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Effects of saffron extract on the frequency of radiation induced chromosomal aberration in G_2-lymphocytes of normal individuals and breast cancer patients

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ABSTRACT

Background: Inherent radiosensitivity associated with elevated chromosomal aberrations (CA) was shown in breast cancer (BC) patients at cellular level. Different agents were used to protect cells against adverse effects of ionizing radiation (IR), mainly antioxidants such as vitamins. The aim of this study was to evaluate the modulating effect of saffron extract on radiation induced CA in lymphocytes of normal individuals and BC patients. ***Materials and Methods:*** G_2 assay was applied on whole peripheral blood lymphocytes received from 5 normal controls and 10 BC patients with luminal A subtype. Blood culture was initiated in complete culture medium, 4h prior to harvesting cells were irradiated with 1 and 2 Gy X-rays. Pretreatment of samples with saffron was done 2 h before irradiation. After metaphase preparation and slide making, slides were stained in Giemsa. Hundred well spread metaphases were scored for presence of chromatid type aberrations with a microscope at a magnification of 1000×. ***Result:*** Results indicated a high and significant frequency of CA both in lymphocytes of normal and BC patient after irradiation. Pretreatment of samples with saffron led to a significant increase in the frequency of breaks in lymphocytes of both normal individuals and BC patients. ***Conclusion:*** Results indicate that despite its antioxidant property, pretreatment of lymphocytes of normal individuals and BC patients with saffron before X-irradiation led to radiosentisizing effect. The way saffron sensitize lymphocytes to X-rays is not known, but it might be possible to inhibit repair of radiation induced DNA strand breaks.

Keywords: *Saffron, breast cancer, luminal A, chromatid aberration, radiotherapy, G2 assay.*

INTRODUCTION

Breast cancer, a common type of malignancy in women occurs with different subtypes sporadically and familial in an increasing rate worldwide and in Iran [1]. About 80 % of patients with BC receive radiotherapy alone or in combination with chemotherapy. It seems that most patients tolerate RT, but some suffer from severe adverse effects of radiation [2, 3]. This variability of response may be caused by several factors, such as age, life style, oxidative stress, genetic predisposition and various genes involved in the response to radiation induced DNA damage. However, genome instiability might be the main cause of this variable response to ionizing radiation in BC patients. Genome instability leading to inherent radiosensitivity in BC patients has been shown previously and with different experimental approaches [4-7].

Exposure to ionizing radiation causes various

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types of cellular and molecular damages directly or indirectly via formation of free radicals or reactive oxygen species (ROS) [8]. Various types of DNA damages are produced in DNA following interaction with ROS, the main of them double strand breaks (DSB). Formation of DSB triggers various types of DNA repair mechanisms [5]. Failure to repair radiation induced damages might lead to adverse effects of radiotherapy and elevated radiosensitivity. Adverse biological effects such as immunologic complications, skin erythema and fibrosis has been reported for BC patients following radiotherapy [9]. Elevated cellular radiosensitivity has been shown for over 40% of BC patients [10-12].

To overcome adverse effects of ionizing radiation at cellular and molecular levels, it is possible to use chemical synthetic or naturally occurring radioprotectors [13]. The main mechanism of action proposed for the most known radioprotectors including aminothiols and antioxidants is to scavenge free radicals [14]. Using agents occurring naturally with low toxicity to human and also having potential for oral administration has always been main concern of researchers in this field. Therefore with these aims, we have chosen saffron to investigate its potential for modulating radiation effects on lymphocytes of breast cancer patients and normal healthy individuals.

Extract of saffron (*rocus sativus*) contains different fractional structures such as crocin, crocetin, carotene and lycopen [15] with potential antioxidant effects [16, 17]. Saffron, usually used as coloring herb and spice in food industry, is also a medicinal plant used for treatment of various types of diseases in traditional and modern medicine. It has been shown that saffron exerts antitumor [18], antinociceptive and anti-inflamatory [19] effects. Teratogenic effects of saffron has also been reported [20] indicating killing effects of this herb in embryonic cells.

