

Cell cycle dependent modulating effects of melatonin on radiation induced cytogenetic damages in lymphocytes of normal individuals and breast cancer patients

H. Mozdarani^{1*}, S. Mozdarani², E. Samei³, S. Mozdarani², F. Pakniat⁴, H. Nosrati⁵

¹Department of Medical Genetics, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran

²Department of Cyto genetics, Cytogenome Medical Genetics laboratory, Ale-Ahmad High way, Chamran Medical Building, Tehran, Iran

³Department of Biology, Science and Research Branch, Islamic Azad University, Tehran, Iran

⁴Department of Medical Physics, Shahid Beheshti University of Medical Sciences, Tehran, Iran

⁵Cancer Institute, Department of Radiotherapy, Tehran, Iran

ABSTRACT

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***Corresponding author:**

Hossein Mozdarani, Ph.D.,

E-mail:

mozdarani@modares.ac.ir

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Background: The use of radioprotectors to ameliorate the deleterious effects of radiation could be an option to reduce normal tissue radiotoxicity. In this study, melatonin as an efficient naturally occurring antioxidant was used to explore its modulating effects on radiation induced cytogenetic damages at G0 and G2-lymphocytes of control and breast cancer (BC) patients. **Materials and Methods:** Whole blood samples were collected from 20 BC patients, and also 10 healthy individuals. Lymphocyte culture was initiated, irradiation was applied in G0-lymphocytes 1 hour before culture initiation using a 6 MV linear accelerator at dose of 3Gy and 4 hours before harvesting for G2-lymphocytes at a dose of 1Gy in the presence or absence of melatonin (800 µg/ml). Cytogenetic damages were assessed using cytochalasin-B blocked micronucleus (CBMN) assay and chromosomal aberrations were analyzed using G2-assay according to standard methods. Data were statistically analyzed and P-value <0.05 was considered as significant difference. **Results:** Results obtained for G0-irradiated lymphocytes in the presence of melatonin, showed a profound reduction in the frequency of CBMN in both lymphocytes of normal control and BC patients compared to radiation alone (p<0.05). Conversely, a significant increase in the frequency of radiation induced chromosomal aberrations was observed in both groups (p<0.05). **Conclusion:** Apparently melatonin exerts its modifying effect on radiation induced cytogenetic damages in a cell-cycle dependent manner. It acts as a radioprotector when lymphocytes have been irradiated in G0-phase of the cell cycle. Conversely it acts as radiosensitizer when used for lymphocytes exposed at G2-phase of the cell cycle.

INTRODUCTION

Breast cancer (BC) is one of the most common malignancy occurring in women that ranks as the fifth cause of death from all cancers. The prevalence of BC in Iran is increasing and affected people are relatively younger compared to other countries (1, 2). About 15% of BC is familial and the rest (85%) is sporadic which express as different subtypes with a vast range of risk factors including, genetic, hormone therapy, physical and chemical environmental agents, life style as well as circadian disruption. Genomic instability has also been described for various hereditary cancers including BC (3, 4). About 50% of patients with malignant breast tumors receive radiation therapy (RT) as an efficient treatment modality. Most patients seem tolerate RT, but some suffer severe adverse effects such as fibrosis or secondary cancers in the radiotherapy area (4, 5).

Therefore, prevention and prediction of adverse complications associated with radiotherapy in BC patients has been the subject of several studies (3).

Recent studies have highlighted the role of oxidative stress in mediating IR-induced damage. IR generates reactive oxygen species (ROS), which cause DNA strand breaks, leading to genomic instability and cell death (3, 6). The creation of ROS also might act as a signaling event leading to the release of cytokines or epigenetic changes or trigger DNA repair machinery (7). Ionizing radiation can cause cell cycle arrest which allows time for DNA repair and prevents the progression of damaged cells from the G2 phase into mitosis (8). Radiosensitivity has been extensively studied in BC patients (7, 9-14). It has been shown that about 45% of an unselected group of BC cases were found to be radiosensitive (10, 13). Some researchers found a high sensitivity and a reduced repair capacity in peripheral blood cells from breast cancer patients

when exposed to X-rays, gamma (7,15,16) by means of the comet assay. Therefore, normal tissue radiosensitivity of breast cancer patients has been shown using various cellular and cytogenetic end points. Accordingly, implementing an appropriate approach to mitigate the radiotherapy side effects in BC patients due to inherent sensitivity of their cells or tissues to ionizing radiation is a priority (17).

