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Cytogenetic and cytotoxic effects of melatonin and saffron on lymphocytes of luminal A and luminal B breast cancer patients irradiated *in vitro*

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► ***Original article***

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ABSTRACT

Background: Due to high radiation- sensitivity of breast cancer (BC) patients, use of radioprotectors to ameliorate the deleterious effects of radiation could be a priority. This study aimed to evaluate the reducing effects of saffron and melatonin on ionizing radiation-induced damages in lymphocytes of luminal A & B BC patients using cytome assay. ***Materials and Methods:*** Whole blood samples were collected from BC patients as well as healthy individuals. Cells were treated with either melatonin or saffron two hours prior to irradiation of G0 lymphocytes using a 6 MV linear accelerator, at a dose of 3Gy. Thirty-six hours after PHA stimulation, Cytochalasin-B was added to the cultures. Cell harvesting and staining was performed using standard method. 1000 binucleate cells were scored per sample for the frequency of micronuclei (MN) or apoptosis under a 1000x light microscope. ***Results:*** The frequency of background MN was significantly lower in normal individuals compared to BC patients. Pretreatments of "saffron and melatonin" alone made no significant changes in the frequency of MN in all groups. However, after 3Gy-irradiation the frequency of MN elevated dramatically ($p<0.01$). Pretreatment of lymphocytes with melatonin and saffron led to a decrease in the frequency of MN in all treatment groups ($p<0.05$). In terms of apoptosis induction, only melatonin was found to exert reducing effect on radiation induced apoptosis. No radioprotection was observed for saffron in lymphocytes of BC patients. ***Conclusion:*** Obtained results elucidated more pronounced role of melatonin in decreasing MN and apoptosis frequencies post irradiation in lymphocytes of BC patients.

INTRODUCTION

Breast cancer (BC) is by far one of the most common malignant tumors and cause of death among women globally. Breast cancer is a heterogeneous disease with luminal A and luminal B subtypes accounting for a significant proportion of cases. These subtypes are characterized by distinct molecular profiles, treatment responses, and prognoses. Luminal A and B are defined as molecular subtypes of BC based on the expression of the estrogen receptor (ER), progesterone receptor (PgR), HER2 and Ki-67 [1]. Luminal A tumors are typically estrogen receptor-positive (ER+) with low proliferation rates, while Luminal B tumors, also ER+, exhibit higher proliferation and greater genomic instability, often leading to poorer outcomes [2]. Amongst main BC treatment modalities including surgery, radiotherapy, chemotherapy and hormone therapy, the conventional radiation therapy is ranked as a predominant modality with about 50%. Despite all advantages of radiotherapy, its use is associated with significant cytotoxic and cytogenetic effects, including DNA damage, chromosomal aberrations, and cell death, which can compromise treatment efficacy and patient's quality of life [3]. Therefore, alleviating adverse complications associated with radiotherapy has been the subject of a plethora of studies [4].

High radiation sensitivity of lymphocytes of BC patients has been previously reported and even an increased chromosomal radiosensitivity as a marker of breast cancer predisposition has been introduced [5]. The radiosensitivity and/or radio-susceptibility could be attributed to non-cancer and cancer effects vulnerability, respectively, as a result of unrepaired or mis-repaired DNA damages [6] and from the cytogenetic viewpoint, it has been linked with the number of micronuclei and chromosomal breaks [7]. Although there is no definite correlation between

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cancer type and radiosensitivity, breast cancer has been recognized as related malignancy in particular in the case of heterozygous BRCA1/BRCA2 mutations [7]. An elevated radiosensitivity has been shown in more than 45% of BC patients [8] and genome instability defined as a high number of chromosomal breakpoints, is proposed in terms of a prognostic indicator for early stage luminal breast carcinoma [4]. 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 (IR) is a priority [8]. IR generates reactive oxygen species (ROS), which cause various type of DNA damages, lipid peroxidation, and protein damage, leading to genomic instability and cell death [9]. Lymphocytes, due to their high sensitivity to radiation, are often used as a model to study these effects.