Thus, in light of antioxidant properties and the controversies over the effect of saffron, in the present study its possible radioprotective effects was investigated against radiation induced chromosomal aberrations induced in G_2 phase lymphocytes of normal individuals and BC patients with luminal A subtype. Luminal A breast cancer is low grade, hormone-receptor positive (estrogens-receptor and/or progesterone-receptor positive), HER2 negative, and has low levels of the protein Ki-67. G_2-assay, a sensitive and reliable method, has been used for evaluation of cellular radiosensitivity or radioprotection against ionizing radiation previously [21, 22].

MATERIALS AND METHODS

Saffron extraction and DPPH assay

Ethanolic saffron extraction was done using natural saffron stigma powders according to Hadizadeh *et al*. [23]. The final solution was incubated in a dark glass container at 55 -65°C for 20 days. Different concentrations of saffron were prepared by dissolving saffron powder in sterile distilled water. Antioxidant properties of saffron was evaluated with the use of DPPH (2,2- Diphenyl-1- picrylhydrazyl) assay. To do this, various concentrations of saffron (100-1200 µg/ml) was prepared and DPPH assay was performed based on standard method [24].

Study subjects

Whole blood sample was collected from 10 breast cancer patients with the mean age of 53.3+10.3 (age range 37-78) and 5 healthy normal individuals with mean age of 39.6 + 11.6 (aged 28-63) in heparinized tubes. All patients were categorized as luminal A breast cancer subtype. The type of cancer was ductal carcinoma, with grades 1-2, hormone-receptor positive [estrogen-receptor (>90%) and/or progesterone-receptor (>88%)], HER2 negative, and had low levels of the protein Ki-67 (12%)]. The study was approved by the NIMAD ethical committee, IR.NIMAD.REC.1397.069. All blood donors gave their informed written consent and completed a written questionnaire to give information related to their personal life style including dietary habits, medical history and exposure to chemical and physical agents. To limit confounding factors, samples from individuals with radiation exposure, antibiotic

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consumption and virus infection at least one month prior to sampling were excluded. Patients with breast cancer were diagnosed as new cases and did not receive any chemo or radiotherapy before sampling.

Lymphocyte culture, saffron treatment and irradiation

Whole blood samples from patient or healthy normal individual was divided into four parts as control (without any treatment), treated with saffron alone (M), radiation alone (R), and combined radiation+ saffron (R+S) treatment. Lymphocyte cultures were initiated with adding 0.4 ml blood to 4.5 ml RPMI-1640 medium, supplemented with 15% fetal bovine serum, 1% L-glutamine and antibiotics (penicillin 100 iu/ml and streptomycin 100 µg/ml) (all reagents from Gibco BRL).

The lymphocytes were stimulated to divide with 1% phytohemagglutinin (PHA)(Sigma). Whole blood cultures were incubated at 37°C. Saffron was added to cultures 2 hours prior to irradiation at a concentration of 800 µg/ml. After 2 hours, culture vessels were exposed to doses of 1 and 2 Gy X- rays generated from a 6 MV linear accelerator (Linac, Varian Unique, USA) at a dose rate of 1 Gy/mim and at ambient temperature. Culture vessels were incubated for four hours after irradiation. Colcemid (final concentration 4 µg/ml, Sigma) was added to lymphocyte cultures 1.5 h prior to harvesting in order to arrest cells at metaphase stage. Cells were harvested with standard procedure, slides were made, air dried and stained in 4% Giemsa for 10 min. Chromatid type breaks were scored and analyzed in well spread metaphase cells under ×100 oil immersion light microscope. 100 cells were scored per sample. Figure 1 shows sample metaphase spreads with and without chromatid aberration.

Statistical analysis

Statistical analysis was done on obtained data using SPSS (version 18, Chicago, IL, USA). Non- parametric Mann-Whitney U-test 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.