Antioxidants are compounds, which combat the free radicals produced during radiation therapy (8). Several antioxidants including vitamins have been introduced as potent radioprotectors of normal tissues such as vitamins E and C (18,19). Melatonin (N-acetyl -5 methoxy tryptamine), an indoleamine secreted mainly by the pineal gland during the dark hours at night, has emerged as a promising radioprotector with potent antioxidant, anti-inflammatory, and DNA-protective properties (20, 21). Melatonin may postpone the inhibition of the repair enzymes, hence allowing the repair of induces damage and the use of higher doses of radiation may supply better therapeutic value (20). Experimental research established the protective effect of melatonin against the genetic damage in blood, bone marrow and mortal effect of whole-body radiation in mice (22). Melatonin have properties of antioxidant reduces the side effects of radiotherapy by scavenging reactive oxygen and nitrogen species (23).

A growing body of evidences suggest that protective efficacy of melatonin against radiation-induced cytogenetic damage may vary across the cell cycle, likely due to stage-specific differences in oxidative stress, DNA repair fidelity, and checkpoint activation. For instance, melatonin pretreatment of human lymphocytes differentially reduced micronuclei and chromosomal aberrations depending on the cell cycle phase at irradiation, with notable protection in G2-phase cells (24). Research indicates melatonin can modulate key cell cycle checkpoints (G1/S, intra-S, G2/M), potentially allowing for more accurate DNA repair before progression (25). Furthermore, it has been shown that melatonin ameliorates radiation-induced DNA damage in bone marrow while modulating circadian clock genes that influence cell cycle progression, adding a layer of chronobiological regulation (22, 26). Cytogenetic studies shown different modulating effects of melatonin on irradiated breast cancer lymphocytes (27- 29).

In this study, the cytokinesis block micronucleus cytochrome (CBMN Cyt) assay in peripheral blood lymphocytes as an effective method with acceptable features of sensitivity, specificity, and reproducibility was used to assess the modulating effect of melatonin on ionizing radiation induced cytogenetic damage in G0 lymphocytes (30-32). Similarly, G2-assay, a well established method with a high sensitivity for chromosomal aberration studies (10, 33) was used for assessment of G2-chromosomal aberrations induced by ionizing radiation in the presence or absence of

melatonin. Because of the controversies over the effect of melatonin, the aim of this study was to investigate the effect of melatonin on G0 and G2 lymphocytes of the same breast cancer patients and normal healthy individuals after X-ray irradiation.

MATERIALS AND METHODS

Study subjects

Whole blood sample was collected from 10 healthy normal individuals with mean age of 40.6 ±10.6 and 20 breast cancer patients with the mean age of 60.2 ±7.6 in heparinized tubes. The study was in accordance with ethical committee of the National Institutes for Medical Research Development (NIMAD, IR.NIMAD.REC.1397.069). In addition, all donors consciously expressed their consent and completed a written questionnaire associated with their personal life style and medical history. The demographic information of patients is presented in table 1. To limit confounding factors, all samples were scrutinized to exclude former radiation exposure, antibiotic therapy and virus infection at least one month prior to sampling. Patients with breast cancer were not under chemo or radiotherapy treatment and all were diagnosed as new cases.

Table 1. Demographic information of studied healthy control and breast cancer patients.

Control	No. of cases	10		
	Mean age ± SD	40.6 ±10.6		
Breast Cancer Patients	No. of cases	20		
	Mean age ± SD	60.2 ±7.6		
	Age at Onset ± SD	59 ± 7.5		
	L/R	Left	5 (25%)	
		Right	15 (75%)	
	Type	Ductal	15 (75%)	
		Luminal	5 (25%)	
	Grade	I	4 (20%)	
		II	15 (75%)	
		III	1 (5%)	
	Stage	I	6 (30%)	
		II	10 (50%)	
		III	4 (20%)	
	Estrogen Receptor	91 ± 10 (%)		
Progesterone Receptor	85 ± 12 (%)			
HER2	Negative	19 (95%)		
	Positive	1 (5%)		
Ki67	37 ± 27.3 %			