Antioxidants, which neutralize ROS and mitigate oxidative stress, have gained attention as potential radioprotectors. Accumulating research has revealed the capability of large number of radioprotectors including antioxidant nutrients such as vitamin A, C and E, and herbal products to ameliorate the deleterious effects of radiation [12, 13]. However, for a variety of reasons, their efficacy in BC patients, particularly in luminal A and B subtypes, remains underexplored. Given the differences in genomic stability and proliferation rates between these subtypes, it is plausible that their responses to IR and antioxidant interventions may vary.

Melatonin (N-acetyl-5-methoxytryptamine) synthesized from tryptophan mainly in the pineal gland with its promising antioxidant, anti-inflammatory and even anti-tumor properties has attracted the attention of radio-protector researchers during recent years [13, 14]. Different studies have been performed to show antitumor activity of melatonin, including BC [15-17]. Melatonin and its metabolites were found to scavenge free radicals directly [18-20]. Therefore, its use as a radioprotector and anti-cancer agent has been proposed [21]. Furthermore, according to the pharmacological studies saffron as a traditional herbal compound with multifactorial characteristics including anti-anxiety, antidepressant, antioxidant, anti-inflammatory and anti-tumor functions [22] could be appropriate alternative to ameliorate radiotherapy toxicities. Saffron contains different fractional structures such as crocin, crocetin, carotene and lycopen [23] is shown to be a potent antioxidant. It was reported that saffron and its constituents was able to reduce lipid peroxidation in various tissues [24-26].

The cytokinesis block micronucleus cytome (CBMN Cyt) assay in peripheral blood lymphocytes as an effective biodosimetry technique of ionizing radiation with acceptable features of sensitivity, specificity, transportability, and reproducibility could be implemented to measure genomic damages reduction post radioprotective agents application [10, 11, 27]. Since the CBMN Cytome assay could score not only the micronuclei (MN) in binucleated cells, but also the apoptosis, it is defined as reliable approach to measure DNA damage, and cytotoxicity of ionizing radiation [10, 11, 27]. The current study aimed to evaluate the reducing effects of melatonin and saffron on IR-induced cytogenetic and cytotoxic damages in lymphocytes of luminal A & B breast cancer patients using cytome assay.

MATERIALS AND METHODS

Preparation of saffron & melatonin

The active ingredient of melatonin was obtained from Ramofarmin Pharmaceutical Company (Tehran, Iran) and pure saffron was prepared (Birjand, Iran). Ethanolic saffron extraction was implemented using saffron stigmas powder according to Hadizadeh *et al*. [28]. The resulting solution was then incubated in the dark at 55 -65°C for 20 days. Finally, the dried saffron extracts were diluted with sterile distilled water and different concentrations were produced. Melatonin was also diluted using ethanol and then sterile distilled water at appropriate concentrations.

Study subjects

Whole blood samples were collected from 10 luminal A breast cancer patients with the mean age of 61.2+8.95 (age range of 45-70) and 10 luminal B breast cancer patients with the mean age of 59.1+5.7 (age range of 49-67), who have not received any chemotherapy or radiotherapy. The mentioned molecular subtypes of Breast cancer are identified as: Luminal A (ER positive, HER2 negative, Ki-67 low, and PR high); Luminal B (ER positive, HER2 negative, and either Ki-67 high (>14) [29] or PR low [30]). Blood sampling was also performed from 10 healthy normal individuals with mean age of 40.6+10.6 (age range of 36-63). 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 inclusion criteria included no history of medical radiation exposure, antibiotics consumption or virus infection at least during the last month prior to sampling. Demographic information of BC patients is presented in table 1.

Saffron & Melatonin administration and irradiation

Whole blood samples from luminal A & B, BC patients or healthy normal individuals were divided into six groups as control (without any treatment), treated with saffron alone (Sa, 800 µg/ml), treated with melatonin alone (Mel, 800 µg/ml), radiation alone (Rad), combined radiation+ saffron treatment

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(Rad+Sa), combined radiation+ melatonin treatment (Rad+Mel). Optimum concentrations of saffron and melatonin was determined using DPPH (2,2-Dipheny-1 picrylhdrazyl, Sigma) assay after using different concentrations of each compound from 200-1200 µg/ml. Chemical treatments were performed 2 hours prior to irradiation of lymphocytes. Irradiation of G0 lymphocytes with a dose of 3 Gy was performed 2 hours before cell culture initiation 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.

