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The effects of melatonin on the frequency of micronuclei induced by ionizing radiation in cancerous and normal cell lines

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► ***Original article***

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ABSTRACT

Background*:** Melatonin is a natural antioxidant that is produced by the pineal gland. In this study was evaluated antioxidant and possible protective effects of melatonin on frequency of micronucleus (MN) formation in human cell lines exposed to γ-radiation. ***Materials and Methods*:** To achieve the best concentration for antioxidant activity of melatonin DPPH (1,1-diphenyl-2-picrylhydrazyl) assay was used. Hela and MRC5 cells were cultured and treated with optimum concentration of melatonin (1200µg/ml). After 2h, cells were exposed to 2Gy-gamma ray. For each cell group, one flask was considered as control. Immediately after irradiation, cells were exposed to cytochalasin B to arrest cells at cytokinesis. Then the frequency of MN induced by radiation alone or in the presence of melatonin was evaluated. ***Results: By DPPH assay, the optimum concentration of melatonin for its antioxidant activity was determined 1200µg/ml. Our results showed that the frequency of micronuclei increased in irradiated cells compared to the control groups ($p<0.05$). Conversely, pre-treatment of cells with melatonin significantly reduced the number of MN produced both in MRC5 and Hela cells ($p<0.05$). ***Conclusion:*** Results indicated that γ-radiation induced MN in the cells. The protective effect was achieved when melatonin was present in the cellular environment pre-irradiation. Indeed, melatonin with scavenging and antioxidant ability neutralizes toxic reactants and stimulates DNA repair pathways. Moreover, the results indicate that protective effect of melatonin is higher in MRC5 cells than in Hela cells. Therefore, it can be concluded that other mechanisms such as induction of cell cycle arrest by melatonin might be exist only in MRC5 cells. However, the radio-protective mechanism of melatonin is not clearly known.

Keywords: *Radioprotection, melatonin, CBMN, γ- radiation, micronucleus, DPPH assay.*

INTRODUCTION

There are various treatment options for cancer. Chemotherapy and radiotherapy are two common treatments for cancer which increases the chance of treatment. But these methods are not completely successful, and can induce *de novo* genetic damages or severe side effects that may lead to new cancers [1, 2].

Approximately 50% of all cancer patients receive radiotherapy during the course of treatment [3, 4]. The goal of radiation therapy is to create damage to the genetic material and blocking the ability to divide cancer cells [5].

Radiotherapy uses ionizing radiation for cancer treatment. Ionizing radiation damage DNA directly or indirectly via free radical formation. This effect is not limited to cancer cells, but it can effect on normal cells. The degree of damage is dependent on the volume and type

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of tissue irradiated and radiation dose [6, 7].

Recently, to increase the efficiency of radiotherapy and reduce its side effects on normal cells use of radioprotective compounds is suggested [8].

Since ionizing radiations led to the production of free radicals in the cell and these radicals mediate DNA damages to the cells, molecules with radical scavenging properties are considered as radioprotectors [9-11].

Melatonin (N-acetyl-5-methoxytryptamine) is a potentially radioprotector. It is a natural antioxidant that mainly produced by the pineal gland [12-14]. This hormone discovered by Lerner in 1958 [15]. Except the pineal gland, melatonin is synthesized in several organs, like the retina, gastrointestinal tract, skin, bone marrow, and lymphocytes [16]. Melatonin is a darkness hormone and light reduces melatonin levels in the body [17].

Chemically, melatonin with a formula of C13H16N2O2, is highly lipophilic and somewhat soluble in water [18]. By this way, it can be well dispersed in the cells and with its antioxidant properties reduce the amount of damages of free radicals [19]. The ability of melatonin to free radical scavenging is directly mediated through electron donation [20-22]. This hormone acts as an antioxidant indirectly by stimulating antioxidant enzyme activity and inhibiting the activity of pro -oxidative enzymes [23, 24].

A study in 2018 demonstrated melatonin can inhibit the cell growth in colorectal cancer, which are resistant to 5-FU [25]. In other studies on melatonin was revealed the use of melatonin alone or in combination with other therapies can increase apoptosis, mitophagy, inhibit cell migration and cell invasion [26, 27].