Blood culture, melatonin treatment and irradiation

Lymphocyte cultures were set up in culture vessel under a laminar flow hood, containing 0.5ml whole blood in 4.5 ml RPMI1640 medium supplemented with 15% fetal bovine serum (FBS), 0.2mM L-glutamine and 1% penicillin/streptomycin (all reagents from Gibco BRL). 100µl of phytohaemagglutinin (PHA)(Sigma) with a final concentration of 5µg/mL was added to stimulate lymphocytes division. Melatonin powder (Chemodaru pharmaceutical, Tehran, Iran) was dissolved in RPMI

medium, then added to culture vessels 2 hours prior to irradiation at concentrations of 800 µg/ml. Irradiation of G0 lymphocytes with a dose of 3 Gy was performed 2 hours before cell culture initiation and for G2 lymphocytes 4 hours before harvesting at a dose of 1 Gy, using a therapeutic 6-MV medical linear accelerator (LINAC, Elekta, Stockholm, Sweden) with a dosimetric calibration based on the IAEA TRS 398 procedure. The sample-source distance was set at 80 centimeters. The field size was set at 15 cm×15 cm, and the dose-rate at the position of the cells was 1 Gy/min. Irradiation was done at ambient temperature (23±2 °C). Lymphocyte cultures were incubated in an incubator providing physiologic 37 °C temperature and 5% CO₂ in air.

G0 cytokinesis blocked MN assay

For G0 MN assay lymphocyte cultures were treated with melatonin 2 hour prior to PHA stimulation and irradiated at a dose of 3 Gy (dose rate of 1 Gy/min), X-rays. Forty-four hours after culture initiation, the cytochalasin B (with a final concentration of 4-6 µg/ml, Sigma-Aldrich, Germany) was added to block the cells at cytokinesis phase, and then the cells were incubated at 37°C for further 24 to 48 h. Harvesting was done according to the standard procedure including the centrifuge (1000-1200 rpm, 5 min), subjecting the cells to a short hypotonic treatment (KCl, 0.075 M), fixation with Methanol: Acetic Acid (3:1 v/v), at room temperature (15min), and washing with the cold fixation solution (Methanol: Acetic Acid, 6:1 v/v). Slide were prepared, air dried and stained in 4% Giemsa for 10 min. 1000 binucleate cells were scored per sample and analyzed under ×100 oil immersion light microscope according to the criteria described previously for scoring micronucleus in binucleate cells (30, 32). Figure 1 shows sample binucleate lymphocyte with and without micronucleus. To ensure influence of cytostatic effect of melatonin and radiation on the process of cell cycle and division for uniform MN scoring, Nuclear Division Index (NDI) was calculated for all treatments according to equation 1:

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

Where; M1, M2, M3 and M4 indicate the number of cells with one, two, three and four nuclei and N is the total number of cells analyzed. NDI demonstrates the irradiation or melatonin treatment have no effect on the cell proliferation and no significant difference between studied groups was observed ($p > 0.05$) (results not shown).

G2 chromosomal aberration assay

For G2-assay, sixty-six hours after whole blood culture initiation, melatonin (800 µg/ml) was added to some samples. After 3 hours culture vessels were exposed to X- rays at a dose of 1 Gy. Slides were made, dried and stained in 4% Giemsa for 10 min.

One hundred well spread mitoses were analyzed and scored under X100 oil immersion light microscope for the presence or absence of chromatid gaps, deletion and various types of exchanges for each sample cells (10, 33). Figure 2 shows sample metaphase spreads with and without chromatid aberration.

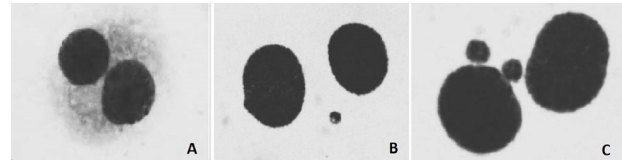


Figure 1. Sample photomicrographs of binucleate lymphocytes. **A;** binucleate without micronucleus. **B;** binucleate cell with one micronucleus. **C;** binucleate cell with two micronuclei. Magnification x1000.

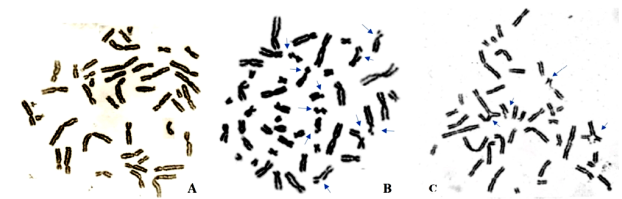


Figure 2. Sample photomicrographs of mitoses analyzed for chromosomal aberrations. **A;** Normal metaphase. **B;** Metaphase with several simple chromatid deletions (breaks) (arrows). **C;** Metaphase with simple chromatid deletion and exchanges, triradial and quadriradial. Magnification x1000.

