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Interindividual differences in radiation-induced apoptosis of peripheral blood leukocytes in normal individuals and breast cancer patients

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Background: Quantification of radiation-induced apoptosis in peripheral blood lymphocytes (PBLs) has been proposed as a possible screening test for cancer-prone individuals and also for the prediction of normal tissue responses after radiotherapy. ***Materials and Methods:*** The neutral version of the comet assay (single-cell gel electrophoresis) was used 24, 48, 72 hours after irradiation with 8 Gy gamma rays to assess interindividual differences in gamma rays-induced apoptosis in peripheral blood leucocytes between a panel of 30 normal individuals, and 30 breast cancer patients who hadn't received radiotherapy or chemotherapy previously. Slides were stained with ethidium bromide and comets were assessed using visual and computer analysis. ***Results:*** In all incubation times, the baseline and radiation induced apoptosis values were higher in breast cancer patients compared to normal individuals which were significantly different ($p<0.01$). Although, both baseline and radiation induced values were higher in young breast cancer patients, the age did not seem to have a significant effect on apoptosis values both in cancer cases and controls. ***Conclusion:*** The increased rate of apoptosis observed in the leukocytes of breast cancer cases might be associated with their deficient DNA repair mechanisms. This finding might indicate that the radiation induced apoptosis could have some predictive potential. However, large interindividual variation has been a drawback. More studies are required to investigate the causes of interindividual variation and how it might be minimized. **Iran. J. Radiat. Res., 2012; 9(4): 237-244**

Keywords: *Breast cancer patients, apoptosis, leukocytes, gamma rays, comet assay.*

INTRODUCTION

The most significant molecular events that cause promotion and progression of cancer are DNA damage, repair deficiency and disregulation of apoptosis [1-3]. Apoptosis is a natural process also known as programmed-cell death, can occur by DNA damage that is induced by mild cellular stress such as low levels of ionizing radiation and physical or chemical agents. This is the most common mechanism that body eliminates damaged cells. Cells that are undergoing apoptosis show special patterns like cell shrinkage, condensation and fragmentation of nucleus to 200 base pairs and bubbling of the plasma membrane known as blebbing [4-6]. Although mitotic death is considered as a major cell killing effects of ionizing radiation, however, radiation induced apoptosis has been documented in normal tissues as well as human leukocytes [7-10]. Mutation in genes that control apoptosis can reduce treatment sensitivity. Apoptosis links cancer genetics to cancer therapy [11].

Radiotherapy which is used extensively for cancer treatment is known as one of the most important causes of DNA damage [12-18]. Normal tissue injury is one of the side effects of radiotherapy. Radiation-dose is limited by tolerance of patient's normal tissue. Tumor response to radiotherapy varies depending on a number of factors such as cell-cycle position, cellular oxygen level, tumor type, oncogen and growth factor expression. Therefore a detecting test that

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can precisely predicts individual radiosensitivity would be a valuable assay in the cancer clinic [4, 19-21]. Prediction of radiation-induced toxicity can help physicians to select the appropriate treatment strategy for each patient. Different investigators described some predictive factors such as initial DNA damage, gene expression pattern and apoptosis. It has been shown that cellular apoptosis detection can be used as appropriate approach for the prediction of normal cell response after radiotherapy [4, 11, 22, 23].

Various techniques are used for apoptosis analysis, such as flow cytometery, TUNEL assay, DNA laddering agarose gel analysis, ELISA, PCR, *in vitro* and *in vivo* DNA end-labeling and comet assay. The later found one of the best selective methods because of its high sensitivity, simplicity, velocity, requirement a few number of cells and DNA damage-detecting in individual cells [4, 24-30]. Among studies focusing on radiosensitivity some showed no difference for the repair of radiation induced- DNA damage between cancer patients and healthy groups [28]. However some studies stated that repair process might be different among these groups [21]. In this study, we compared the radiosensitivity of breast cancer patients and healthy volunteers by measuring basal and induced apoptosis level in peripheral blood leukocytes by means of neutral comet assay.

MATERIALS AND METHODS

Blood sampling and irradiation

Thirty breast cancer patients aged between 26 and 81 years (mean age 57.06 ± 14.82) were recruited at Imam General Hospital in Tehran before mastectomy. None of them had been treated with chemotherapy or radiotherapy. Thirty normal volunteers aged between 25 and 89 years (mean age 52.86 ± 19.17) were selected as a control group. The study was approved by the Ethical Committee of the School of Medical Sciences of the Mazandaran University of Medical Sciences, Sari, Iran. Patients gave their informed written consent. All donors completed a written questionnaire to obtain information related to their life style, such as dietary habits, medical history and exposure to chemical and physical agents. All normal volunteers and cancer patients were non smokers without infectious disease, antibiotic consumption and X-rays during the last month prior to sampling. Five mL of venous blood was obtained from each individual in a heparinized syringe. Mononuclear cells were separated from heparinized blood samples by Ficoll-hypaque (supplied by Blood Transfusion Organization of Iran, Tehran) centrifugation (2000 revolutions per minute (rpm), 20 min, 20°C), washed in phosphate buffered saline and resuspended in RPMI-1640 medium (Gibco, BRL, Long Island, NY, USA) containing 20% fetal calf serum (Gibco, BRL) for 1 day. Such a strategy is advisable because the isolation stress itself is sometimes sufficient to induce DNA damage that can be detected in the comet assay. Lymphocytes were suspended at 3×10^5 cells/mL and cultured in a 5% CO_2 incubator at 37 °C. Cells were irradiated on ice with 8 Gy of gamma rays generated from a 60Co source (Theratron II 780C, Canada, AECL, Ontario, Canada) (at source to sample distance = 80 cm, room temperature 23 ± 2°C) with a dose rate of 2.77 ± 0.11 Gy/min.