Table 1. Demographic information of individuals involved in the study. SD (standard deviation); L (left); R (right); ER (estrogen receptor); PR (progesterone receptor).

subjects	No. of cases	Mean age ± SD		Demographic information							
Normal	10	40.6±10.6									
			Age at Onset	L/R	Type	Grade	Stage	ER	PR	Her2	Ki67
Luminal A	10	61.2±8.9	60.2±8.9	L (30%) R (70%)	L (10%) D (90%)	1 (40%) 2 (60%)	1A (30%) 2A (60%) 3A (10%)	93.5±10	89±11.4	N (100%)	12±7.5
Luminal B	10	59.1±5.7	57.8±5.5	L (20%) R (80%)	D (100%)	2 (90%) 3 (10%)	1A (30%) 2A,B (40%) 3A (30%)	89±10.4	80.5±10.6	N (90%) P (10%)	62±13.9

Lymphocytes culture and cytome assay

Separate lymphocyte cultures were set up in a culture vessel under a laminar flow hood, containing 0.5 ml blood (irradiated alone or in the presence of melatonin or saffron in 4.5 ml RPMI1640 medium supplemented with 15% fetal bovine serum (FBS), 0.2 mM L-glutamine and 1% penicillin/streptomycin (all reagents from Gibco, USA). 100µl of phytohemagglutinin (PHA)(Sigma-Aldrich, Germany) with a final concentration of 5 µg /ml was added to stimulate lymphocytes division. 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.

The cell harvesting was applied according to the standard procedure including the centrifuge (1000-1200 rpm, 5 min), subjecting the cells with the hypotonic solution (KCl, 0.075 M) and the prefix solution comprising of the fixative (Methanol: Acetic Acid, 3:1 v/v)(Merck, Germany), keeping at room temperature (15min), secondary centrifuge, washing with the cold fixation solution (Methanol: Acetic Acid, 6:1 v/v). Subsequently, after slide preparation, they were air dried. and stained in 4% Giemsa for 10 min. 1000 cells were scored per sample and analyzed under ×100 oil immersion light microscope for presence of micronucleus or apoptotic cells. Moreover, nuclear division index (NDI) was calculated according to the equation 1 for each treatment group to assess the cytostatic effect of radiation alone or in combination with melatonin or saffron on the process of cell cycle and division:

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

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.

Statistical analysis

Statistical analysis was performed using SPSS software (version 18, Chicago, IL, USA). All data were expressed in terms of mean values ± SEM (standard error of the mean), which were then analyzed statistically by the one-way analysis of variance (ANOVA) test. The P-values less than 0.05 was considered as significant level. Graph-pad Prism (version 4.0) was used for drawing figures.

RESULTS

Nuclear division index

Calculation of nuclear division index (NDI) clearly indicated that 3 Gy-irradiation in combination with either saffron or melatonin treatment had no effect on the cell proliferation and no significant difference between studied groups was observed ($p > 0.05$) (results not shown).

Frequency of micronuclei in binucleate cells

Figure 1 shows result of background MN and MN induced after irradiation of lymphocytes of control individuals, luminal A and B, BC patients. As seen, the value of background MN is significantly higher in BC patients compared to normal individuals ($p<0.05$) and there is no definite distinction between two groups of BC patients. However, 3Gy-irradiation increased the frequency of MN in lymphocytes of normal individuals and BC patients dramatically ($p<0.01$). Frequency of radiation induced MN was not significantly different for luminal A and B BC patients ($p>0.05$).

Antioxidant treatments

Saffron

As illustrated in figure 2, there is no obvious difference between background MN in lymphocytes of the normal control and BC patients after saffron

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treatment alone (P>0.05). However, presence of saffron at the time of irradiation led to decreased frequency of MN in BC patients significantly (p<0.05). There was no significant difference between the frequency of MN induced by radiation alone or in the presence of saffron for normal control groups (p>0.05). No significant difference for MN induced was also seen for BC subtypes after saffron treatment.