Statistical analysis

SPSS software (version 18, Chicago, IL, USA) was used for statistical analysis. Non- parametric Mann-Whitney U-test and one way analysis of variance (ANOVA) was used to test the significant difference between studied groups. P-value less than 0.05 was regarded as significant level. Graph-pad Prism (version 4.0) was used for drawing figures.

RESULTS

Results of G0- irradiated lymphocytes

Results obtained with CBMN-assay for G0-lymphocytes treated with melatonin alone or in combination with 3 Gy radiation is summarized in table 2 and shown as figure 3 both for normal subjects and breast cancer patients. As seen, there was no significant difference between the frequency of background MN in lymphocytes of normal subjects and those treated with melatonin alone ($P > 0.05$). However, irradiation of 3Gy X-rays led to a pronounce increased frequency of micronuclei in lymphocytes of normal cases ($p < 0.01$). Combination of melatonin with radiation led to a statistically significant reduction in the frequency MN in control healthy individuals and BC patients ($p < 0.05$) (figure 3).

As shown in the figure 3, the value of background frequency of MN is significantly higher in BC patients compared to normal individuals ($p < 0.05$). Similarly, radiation induced micronuclei in lymphocytes of BC

patients is much higher than those of normal individuals ($p < 0.05$). Treatment of lymphocytes with melatonin prior to irradiation led to a significant

decrease of the frequency of micronuclei in lymphocytes of BC patients as well ($p < 0.05$) (table 2, figure 3).

Table 2. Radiation-induced micronuclei and chromosomal breaks in G0 and G2-normal and breast cancer patients' lymphocytes in the presence or absence of melatonin. G0= G0 phase of cell cycle; G2 + G2 phase of cell cycle; CBMN= cytokinesis blocked micronucleus; MN= micronucleus; Bc= breast cancer; Sd= standard deviation of mean values.

Subjects	No. of cases	Mean age \pm SD	Number of cells analyzed	Treatments				
				No treat. (0)	Melatonin alone	Radiation alone	Radiation + Melatonin	
G0-CBMN assay (Mean frequency of MN / 1000 CB cells \pm SD)	Healthy control	10	40.6 \pm 10.6	10000	18.3 \pm 6.0	14 \pm 2.7	140.1 \pm 50.9	116.4 \pm 36.7
	BC patients	20	60.2 \pm 7.6	20000	46.6 \pm 16.2 $P < 0.05$	41 \pm 9.8 $P < 0.05$	188 \pm 56.8 $P < 0.05$	143.7 \pm 37.5 $P < 0.05$
G2-assay (Mean frequency of Chromatid breaks / 100 cells \pm SD)	Healthy control	10	40.6 \pm 10.6	1000	1.7 \pm 0.75	2.5 \pm 1.0	18 \pm 5.3	19.2 \pm 3.7
	BC patients	20	60.2 \pm 7.6	2000	6.1 \pm 1.5 $P < 0.05$	14.2 \pm 5.3 $P < 0.05$	74 \pm 18.3 $P < 0.01$	108 \pm 26.7 $P < 0.01$

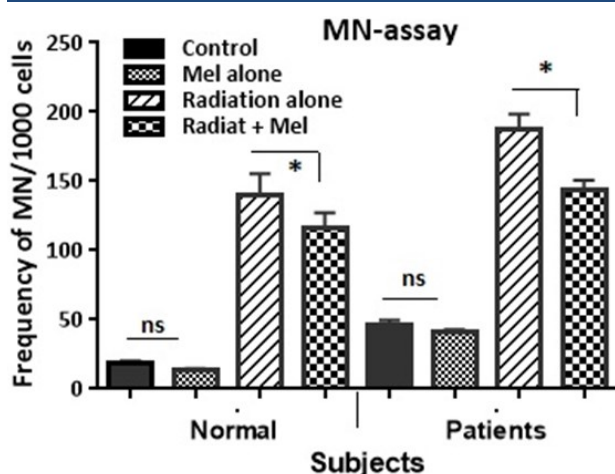


Figure 3. Frequency of micronuclei scored in binucleate lymphocytes for various treatment groups of normal and breast cancer patients irradiated at G0-phase of the cell cycle in the presence or absence of melatonin. *, denote p -value < 0.05 ; ns, denote, not significant.