With respect to previous studies on melatonin and its roles as cancer inhibitor, but few studies have been done on the effects of combination of melatonin and γ- radiation on the DNA of the cells. So in the present study, we used melatonin as an antioxidant compound and assessed gamma radiation genotoxic effects alone and combined with melatonin in MRC5 and Hela cells by using the cytokinesis- block micronucleus (CBMN) assay.

Indeed, CBMN assay is a cytogenetic assay to evaluate the amount of DNA damages. In this assay, cells are treated with cytochalasin-B (Cyt-B) and thereby prevented cytokinesis. As a final result of this effect, after one nuclear division in each cell appears a binucleated form. The appearance of micronuclei (MN), the small nucleus, in binucleated cells is related to chromosome breakage or whole chromosome loss. Thus, frequency of micronuclei (MN) can be used as an index of genotoxic effects [28-30].

MATERIALS AND METHODS

DPPH assay

Before anything, to get the best concentration for antioxidant activity of melatonin we used DPPH (1, 1- diphenyl-2-picrylhydrazyl) assay. DPPH assay was conducted based on Brand-Williams methods [31]. Different concentration of melatonin (200, 800, and 1200µg/ml) reacted with a stable DPPH radical solution. Each reaction included; 0.5 ml of melatonin solution, 3 ml of absolute ethanol and 0.3 ml of DPPH radical solution (0.5 mM in ethanol). When DPPH reacts with an antioxidant compound, it decreases. This reaction leads to a change in color (from deep violet to light yellow) that was read at 520 nm by UV–Vis spectrophotometer (ELX 800) after 60 min. The control solution was prepared by adding 3.5 ml of ethanol and 0.3 ml of DPPH radical solution. For each concentration of melatonin, DPPH assay was done in triplicate.

The inhibition percentage was determined according to the equation 1 [32]:

% Inhibition = (Control OD – Sample OD) / Control OD×100 (1)

Cell lines and cell culture

Hela cell (as a cancer cell line) and MRC5 (as a normal cell line) were obtained from the Cell Bank of Pasteur Institute of Iran (Tehran, Iran). Cells were cultured with RPMI-1640 medium (Bio idea), 10% fetal bovine serum (Bio idea) and antibiotics (100µg /ml penicillin and 100µg /ml streptomycin, Sigma). Cells were placed at 37°C and 5% CO2 and were passaged

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every 3-4 days after trypsinization with trypsin/ EDTA. For each cell group, three cellular flasks were prepared. We used from these cells for CBMN assay, When 80% of flasks were covered by cells.

RPMI-1640 medium stored at 4°C, FBS and antibiotic solution stored at -20°C before use.

Cell radiation

After cell culture and insure about cell adhesion, one cellular flask in each group was treated with the best concentration of melatonin (1200µg/ml) and incubated for 2h at 37°C. after spending this time, two cellular flasks in each group (melatonin treatment, Non- melatonin treatment) were irradiated with dose of 2Gy of γ - radiation with a cobalt source (^{60}Co therapeutic machine; Theratone 780 ACEL, Canada) at a dose rate of 0.85cGy/min at room temperature and a source to sample distance (SSD) of 80 cm. For each cell group, one flask was considered as a control (No- radiation, No- melatonin treatment) and kept in the same condition.

CBMN assay

In order to study the antioxidant effects of melatonin on DNA damages induced by γ- radiation cytokinesis block micronucleus assay (CBMN) was used. This assay was performed according to standard protocol that is explained by Fenech and Moley [33]. Immediately after radiation, the cells were blocked in cytokinesis by adding 100µl of cytochalasin B to each cellular flask (Sigma, St. Louis, MO; final concentration 4 µg/mL) and were incubated for 48 h at 37°C. After the end of the incubation period, the cells were treated with a chilled hypotonic solution of potassium chloride (0.4%). Then instantly cells were centrifuged and fixed in 3:1 methanol/acetic acid. Fixation step was repeated twice. After that, cell samples were dropped onto clean microscope slides. Slides were dried at room temperature and stained with Giemsa solution (10%) for 10 min. then the slides were washed with distilled water, dried and analyzed with the microscopic.

