamide (CP)-induced genotoxicity in murine bone marrow cells was tested using micronuclei as an index of induced chromosomal damage.

MATERIALS AND METHODS: Mice were pre-treated with four different doses of melatonin (2.5, 5, 10 and 20 mg/kg by weight, b.w.) via intraperitoneal injection for five consecutive days followed by injection with CP (60 mg/kg b.w.) 1 hr after the last injection of melatonin on the fifth day. After 24 hr, mice were euthanized by cervical dislocation to evaluate micronucleated polychromatic erythrocytes (MnPCEs) and the ratio of polychromatic erythrocyte/polychromatic erythrocyte+normochromatic erythrocyte [PCE/(PCE+NCE)]. Histological examination of the bone marrow was also performed.

RESULTS: Treatment with melatonin significantly reduced the number of MnPCEs induced by CP at all doses ($p < 0.0001$). At 20 mg/kg, melatonin had a maximum chemoprotective effect and reduced the number of MnPCEs by 6.93 fold and completely normalized the PCE/(PCE+NCE) ratio. Administration of 20 mg/kg of melatonin led to marked proliferation and hypercellularity of immature myeloid elements after mice were treated with CP, as well as mitigated bone marrow suppression induced by CP.

CONCLUSIONS: Our study revealed that melatonin has a potent antigenotoxic effect against CP-induced toxicity in mice, which may be due to the scavenging of free radicals and increased antioxidant status. Because melatonin is a safe, natural compound, it could be used concomitantly as a supplement to protect people undergoing chemotherapy.

Key Words:

Chemoprotective, Melatonin, Genotoxicity, Cyclophosphamide, Bone marrow

Introduction

Hazardous environmental chemical agents can produce toxic substances such as free radicals and reactive oxygen species (ROS) in tissues. Oxidative stress potentially attacks critical macromolecules such as DNA, RNA, lipids and proteins. DNA damage is involved in enhancing malignant proliferation of normal cells toward cancer¹. The consumption of vegetables and fruits has been an effective strategy in reducing the genotoxic and carcinogenic effects induced by hazardous chemicals^{2,3}. The incidence of cancer is significantly reduced by the consumption of natural products^{4,5}. The preventive effects of natural products are the result of their antioxidant and free radical scavenging activities, which can affect cellular signaling pathways^{1,6}.

Melatonin (N-acetyl-5-methoxytryptamine), a pineal secretory product, influences circadian rhythmicity by acting on the suprachiasmatic nucleus⁷⁻⁹. Melatonin also influences various physiological activities such as neuroendocrine function⁷, regulation of seasonal reproduction¹⁰, sexual maturation¹¹, immunoregulation¹² and thermoregulation¹³. Melatonin may also have an effect on some aspects of aging¹⁴.

Apart from the above functions, melatonin possesses strong antioxidant activity by which it protects cells, tissues and organs from the oxidative damage caused by ROS, especially the hydroxyl radical ($\bullet\text{OH}$), which attacks DNA, proteins and lipids and causes pathogenesis¹⁵. Melatonin can scavenge the $\bullet\text{OH}$ generated *in vitro* by ultraviolet (254 nm) irradiation of H_2O_2 ¹⁶ as well as quench the peroxy radical, hypochlorous acid and singlet oxygen, all of which cause cell dam-

age¹⁷⁻¹⁹. Melatonin protects against age-related oxidative damage in the central nervous system^{15,20}, oxidative damage of neuroblastoma cells by amyloid b protein, which is characteristic of Alzheimer's disease²¹, 1-methyl-4-phenyl-1,2,4,6-tetrahydropyridine (MPTP)-induced Parkinson-like neurodegenerative changes²² and free radical damage to the outer hair cells in the organ of Corti²³. Melatonin also prevents oxidative damage of the liver induced by chemia-reperfusion²⁴ as well as lung and brain damage induced by hyperbaric oxygen²⁵. Recently, melatonin was shown to reduce cardiac arrhythmias; this action was attributed, at least in part, to its radical scavenging activity²⁶. The ability of melatonin to inhibit lipid peroxidation induced by d-aminolevulinic acid (which occurs in experimental porphyria²⁷) as well as cataractogenesis induced by buthionine sulfoximine (a glutathione synthesis inhibitor) in newborn rats²⁸ has also been documented.