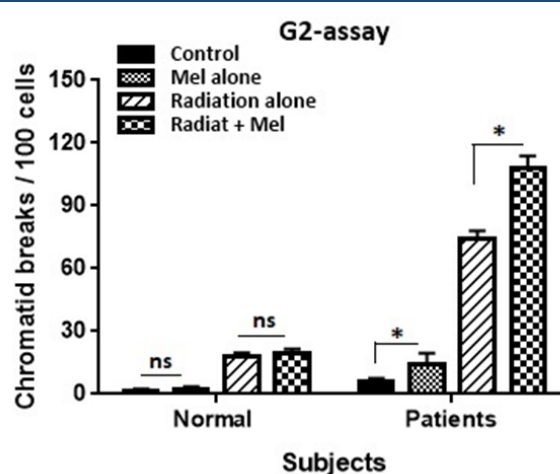


Figure 4. Frequency of chromatid breaks scored for various treatment groups of lymphocytes of normal and breast cancer patients irradiated at G2-phase of the cell cycle in the presence or absence of melatonin. *, denote p -value < 0.05 ; ns, denote, not significant.

Results of G2-irradiated lymphocytes

Detailed results obtained for G2 lymphocytes exposed to 1 Gy X-rays or in combination with melatonin is also summarized in table 2 and shown as figure 4.

Low frequency of chromatid breaks in control and melatonin treated samples was observed ($p > 0.05$). Frequency of chromatid breaks in control samples after exposure to radiation alone increased significantly different from unirradiated control samples ($p < 0.05$). No significant difference in the frequency of chromatid breaks was observed between lymphocytes of control subjects irradiated alone or in combination with melatonin ($p > 0.05$) (figure 4).

As seen in figure 4, background frequency of chromatid breaks in lymphocytes of BC patients was higher than those of normal subjects, statistically significant ($p < 0.05$). Moreover, slight increase or chromatid breaks was observed for melatonin treated cells compared to non-treated BC lymphocytes. The frequency of radiation induced chromatid breaks in lymphocytes of BC patients was

significantly higher than control ($p < 0.01$). Moreover, pretreatment of BC lymphocytes with melatonin led to an increased frequency of radiation induced chromosomal aberrations compared to normal control individuals ($p < 0.01$) (table 2, figure 4).

DISCUSSION

The cytokinesis-block micronucleus (CBMN) assay has been considered as a cytogenetic endpoint method with high potential for detection of cytogenetic abnormalities. With the use of MN assay, we have shown higher background MN in BC patients compared with the normal subjects and also a remarkable frequency of radiation-induced MN in lymphocytes of BC patients ($p < 0.01$). These results comply with a substantial body of evidences reported earlier that highlighted the genomic instability of normal tissue of BC patients (3, 12-15). Predisposing factor for radiation vulnerability of lymphocytes of BC patients expressed as a higher mean value of MN observed in this study, in agreement with several

published reports, could be the deficiency or impairment in DNA repair capacity (7). Radiation therapy (RT) prescribed for about half of the BC patients, may end up in normal tissues toxicity which limits the therapeutic efficiency. Therefore, higher normal tissue radiosensitivity of BC patients could often end up in RT-related complications such as skin rash, erythema, lymphedema, radiation dermatitis and fibrosis (34). To overcome radiation induced toxicity in normal tissues, radio-protectors were introduced to ameliorate the deleterious effects of radiation therapy. Natural antioxidant nutrients can have protective properties in this regard but with a weaker effect than the most known synthetic radio-protectors, such as amifostin (8).

In this study, melatonin (N-acetyl-5-methoxytryptamine), a pineal gland hormone has been considered as a highly efficient radio-protector with promising merits including low toxicity and available naturally in our body and has potential to spread in all cellular and subcellular sections (35). Our results as shown in table 2 and figure 3 clearly indicate radio-protective role of melatonin in reducing MN frequencies in lymphocytes of both control individuals and BC patients ($p < 0.05$). This observation is in accord with our previous observation with smaller sample size (36) for G0-lymphocytes and also reduced number of apoptotic cells in irradiated lymphocytes in the presence of melatonin (29). Studies in normal human lymphocytes and breast epithelial cell models indicate that melatonin pretreatment can reduce the frequency of radiation-induced micronuclei and chromosomal breaks in G1 cells. This is attributed to its antioxidant action, which decreases initial DSBs, and its potential to enhance the fidelity of early non-homologous end joining (NHEJ) repair pathway (24). Research using MCF-7, MDA-MB-231, and normal human mammary epithelial cells (HMECs) consistently shows that melatonin reduces clonogenic survival after irradiation in cancer cells while increasing it in normal cells. Cytogenetic analyses in these models confirm a significant decrease in radiation-induced micronuclei and γ -H2AX foci (a marker of DSBs) across all cell cycle phases, with the magnitude of protection varying (37).