Melatonin

As shown in figure 3, there is no obvious difference between background MN in lymphocytes of the normal control and BC patients after melatonin treatment alone (P>0.05). However, presence of melatonin at the time of irradiation led to significant decreased frequency of MN in lymphocytes of both normal control and BC patients significantly (p<0.05). No significant difference was seen for MN induced in lymphocytes of BC subtypes after melatonin treatment (p>0.05).

Frequency of apoptosis in binucleate cells

In general, figure 4 implies no significant distinction in apoptosis value following treatment of antioxidant agents alone compared to the background level in all studied groups (p>0.05). However, 3 Gy-radiation results in the remarkable increase in the apoptosis percentage (p<0.01). No significant reducing effect was seen following saffron treatment in all studied groups (p>0.05). Noteworthy to mention that the melatonin utilization reduced the apoptosis value significantly compared to the irradiation alone or radiation conjointly with saffron treatment (p<0.05).

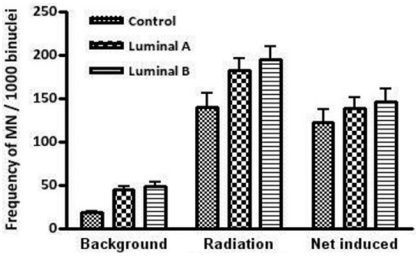


Figure 1. Frequency of micronuclei scored in in binucleated cells of normal control individuals, luminal A and B BC patients before and after irradiation. Error bars indicate standard error of mean values.

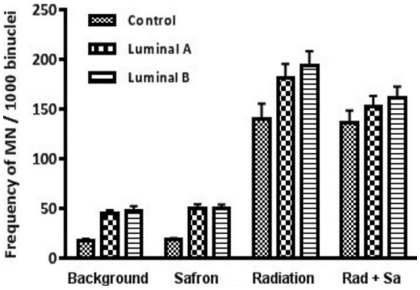


Figure 2. Frequency of micronuclei scored in binucleated cells of normal control individuals, luminal A and B BC patients before and after saffron treatment. Sa: Saffron; Mel: Melatonin; Rad: Radiation.

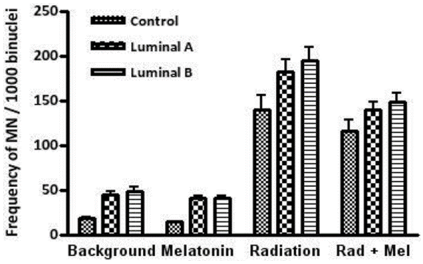


Figure 3. Frequency of micronuclei scored in binucleated cells of normal control individuals, luminal A and B BC patients before and after melatonin treatment. Sa: Saffron; Mel: Melatonin; Rad: Radiation.