Cyclophosphamide (CP) is a commonly used chemotherapeutic drug and well-known mutagen and clastogen²⁹. It is an alkylating agent that produces the highly active carbonium ion, which reacts with the electron-rich area of nucleic acids and proteins³⁰. CP is widely used as a genotoxic agent because it and its metabolites can bind DNA, causing damage that may result in chromosome breaks, micronucleus formation and cell death^{31,32}. Natural products exerted protective effects against genotoxicity induced by CP in bone marrow cells of mice when these compounds were administered prior to CP treatment. Antioxidant activity is the proposed mechanism for the chemoprotective effects of these natural products^{33,34}.

Because melatonin has excellent antioxidative properties, there is a likely possibility that melatonin would protect against the toxicity of cyclophosphamide, i.e., an elevated level of melatonin in body may act as a prophylactic against damage. Therefore, this study was undertaken to assess the effects of melatonin against the genotoxicity induced by CP in murine bone marrow cells using the micronucleus test.

Materials and Methods

Animals

The protocol for the study was approved by the Research Committee of Mazandaran University of Medical Sciences, Sari, Iran. Male Naval Medical Research Institute (NMRI) mice weigh-

ing 25 ± 3 g were purchased from the Pasteur Institute of Iran (Amol). The mice were kept in good condition at the university animal facility and were given standard food pellets and water *ad libitum*. All of the animals were maintained under a controlled 12 hour light/dark cycle and temperature ($23 \pm 1^\circ\text{C}$). The "Care and Use of Laboratory Animals" was prepared by Mazandaran University of Medical Sciences.

Chemicals

CP (Endoxan[®]) was obtained from Baxter Oncology (Westfalen, Germany), and the melatonin was from Sigma Aldrich Co (St Louis, MO, USA). 1,1-diphenyl-2-picryl hydrazyl radical (DPPH) was purchased from Sigma Chemicals Co. (St. Louis, MO, USA). Butylated hydroxytoluene (BHT) was purchased from Merck (Darmstadt, Germany). All other chemicals were either at or purer than analytical grade.

Measurement of Free Radical-Scavenging Activity of Melatonin

The free radical-scavenging capacity of melatonin were determined by bleaching of the stable 1,1-diphenyl-2-picryl hydrazyl radical (DPPH)³⁵. Approximately 1 mL of the various concentrations of melatonin (0.0125-0.4 mg/mL) were added to 3 mL of a methanol solution of DPPH (10 mg/250 mL). After a 15 minute incubation in a dark room at ambient temperature, the absorbance was recorded at 517 nm. The experiment was performed in triplicate; butylated hydroxytoluene (BHT) was used as a potent standard antioxidant agent. The percentage of scavenging was calculated using the following formula: $([\text{Control_Test}]/\text{Control}) \times 100$.

Experimental Treatment

For the micronucleus assay, animals were divided into six groups (groups 1-6, n=5 for each group), which were comprised of the following: group 1 (negative control), mice that received distilled water (10 ml/kg b.w.) via intraperitoneal (i.p.) injection for 5 days; group 2 (positive control), mice that received a single genotoxic dose of CP (60 mg/kg b.w, i.p.)^{33,36} in distilled water (10 ml/kg b.w); group 3, mice that were treated with melatonin (2.5 mg/kg b.w. by i.p. injection) in distilled water (10 ml/kg b.w) per day for 5 days followed by a single i.p. dose of CP 1 h after the last dose of melatonin; group 4, mice that were treated with melatonin (5 mg/kg b.w. by i.p. injection) in distilled water (10 ml/kg b.w) per

day for 5 days followed by a single i.p. dose of CP 1 h after the last dose of melatonin; group 5, mice that were treated with melatonin (10 mg/kg b.w. by i.p. injection) in distilled water (10 ml/kg b.w.) per day for 5 days followed by a single i.p. dose of CP 1 h after the last dose of melatonin; and group 6, mice that were treated with melatonin (20 mg/kg b.w. by i.p. injection) in distilled water (10 ml/kg b.w.) per day for 5 days followed by a single i.p. dose of CP 1 h after the last dose of melatonin.