The frequency of MN in each sample was evaluated by counting at least 1000 binucleated cells using a light microscope at ×400 magnification. The scoring criterion was performed based on Fenech *et al.* outline [34]. To calculate nuclear division index (NDI) for each sample, the frequencies of mono-bi-tri and tetra nucleate cells was evaluated for each cell group. A typical photomicrograph of binuclei with and without micronuclei is shown in figure 1.

Nuclear division index (NDI) was calculated by using equation 2 (Eastmond and Tucker's proposed formula) [35]:

$$NDI=[(1\times M1)+(2\times M2)+(3\times M3)+(4\times M4)]/N \quad (2)$$

In equation 2, M1 to M4 represent the number of cells with one, two, three and four nuclei, respectively, and N is the total number of viable cells.

Statistical analysis

The results were expressed as mean and standard deviation (SD).The data were analyzed using SPSS software, version 16. Statistical analysis was performed by *t-test*. The significant statistical level was expressed at $p<0.05$.

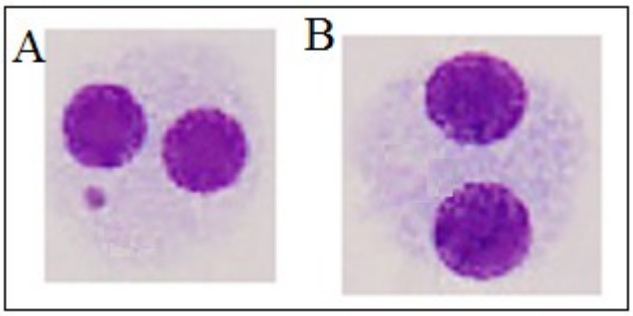


Figure 1. A) a binucleated cell with one micronuclei. **B)** a binucleated cell without micronuclei. stained in Giemsa.

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RESULTS

DPPH assay

At the first step, according to the previous studies we selected different concentrations of melatonin (200, 800, and 1200µg/ml). And using DPPH assay was found the best concentration for the antioxidant activity of melatonin. Finally, among various concentrations of melatonin, the concentration of 1200µg/ml was determined as the best of concentration for antioxidant activity of melatonin [figure 2]. So, the other experiments were conducted using this concentration of melatonin.

Evaluation of effects of γ- radiation alone and in combination with melatonin on Hela and MRC5 cells by the CBMN assay

The results of the CBMN assay are summarized in table 1 and shown in figure3. As shown in figure 3 and table 1, there is a significant increase in the number of micronuclei in irradiated cells compared to the control group (without irradiation) ($p<0.05$; t-test). As shown in table 1, for control groups, the mean of MN per 1000 binnucleated cells were 34.5 in MRC5 cells and 25.5 in Hela cells. When these cells were exposed to γ- radiation, the mean of MN increased significantly (117.5 for MRC5 cells and 211 for Hela cells).In contrast, cell treatment with melatonin before irradiation decreases the amount of radiation-induced MN in both cell groups (MRC5 and Hela cells) ($p<0.05$; *t-test*).

The difference between total numbers of MN in different conditions is recorded in table 1. As seen, treatment of cells before radiation with melatonin decreases the number of MN produced 47% in MRC5 cells and 17 % in Hela cells compared to only radiation. According to our studies, it was found that melatonin has protective effects against gamma radiation for Hela and MRC5 cells.

To evaluate cell proliferation capacity under different conditions (presence and absence; melatonin and γ-radiation) Nuclear Division Index (NDI) was calculated. Table 1 shows, the values of NDI in MRC5 and Hela cells at different conditions.

No significant difference in the NDI was observed between cells treated with γ-radiation alone compared to cells treated with radiation and melatonin in both cell groups (Hela and MRC5) ($p>0.05$; *t-test*). This means that cell treatment with melatonin before radiation couldn't induce nuclear cell arrest and also couldn't reduce cell division compared to radiation alone.

Among different cellular conditions, only Nuclear Division Index of MRC5 cells treated with radiation and melatonin was statically lower than MRC5 cells without any treatment ($p<0.05$; *t-test*).

According this experiment was found a significant reduction in the number of radiation-induced MN in the presence of melatonin compared to radiation alone ($P<0.05$; *t-test*). Therefore, it can be seen the protective effect of melatonin against γ-radiation in melatonin-treated cells.