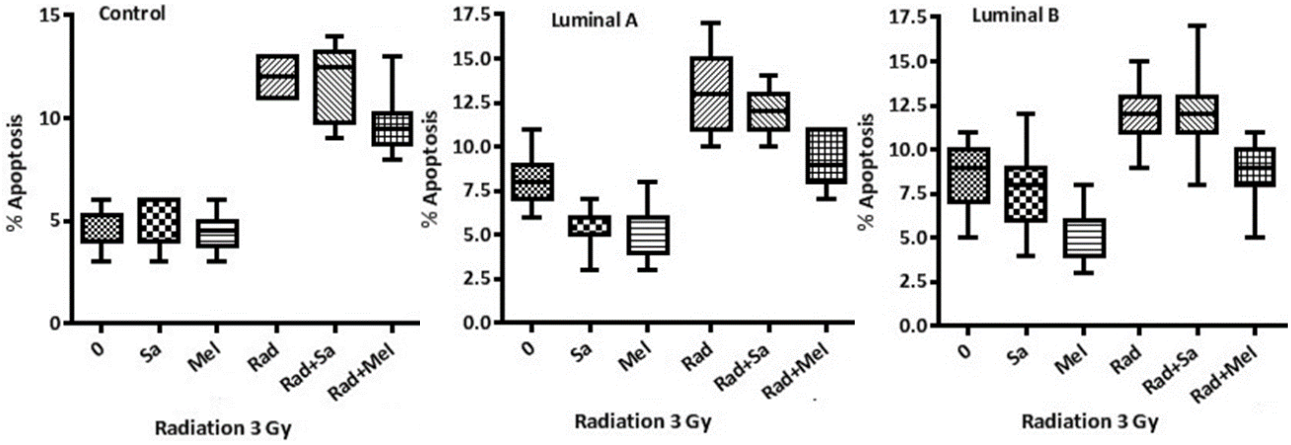


Figure 4. Apoptosis percentage scored in normal control individuals, luminal A and B BC patients in all studied groups. The boxes extend from the 25th percentile to the 75th percentile, with a horizontal line at the median (50th). Sa: Saffron; Mel: Melatonin; Rad: Radiation.

DISCUSSION

In the current study cytome assay was used to evaluate the reducing impacts of antioxidant agents including melatonin and saffron on ionizing radiation induced cytotoxicity and cytogenetic endpoints in lymphocytes of luminal A & B breast cancer patients compared to the normal individuals. The obtained results confirmed that although the use of "saffron and melatonin" alone makes no significant changes of micronuclei in lymphocytes of all studied groups, the use of antioxidant agents in combination with radiation can lead to the statistically significant reduction in the MN frequencies.

The cytokinesis-block micronucleus (CBMN) cytome assay has been considered as a multi-endpoint cytogenetic method with high potential for detection of various nuclear abnormalities. The

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cytome assay is now identified as an evolved system assay scoring DNA damages, cytostasis, and cytotoxicity that could overcome the restrictions of the original cytokinesis-block micronucleus (CBMN) [10, 27, 31]. Using cytome approach in the current study we appraised the distinction between lymphocytes of luminal A & B breast cancer patients in response to the antioxidant agents and radiation. The clear contrast in the background MN is elucidated in BC patients compared with the normal subjects and also the numbers of radiation-induced MN of BC patients have been elevated remarkably. These results comply with a substantial body of evidence that highlighted the genomic instability of BC patients [4, 32-35]. Breast cancer as the leading cause of nearly 14% mortality amongst all malignancies in developed countries [36] could be considered as a disease with elevated tendency to the genetic alterations. Accumulating evidences demonstrated the radiosensitivity of BC patients [34, 35, 37-40] in which the susceptibility to the induction of chromosomal damage by ionizing radiation was higher than the healthy individuals. Predisposing factor for radiation vulnerability of BC patients as a higher mean value of MN observed in this study (figure 1), in agreement with several published reports, could be the deficiency or impairment in DNA repair capacity [35]. The definite mechanism of higher radiosensitivity of BC patients has not been obvious but genome instability and defective repair of radiation induced DNA double strand breaks (DSBs) was postulated to have a 70% contribution [4]. Owning to the potential of cytome assay for inter-individual variation determination of vulnerability to the genomic effects of ionizing radiation, it could be utilized to provide further insight into BC patients' susceptibility to radiation therapy-induced chromosomal damages [10, 27]. However, as depicted in the figure 2, no statistically significant difference is observed between the studied endpoint in molecular subtypes of BC as Luminal A and B BC individuals in our study.