Micronucleus (Mn) Assay

The Mn test was performed as previously described^{33,36}. The bone marrow Mn test is a well-known *in vivo* assay for the assessment of genotoxicity and DNA damage in animals such as mice and rats. The number of MnPCEs (micronucleated polychromatic erythrocytes) is increased in rodent bone marrow cells exposed to chemical hazards and chromosome-breaking agents. A micronucleus is round with a diameter of approximately 1/20th to 1/5th of an erythrocyte. The ratio of polychromatic erythrocytes (PCE) to normachromatic erythrocytes (NCE) in bone marrow preparations is useful in estimating any perturbations in hematopoiesis as a result of treatment in exposed animals^{37,38}.

Mice were pretreated with solutions of melatonin at four different doses (2.5, 5, 10 and 20 mg/kg b.w.) via i.p injection for five consecutive days followed by injection of CP (60 mg/kg b.w.) on the fifth day 1 hr after the last injection of melatonin. Mice were then sacrificed by cervical dislocation 24 h after CP injection. The bone marrow of both femurs was removed in the form of a fine suspension into a centrifuge tube with fetal calf serum (FCS). The cells were dispersed by gentle pipetting and collected by centrifuge at 1500 rpm for 10 min. The cell pellet was resuspended in a drop of FCS, and smears were prepared. The slides were coded to avoid any observed bias. After 48 h of air drying, smears were stained with May-Grunwald/Giemsa. For each experimental point, five mice were used, and 5000 PCEs were scored per each experimental point to determine the percentage of micronuclei in the polychromatic erythrocyte and the PCE/(PCE+NCE) ratio.

Histology of Bone Marrow

For histological examination of myeloid hyperplasia in bone marrow, mice were administered 20 mg/kg of melatonin for 5 days and then

sacrificed 24 h after CP injection. Femurs were immersed in 10% formalin; bones were decalcified and processed with a microtome at micron slides. Routine Hematoxylin and Eosin-staining was performed on 6- μ m paraffin sections, and the slides were evaluated under light microscope.

Statistical Analysis

Data are presented as the mean \pm SD. One-way analysis of variance and Tukey's HSD (Honestly Significant Difference) test were used for multiple comparisons of data. A *p* value < 0.05 was considered to be significant.

Results

After treatment with melatonin, a significant scavenging effect was observed. The scavenging effects of melatonin on DPPH radicals increased in a dose-dependent manner, with 98% inhibition at a dose of 0.4 mg/mL (Figure 1).

The effect of various doses of melatonin on the frequency of MnPCEs in bone marrow cells at 24 h after CP treatment or with no treatment is shown in Table I. The mice treated with melatonin were given doses of 2.5, 5, 10, or 20 mg/kg for 5 days prior to CP treatment. The frequency of micronuclei was increased in the mice treated with CP compared with the control group. In mice treated with the melatonin and CP, the number of MnPCEs was decreased compared with those treated with only CP. All doses of melatonin significantly reduced the frequency of MnPCEs induced by CP treatment (*p* < 0.0001). The frequency of MnPCEs was lower in the melatonin

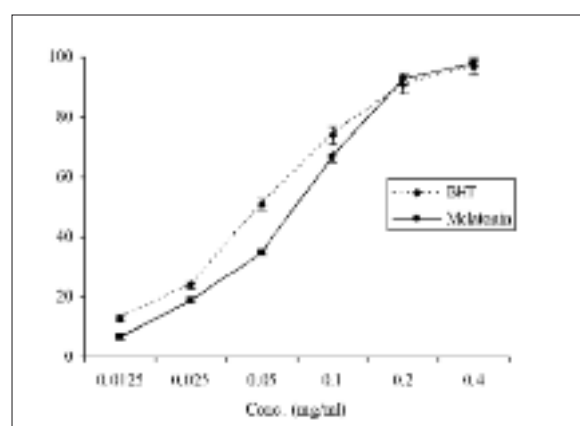


Figure 1. Scavenging effect of melatonin and BHT on the DPPH free radical at 517 nm. Abbreviations: BHT, butylated hydroxytoluene; DPPH, 1,1-diphenyl-2-picrylhydrazyl.