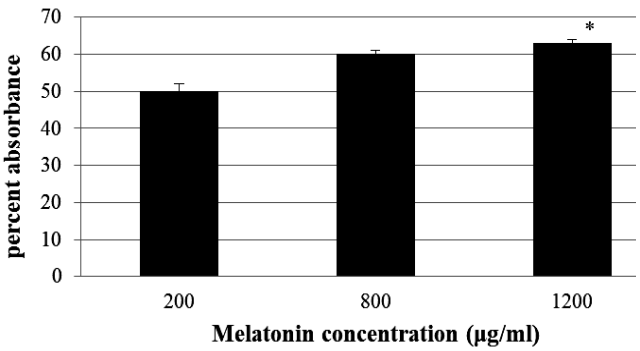


Figure 2. Free radical scavenging activity of melatonin in different concentrations. This activity was measured using scavenging of DPPH radicals. Results are expressed as the mean ± SD.

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Table 1. Results of CBMN assay of MRC5 and Hela cells at different conditions.

		Mean of Binuclear cells		Mean of MN per 1000 Bi cells	Mean of cells with MN				Mean of NDI
		Without MN	with MN		M1	M2	M3	M4	
MRC5	Control	459.5 ± 4.5	31± 1	34.5± 0.5	483±1	490.5±3.5	25±2	0	1.541± 0.01
	2Gy	409±1	91.5± 1.5	117.5± 5.5	480.5± 4.5	500.5± 0.5	17	0	1.535± 0.01
	2Gy+M	386.5± 6.5	51 ± 4	62 ± 1*	555.5± 19.5	437.5± 10.5	6±3	0	1.451±0.01
Hela	Control	588 ± 2	20.5± 3.5	25.5± 5.5	337± 4	608.5± 5.5	25±2	3±1	1.720±0.01
	2Gy	539± 1	160± 2	211±4	295	699±1	5± 1	1±1	1.717 ±0.01
	Control	569± 2	130±4	175± 32*	291.5± 8.5	699± 2	9± 2	0	1.711±0.01

Bi cells (binucleated cells); MN (micronucleus); control (without radiation and melatonin treatment). M1 to M4 represent number of cells with one to four nuclei.*significant when compared with γ- radiation alone ($p<0.05$; t- test).

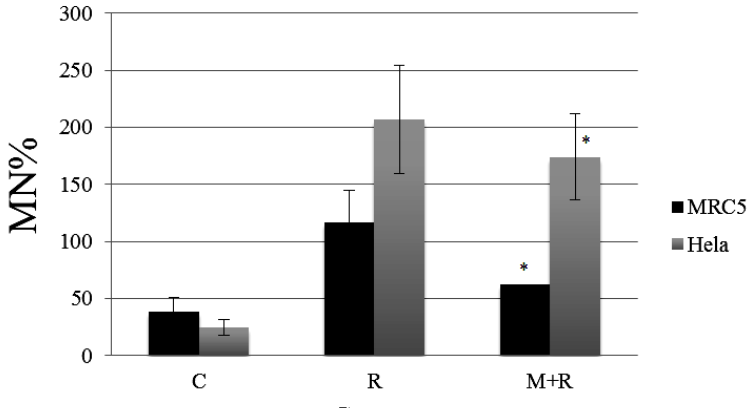


Figure 3. The average precentages ±SD of micronuclei of MRC5 and Hela cells at different conditions. C (control); without radiation and melatonin treatment, R; treated only with 2Gy of γ- radiation, M+R; treated with melatonin and 2Gy of γ radiation. *significant when compared with γ- radiation alone.

DISCUSSION

Melatonin as a hormone produced in the central nervous system has many various functions. Its functional range including from the regulation of the circadian rhythm [36] to act as an antioxidant [37-39]. The antioxidant activity of melatonin is higher than many other antioxidants like vitamin E [40, 41]. Also because, melatonin is a natural compound in the body, so use it in usual does has no significant side effects. This is a melatonin advantage compared to other radio-protective. Several studies have demonstrated the antioxidant property and free radical scavenger of melatonin [42, 43]. However, further *in vitro* and *in vivo* experiments are needed to clarify its mechanisms of action.