Despite observation of radioprotective effect of melatonin in G0-lymphocytes of normal healthy individuals and BC patients, Observation of modulating effects of melatonin on radiation induced chromosomal breaks in G2-lymphocytes of both normal subjects and BC patients was totally different. As seen in figure 4, not only no protective effect has been exerted by melatonin in lymphocytes of normal cases following irradiation, but a sensitizing effect was observed in lymphocytes of BC patients. This observation was reported earlier for a small sample size by our group previously (28). In this study we used a larger sample size of BC patients and control

healthy subjects to apply two different methods for assessment of modulating role of melatonin on radiation induced cytogenetic damages on different cell cycle stages. Although melatonin is considered as an antioxidant agent, particularly as scavenger of ROS (38), our results demonstrate that melatonin could act as a radiosensitizer. Similar results were reported previously. For example, Alonso-Gonzalez (39) reported pretreatment of breast cancer cells with melatonin before irradiation sensitizes cells to ionizing radiation by downregulation of proteins (RAD51, DNA-PKcs) involved in double-strand DNA break repair. It has been shown that melatonin reduced the effectiveness of DNA repair and increased the rate of DNA damage promoted by irinotecan, a camptothecin analog, in human non-small-cell lung cancer and human colorectal adenocarcinoma cell lines (40). Melatonin is known to reinforce the G2/M checkpoint arrest in normal cells, preventing mitosis until DNA is adequately repaired. It reduces radiation-induced micronuclei formation in breast cell lines. A key differential effect is observed between normal and cancerous cells; while melatonin may prolong arrest in normal cells for repair, it can promote mitotic catastrophe or apoptosis in irradiated breast cancer cells, exploiting their defective cell cycle controls (25, 41). One of the most consistent mechanisms is melatonin's capacity to arrest proliferating cancer cells in the G2 and M phases of the cell cycle, which are the most vulnerable to radiation-induced DNA double-strand breaks (42). Melatonin achieves this by downregulating the expression of key cell cycle promoters. For instance, in liver cancer, melatonin suppresses the Wnt/ β -catenin pathway, leading to a significant reduction in cyclin D1 and c-Myc, critical drivers of the G1/S transition (24). This arrest in G2/M creates a larger target pool for radiation, maximizing lethal damage. Radiation's lethality can be mitigated by efficient DNA repair. It has been shown that melatonin downregulates crucial proteins involved in the two primary double-strand break repair pathways: non-homologous end joining (NHEJ) and homologous recombination (HR). By suppressing proteins such as Ku70/80 (for NHEJ) and RAD51 (for HR), melatonin prevents cancer cells from efficiently repairing radiation-induced breaks, leading to the accumulation of irreparable DNA damage and apoptotic cell death (43). Moreover, melatonin by decreasing the ratio of cells in the S and G2 phases decrease the probability to repair the DNA damage by homologous recombination, which occur in these phases of the cell cycle in breast cancer (44). In addition to the antioxidant mechanisms of melatonin and protection of DNA in G0 lymphocytes, our results show that melatonin influence on the radio-sensitivity of G2-lymphocytes of breast cancer patients.

CONCLUSION

Our results support that melatonin exerts significant protective effects against radiation-induced cytogenetic damage in G0-lymphocytes of both normal individuals and breast cancer patients. Its actions are modulated through mechanisms involving oxidative defense, DNA repair enhancement, and strategic cell cycle arrest. Moreover, results of the present study indicate that despite antioxidant property of melatonin, treatment of cells with melatonin before irradiation led to an increased clastogenic effect in lymphocytes of BC patients. The mechanism by which melatonin enhanced radiation effect is not fully understood but may be due to alterations in genes involved in DNA repair process leading to increased chromatid aberrations. More future studies are required to elucidate these differential cell cycle dependent modulating effects of melatonin on radiation induced cytogenetic damages.

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Conflicts of interest: None to declare.

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Ethical considerations: The Ethics Committee of the National Institutes for Medical Research Development (NIMAD,) approved the study with registration number IR.NIMAD.REC.1397.069. All patients and healthy donors provided their informed consent before participating in the study.

Authors' contribution: H.M.; conceived the idea, supervised the research, analyzed and interpreted data, drafted and finalized the manuscript. S.M., E.S., S.M. and F.P. were involved in experimentation and data collection. H.N.; was involved in sampling and irradiation of samples.

AI usage: Ai was used for grammar correction and text editing.

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