Table I. Effects of melatonin on the formation of CP-induced MnPCEs and the PCE/(PCE+NCE) ratio in murine bone marrow cells.

Group	Treatment	MnPCE/PCE (%) ^a	PCE/(PCE+NCE) (%) ^a
1	Control	0.81 ± 0.03	64.76 ± 5.01
2	CP	6.45 ± 0.49 ^b	37.49 ± 2.37 ^b
3	Melatonin 2.5 mg/kg+CP	3.14 ± 0.67 ^c	48.73 ± 3.78 ^c
4	Melatonin 5 mg/kg+CP	1.78 ± 0.26 ^c	57.58 ± 1.52 ^c
5	Melatonin 10 mg/kg+CP	1.01 ± 0.35 ^c	61.08 ± 4.27 ^c
6	Melatonin 20 mg/kg+CP	0.93 ± 0.17 ^{c,d}	65.52 ± 2.91 ^{c,d}

Notes: CP, cyclophosphamide; MnPC, micronucleated polychromatic erythrocyte; NCE, normochromatic erythrocyte. ^aValues are the mean ± standard deviation for each group of 5 mice. ^b $p < 0.0001$ compared to the control; ^c $p < 0.0001$ compared with the CP treated group; ^dNo significant difference compared to the control. The data were analyzed with one-way ANOVA and the Tukey's HSD test.

tonin+CP groups by factors of 2.05, 3.62, 6.38, and 6.93 for the doses of 2.5, 5, 10, and 20 mg/kg, respectively, compared with the CP treated group (Table I). The data showed that melatonin suppresses the action of cyclophosphamide on clastogenic effects.

Calculation of the PCE/(PCE+NCE) ratio in CP treated mice showed a pronounced cytotoxic effect of CP on bone marrow proliferation, and this ratio was significantly reduced in murine bone marrow after CP treatment. Treatment of mice with melatonin arrested the CP-induced decline in the PCE/(PCE+NCE) ratio (Table I). The increase in the PCE/(PCE+NCE) ratio in the melatonin+CP groups was higher than that of CP alone group ($p < 0.0001$). There was a dose-dependent effect of melatonin on the PCE/(PCE+NCE) ratio. Melatonin treatment completely prevented the cytotoxicity induced by CP at the dose of 20 mg/kg in the murine bone marrow and increased ratio of PCE/(PCE+NCE).

Histological examination of the bone marrow showed that administration of CP induced

myelosuppressive effects and hypocellularity of immature myeloid elements (Figure 2). Administration of 20 mg/kg of melatonin led to marked proliferation and hypercellularity of immature myeloid elements after mice were treated with CP, as well as mitigated bone marrow suppression (Figure 3).

Discussion

The results from our study show the ability of cyclophosphamide to induce the formation of micronuclei in PCEs in the bone marrow of mice. The induction of micronuclei is commonly used to assess chromosomal damage³⁹⁻⁴¹.

This study demonstrated that melatonin had potentially chemoprotective effects against the genotoxicity induced by CP in murine bone marrow cells. Administration of melatonin for 5 consecutive days resulted in the inhibition of micronuclei caused by CP, as well as showed protective and anticlastogenic effects. DNA

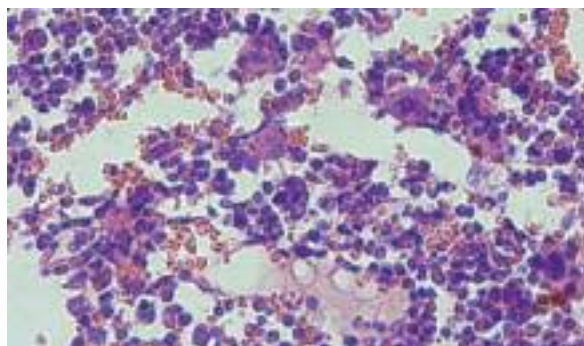


Figure 2. Histological examination (×400 magnification) of the bone marrow showed that administration of CP induced myelosuppressive effects and hypocellularity of immature myeloid elements.