In this study, we focused on the antioxidant properties of melatonin and its ability to control the total number of MN produced by γ- radiation on cells. Because 2Gy of γ- radiation is part of radiation therapy for cancer patients, we examined its effects on the production of MN in different conditions (γ- radiation alone, γ- radiation in combination with melatonin), using CBMN assay.

At the first step, using the DPPH assay, different concentrations of melatonin (200, 800, and 1200μg/ml) were examined and finally, the best concentration for the antioxidant activity of melatonin was determined 1200μg/ml. DPPH (1,1-diphenyl-2-picryl-hydrazyl) is a free stable radical at room temperature and is decreased in the presence of an antioxidant compound [44]. DPPH assay is an easy, rapid and sensitive way to evaluate the antioxidant activity [45, 46].

Using CBMN assay was evaluated genetic instability in different conditions (γ- radiation alone and in combination with melatonin). CBMN assay is a preferred method for detecting chromosome damages [47]. In this test, the number of micronuclei in binucleated cells was

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counted. Change in the frequency of micronuclei can be related to changes in the frequency of chromosomal damage.

As seen in table 1 and figure 3, gamma radiation alone increases the production of the MN in the cells. As demonstrated by the results of this study, the number of MN produced in Hela cells (as cancer cells) after irradiation alone is higher than MRC5 cells (as normal cells), which is related to the greater sensitivity of the cancer cells to radiation. These results are similar to the previous studies [48, 49]. Reported previously in other *in vitro* and *in vivo* studies showed that, the cancer cells have various types of mutations and aberrant expression in genes involved in DNA repair pathways. Inability to DNA repair increases radiosensitivity in these cells [50].

In contrast, our data (table 1 and figure 3) suggest that cell treatment with melatonin before irradiation led to a considerable reduction in the frequency of MN produced in the cells compared to the radiation alone, but this reduction is slightly higher in MRC5 cells, due to the efficient induction of DNA repair pathways.

According this study and previous studies, it was found that gamma radiation alone increases the production of the MN in cells and the use of antioxidant agents before irradiation could reduce DNA damages via their radical scavenging property [51-53].

Here, we showed that melatonin, as an antioxidant and free radical scavenger could reduce the frequency of radiation induced MN in MRC5 and Hela cells. These results are consistent with previous studies about antioxidant and radio-protective effects of melatonin [54, 55].

Vijayalaxmi and colleagues for the first time was confirmed the radio-protective effect of melatonin in 1995[56].Subsequently, several studies demonstrated scavenging ability of melatonin. It could scavenges various spices of reactive nitrogen and oxygen [57]. Monobe and colleagues in 2005, reported giving melatonin to the mice before irradiation have protective effects against intestinal damage [58]. Other studies demonstrated that melatonin with amphipathic property can enter the cells and exert its antitumor effects[59].Cascade reactions created by the second and third metabolites of melatonin increases the efficacy of melatonin at free radical scavenger and reducing oxidative stress [35,36]. Moreover, it was found melatonin through directly and indirectly manner can neutralize a number of toxic reactions and induce DNA repair system [50]. However, all of its radioprotective mechanisms have not been fully elucidated.

On the other hand, in the present study, we evaluated the Nuclear Division Index (NDI) to evaluate the effect of different treatments on cell proliferation. As shown in table 1, no significant difference was found in NDI between cell treated with radiation only and cell treated with radiation and melatonin. Only NDI of MRC5 cells treated with radiation and melatonin was statically lower than MRC5 cells without any treatment ($p<0.05$). This means that melatonin with in selective manner, only could induce nuclear division arrest in MRC5 cells. Such delays could increase the time of DNA repair and prevent them from converted to irreversible mutations [60].

In conclusion, our study showed that cell treatment with the optimum concentration of melatonin (1200µg/ml) significantly reduces the frequency of MN. According to the results, by using of CBMN assay, it can be confirmed the radioprotective effects of melatonin on γ- irradiated MRC5 and Hela cells. In the other words; melatonin protects DNA against ionizing radiation with its radioprotective and antioxidant effects without affecting the cell division rate (compared to radiation alone).

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Conflicts of interest*: *Declared none.

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