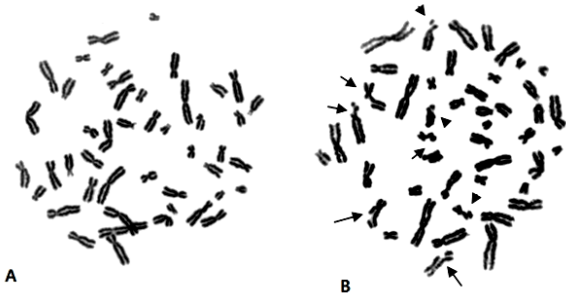

Figure 1. Typical photomicrographs showing normal metaphase **(A)**; metaphase with multiple chromatid breaks (deletions) (arrows) **(B)**. Magnification×1000.

RESULTS

DPPH assay

Result of DPPH assay for different concentrations of saffron is shown in figure 2. Saffron showed moderate antioxidant capacity at concentrations above 400 µg/ml. There was a significant difference between concentration of 100-200 µg/ml and 400 – 1200 µg/ml ($p<0.001$); but the difference between concentrations above 400 µg/ml was not statistically significant ($p>0.05$). Therefore saffron with concentration of 800 µg/ml was used for all experiments with radiation.

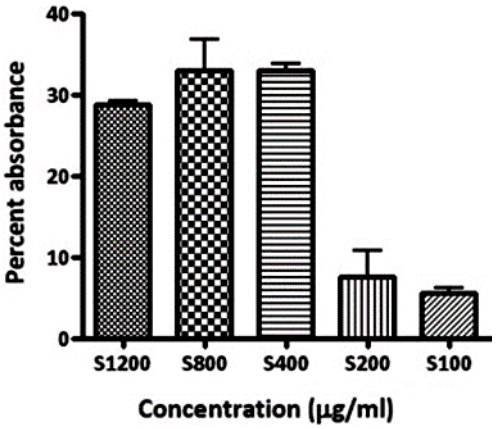

Figure 2. Free radical scavenging activity of saffron at different concentrations evaluated by DPPH assay. Error bars indicate standard deviation from the mean values.

G2 assay

Normal individuals

The results are summarized in table 1 and shown in figures 3-5. Background frequencies of

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chromatid aberrations were low. However, treatment of lymphocytes with saffron alone led to elevated frequency of chromatid type aberrations significantly different with non-treated samples ($p<0.01$). Irradiation of lymphocytes with doses of 1 and 2 Gy gamma-rays led to increase in the frequency of chromosomal aberrations significantly different from the control un-irradiated samples ($p<0.01$); although the yield of breaks was doubled with the dose of 2 Gy (figure 3). Frequency of chromatid aberrations induced by saffron alone was higher than the frequency of aberrations induced by radiation at a dose of 1 Gy. Irradiation of lymphocytes in the presence of saffron led to a significantly higher frequency of chromosomal aberrations both after 1 and 2 Gy irradiation ($p<0.01$) (figure 4).

BC patients

Results obtained for chromosomal analysis of lymphocytes of BC patients are shown in figure 5. As seen, higher background frequency of chromatid breaks was seen in BC lymphocytes compared to control ($p<0.01$) (figure 3). The level of chromatid breaks induced by saffron alone was nearly similar to the background frequency of breaks scored for BC patients ($p>0.05$).

Radiation induced chromatid breaks in lymphocytes of BC patients was more than normal individuals significantly different ($p<0.01$). At radiation dose of 2 Gy, the frequency of breaks was nearly doubled (figure 3). Irradiation of lymphocytes in the presence of saffron led to an increased frequency of breaks for both 1 Gy and 2 Gy doses of radiation (figure 5). Statistical analysis on data obtained from combined treatment of lymphocytes of BC patients with radiation and saffron showed significant difference with data obtained for control individuals for both radiation doses ($p<0.001$).

Table 1. Mean frequency of chromatid breaks scored in blood samples obtained from control individuals and breast cancer patients. ± indicates standard deviation (SE) of mean values.