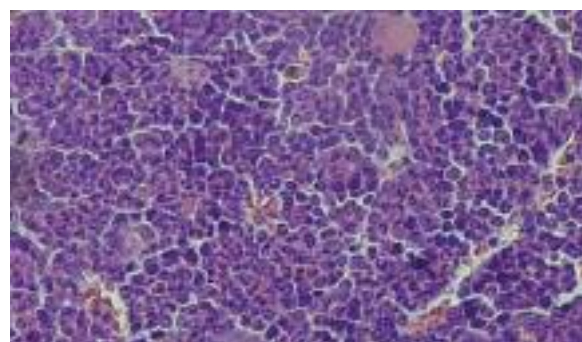


Figure 3. Administration of melatonin (20 mg/kg) led to marked proliferation and hypercellularity of immature myeloid elements after mice were treated with CP and mitigated the bone marrow suppression (×400 magnification).

damage occurs due to the increasing intracellular levels of ROS, which are toxic at high levels and can interact with macromolecules. The free radicals produced from ROS can oxidize nucleic acids, proteins, lipids and DNA as well as initiate degenerative diseases¹. The main characteristic of antioxidants is the ability to trap free radicals. It appears that the primary defense mechanisms of chemoprotective agents is to either directly or indirectly interact with these ROS. In our study, melatonin had dose-dependent protective effects in reducing MnPCEs induced by CP. Administration of 20 mg/kg of melatonin to mice for 5 days prior to injection of CP reduced the frequency of MnPCE approximately 6.93 fold. Melatonin treatment also increased the PCE/PCE+NCE ratio, which declined in mice treated with CP. Because this ratio gives a direct index of cell division, the current study is in agreement with previous studies.

The protective effect of the pineal secretory product melatonin against oxidative damage has been examined in both *in vivo*^{24,42,43} and *in vitro* studies⁴⁴⁻⁴⁷. Melatonin scavenges not only the hydroxyl radical^{16,48} but also the peroxy radical^{17,49} and possibly singlet oxygen¹⁸. Melatonin also stimulates the endogenous antioxidant enzyme glutathione peroxidase^{50,51}, thereby contributing to enhanced cellular defenses against oxidative stress. Moreover, melatonin has been shown to protect DNA from physical agents, ionizing radiation^{41,52}, and lipopolysaccharide (a chemical toxin)¹⁵, the latter two of which are believed to damage the genetic material by free radical mechanisms. In a previous report, melatonin was shown to protect the lung and liver of paraquat-treated rats from oxidative stress and reduce mortality following treatment with herbicide⁴³. In another experiment, co-administration of melatonin⁵⁶ resulted in significant protection against paraquat-induced alterations in lipid peroxidation levels and maintenance of glutathione levels of the tissues. They concluded that the protective effect of melatonin was likely due to the antioxidative properties of the indolamine.

Due to the high lipophilicity of melatonin and its low molecular weight⁵³, it easily passes through cell membranes and provides on-site protection against locally generated free radicals directly at DNA sites. There is evidence suggesting the preferential compartmentalization of melatonin in the nucleus relative to levels in the cytosol and cell membranes^{54,55}. The present data confirm melatonin's protection against CP-in-

duced damage and extend the sphere of melatonin's action from the protection of membrane phospholipids against peroxidative damage^{43,56} to the maintenance of DNA integrity.

Conclusions

Melatonin was shown to reduce the genotoxicity induced by CP in bone marrow cells of mice through antioxidant activity and free radical scavenging properties. However, the exact molecular mechanism of chemoprotective effect of melatonin is not completely clear and further molecular studies are needed for elucidation. With regard to the low toxicity related to its consumption and its provision of on-site protection against locally generated free radicals directly at DNA sites, melatonin is a good candidate to help defend the body against side effects, particularly DNA damage, induced by hazardous chemical agents.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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