Radiation as a potent inducer of oxidative stress and reactive oxygen species (ROS), generates DNA lesions and chromosomal aberrations that might lead to cellular damages including apoptosis, necrosis, or carcinogenesis [41]. Based on the fact that radiation therapy (RT) is prescribed for about half of the BC patients, susceptibility to the RT-related toxicity may result in normal tissue side effects which limit the therapeutic efficiency. This radiosensitivity in BC patients could often end up in RT complications including skin irritation, discoloration or a bruised appearance, lymphedema, radiation dermatitis and fibrosis [42]. As a consequence, radioprotectors attracted attention of the scientific community to ameliorate the deleterious effects of radiation therapy. Natural antioxidant nutrients can have protective properties in this regard but with a weaker effect than synthetic radioprotectors, such as amifostine [13]. One important disadvantage of synthetic radioprotectors in comparison with the natural herbal ones might be their restricted clinical use because of their side effects and toxicities; therefore herbal products containing antioxidants and immune-stimulating compounds can be a promising alternatives [17]. Extract of saffron (*rocus sativus*) including different fractional structures such as crocin, crocetin, carotene and lycopen has been regarded as an impressive antioxidant agent in this field [22]. Antitumor [26], anti-inflammatory, and antinociceptive [43], effect of saffron has also been reported previously. The anti-genotoxic, anti-oxidant and chemo-preventive activity of saffron has been previously indicated in a way that saffron pretreatment impeded the cellular DNA damages of anti-tumor drugs expressing as decreased comet tail length and tail moment [44]. Despite the antioxidant properties of saffron, there is ambiguities about its protective role. Our recently published findings indicated the radio-sensitizing impact of saffron on G2 phase lymphocytes [45]. Pretreatment of lymphocytes of normal individuals and luminal A subtype of BC patients with saffron alone resulted in an elevated number of chromatid type aberrations with an increasing trend when combined with 1 and/ or 2-Gy irradiation. However, its possible function might be the DNA double strand breaks repairs inhibition that appeared as chromosomal aberrations in G2 phase of the cell cycle [45]. Similar findings were reported for melatonin utilization in G2-phase lymphocytes of BC patients irradiated with 1 Gy gamma rays [46]. In the present study as indicated in the figure 4, when this antioxidant agents were combined with radiation, the frequency of apoptosis was decreased (statistically significant in BC patients).

Alternatively, melatonin (N-acetyl-5-methyloxytryptamine), a pineal gland hormone has been considered as a highly efficient radio-protector with promising merits including low toxicity and high amphiphilicity that is available naturally in our body and has potential to spread in all cellular and subcellular sections [13]. Our results (figures 3 and 4) in the current study are consistent with the majority of reports that proposed the radioprotective role for melatonin. The use of this antioxidant with radiation in our study led to a statistically significant reduction in the MN frequencies in lymphocytes of both control individuals and BC patients. These findings could enhance the dramatic role of it in amelioration of radiotherapy toxicities. Melatonin with anti-inflammatory activity has proved its potential to mitigate the onset of inflammation and its persistence post ionizing radiation exposure [20]. Because inflammation was implicated in the acute and long term normal tissues side effects of radiotherapy, melatonin could act as an efficient radioprotector, consequently [13]. The antioxidant

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function of melatonin has been demonstrated as a result of two direct and indirect process; scavenging free radicals including reactive oxygen (ROS) and nitrogen (NO) species and changes in gene transcription and antioxidant enzymes [14]. Melatonin might reduce DNA damage, because of its direct radical scavenging actions of free radicals induced by IR [47]. Melatonin and most of its metabolites have the capability to scavenge free radicals and reactive nitrogen species [18]. It was shown that melatonin increased frequency of the programmed cell death induced by ROS generated by arsenic trioxide, activation of the p38/JNK pathways, and by upregulation of Redd1 expression in human BC cells [48]. The anti-apoptotic role of melatonin has been also previously assessed and increased Bcl-2 gene expression with a significant decrease in Bax gene expression in irradiated spinal cord has been shown [49]. Furthermore, in pursuit of melatonin antioxidant functions, it has been proposed as mitochondria targeting agents to mitigate mitochondria ROS via modulation of radiation induced ROS/NO [13, 14]. Based on the figure 4, the melatonin utilization reduced the apoptosis value significantly compared to the irradiation alone or combined with saffron treatment, indicating the protective role of melatonin against radiation induced DNA damage and cell death. However, further work on gene alteration could clarify the role of melatonin significantly.

CONCLUSION

Current work provides evidence for capability of utilizing cytome assay to demonstrate the impacts of two natural antioxidant compounds including saffron and melatonin in reducing cytotoxicity and cytogenetic effects of radiation in lymphocytes of BC patients. Noteworthy to mention that the significant difference was found in the background MN and apoptosis frequency in lymphocytes of BC patients compared to the normal individuals. Melatonin was found more effective radioprotector against radiation induced MN or apoptosis in lymphocytes of BC patients compared to saffron.

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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, analyzed and interpreted data, drafted and finalized the manuscript. S.M., F.P. and S.M. were involved in experimentation and data collection. H.N.; was involved in sampling and irradiation of samples.

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