Subjects	No. of samples	Mean ±SD age (Range)	No. of cells analyzed	Mean ±SD (SE) background breaks	Mean ±SD (SE) saffron induced breaks	Radiation dose (Gy)	Mean±SD (SE) gamma rays induced breaks	Mean±SD (SE) gamma rays induced breaks + saffron
Control	5	39.6±11.6 (28-63)	500	1.43 ± 1.5 (057)	9.2± 1.7 (0.5)	1	5.43±3.6 (1.3)	12.6 ± 3.3 (1.0)
						2	11.3±6.1 (2.3)	20.8±5.9 (1.8)
Breast cancer patient	10	53.3±10.3 (37-78)	1000	8.34 ± 3.7 (1.1)	9.2± 1.7 (0.5)	1	25.3±12.3 (3.7)	31.2±12.9 (3.8)
						2	49.2±22.1 (6.6)	63.1±24.6 (7.4)

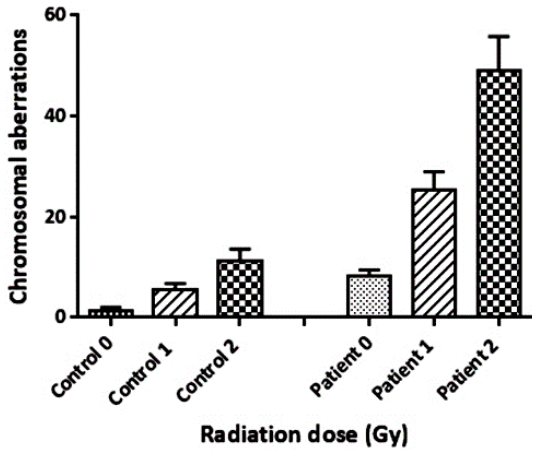

Figure 3. Comparison of background and radiation induced chromosomal breaks between lymphocytes of normal individuals and breast cancer patients. Error bars indicate standard error of mean values.

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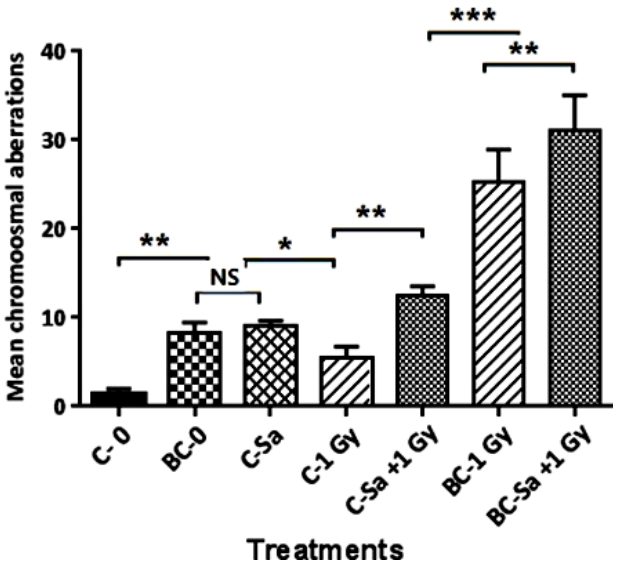

Figure 4. Frequency of chromatid aberrations observed in lymphocytes of normal individuals following irradiation alone or in the presence of saffron (800 µg/ml). Error bars Indicate standard errors of mean values. NS=non-significant; *, p<0.05; **, p<0.01; ***, p<0.001.

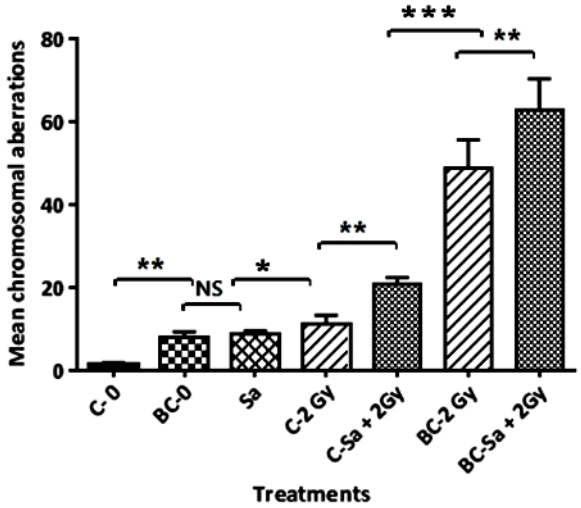

Figure 5. Frequency of chromatid aberrations observed in lymphocytes of breast cancer patients following irradiation alone or in the presence of saffron (800 µg/ml). Error bars Indicate standard errors of mean values. NS=non-significant; *, p<0.05; **, p<0.01; ***, p<0.001.

DISCUSSION

Higher significantly different background frequency of chromatid type aberrations in lymphocytes of BC patients compared to control (p<0.01) (table 1, figure 3) clearly indicates that normal cells in BC patients suffer from genome instability. Genome instability in BC patients, as shown previously [4, 6, 11], expressed as elevated DNA damage and chromosomal aberrations might be associated to repair deficiency in DNA damage repair machinery [5]. The frequency of background chromatid breaks in lymphocytes of BC patients was similar to the frequency of 1 Gy radiation induced breaks in lymphocytes of normal individuals. Repair deficiencies in lymphocytes of BC patients make them vulnerable to experience higher frequency of chromosomal aberrations following exposure to ionizing radiation. As shown in figure 3, statistically higher frequencies of chromatid breaks were induced in lymphocytes of BC patients following 1 and 2 Gy irradiation compared to control (p<0.001). The elevated frequencies of chromosomal aberrations seen in lymphocytes of BC patients might lead to adverse biological effects in BC patients in response to radiotherapy expressed as skin erythema, fibrosis or immunologic complications [2, 3].

There has been a long time effort to use chemical radioprotecters to reduce radiation induced biological damage since 1949 [25]. Compounds such as aminothiols were found to be effective because of their radical scavenging potentials [26]. Similar observations were reported for various naturally occurring or dietary antioxidants [27]. Natural antioxidants such as vitamins E, C and melatonin were shown to reduce radiation induced chromosomal abnormalities in normal murine or human cells [28-30]. Therefore it is expected that any agent with radical scavenging potential would be able to reduce clastogenic effects of IR [31, 32]. As seen in figure 2, saffron showed to be a moderate antioxidant as evident by DPPH assay. Other investigators have also shown antioxidant properties for saffron [16, 17]. However, results show at least for G_2 lymphocytes the opposite effect, i.e. radiosensitisizing instead of being a radioprotector. Saffron treatment alone induced a high frequency of chromatid breaks both in lymphocytes of normal and BC patients. Combination of saffron with radiation led to an elevated frequency of aberrations following 1 and 2 Gy exposure in lymphocytes of normal

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individuals (figure 4). This effect was more pronounced for lymphocytes of BC patients, significantly different with normal samples ($p<0.001$). Similar effect has been reported for melatonin when used G_2 lymphocytes of BC patients [33]. The reason that saffron with antioxidant property increase the clastogenic effects of ionizing radiation in G_2 lymphocytes of both normal and BC patients is not known. However it is possible that saffron inhibits nucleic acid synthesis by impairment of topoisomerase II function involved in DNA synthesis [34]. Mis-repaired or unrepaired DNA damages may be converted to chromosomal aberrations in G_2 phase of the cell cycle [35].

In conclusion, results indicate that despite antioxidant property of saffron, treatment of cells with saffron before and after irradiation led to an increased clastogenic effect in lymphocytes of normal individuals and BC patients. The mechanism by which saffron enhanced radiation effect is not fully understood but may be due to alterations in genes involved in DNA repair process in G_2-phase of the cell cycle that lead to elevated frequency of chromatid aberrations.

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***Conflicts of interest*:** *Declared none.*

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