Apoptosis was assessed at 24, 48, and 72 h after irradiation by keeping cells at 37 °C and 5% CO_2 before start of experiments. Cryopreserved lymphocytes of a healthy individual were used as an internal standard and were assayed at several experimental dates.

Slide preparation and comet assay

Briefly, the samples were centrifuged for 5 min at 2500 rpm, and the supernatant was removed, 105 cells were mixed with 140 µL of 0.75% low melting point (LMP) agarose (Fermentas) in phosphate buffer saline (PBS). Seventy µL of the resulting suspension was layered on top of each window of

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microscope slides precoated with a supporting layer of 1% normal melting point (NMP) agarose (Fermentas) in distilled water, then covered with coverslips and kept in 4 °C for about 5 min in order to solidify the gel. The cover-slips were removed and the slides were then soaked in freshly prepared lysing solution (2.5 M NaCl, 0.1 M EDTA, 10 mM tris-base, 1% N-lauryl sarcosine, 1% triton x -100, 10% dimethyl sulphoxide (DMSO) for about 30 minutes at 4 °C in the dark to remove DNA-bound proteins to prevent migration in the electric field. All materials used for preparation of the lysis solution were supplied by Merck, (Germany). The final pH of the lysis solution was adjusted to about 10. After lysing, the slides were washed three times in electrophoresis buffer consisting of 90 mM tris base, 90 mM boric acid and 2.5 mM Na_2EDTA (Merck, Germany), at a pH of 8.3 - 8.4. Slides were then transferred onto a submarine horizontal electrophoresis chamber containing a fresh electrophoresis buffer. Electrophresis was performed at 20 volts (0.8 V/cm) and 8 mA for 15 min. The slides were then washed with distilled water for 5 min and then fixed in ethanol for 5 min at room temperature. The air dried slides were stained with ethidium bromide solution (20 mg/mL) and covered with cover-slips before analysis. Cells were analysed using a fluorescent microscope (Nikon/Japan) equipped with an excitation filter (510 – 550 nm) and barrier filter (590 nm), at 200× magnification, for the presence of apoptotic and non apoptotic cells. A typical photomicrograph of normal and apoptotic cells is shown in the figure 1. A total number of 500 cells were randomly analysed for each slide. For each sample, at one run, at least 1000 cells were analysed.

All statistical analyses were carried out using Graph Pad Prism software version 4. Differences between means of initial radio-induced DNA damage between groups were tested for significance with the two-sided, unpaired Student's *t-test*. To analyze the results of residual DNA damage between groups the non-parametric Mann–Whitney U-test was also used. Figures were drawn using Sigma Plot 2004 for Windows, version 10.0.

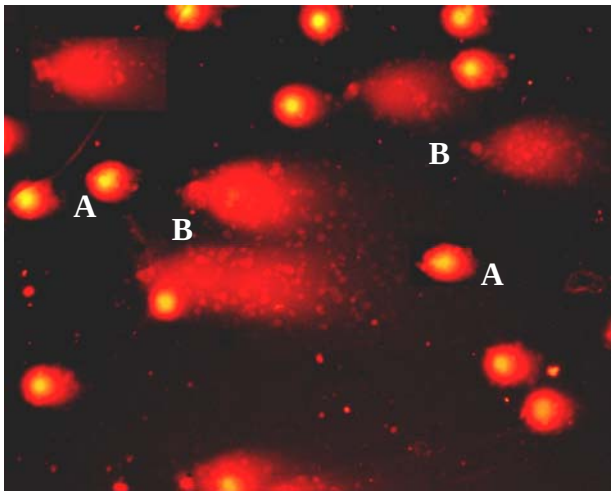

Figure 1. Normal (A) and apoptotic (B) cells after 24 hours gamma irradiation detected by neutral comet assay.

RESULTS

Appropriate dose determination for radiosensitivity tests

Radiation dose that created the average response of apoptosis in leukocytes has been needed for this test. A blood sample of normal individual, irradiated to different doses of gamma radiation from 4 to 32 Gy and induced apoptosis was analyzed by neutral comet assay. Results showed that the dose of eight Gy gamma rays induced moderate number of apoptosis at all of the incubation times (figure 2), so we selected this irradiation dose for all steps of our research.

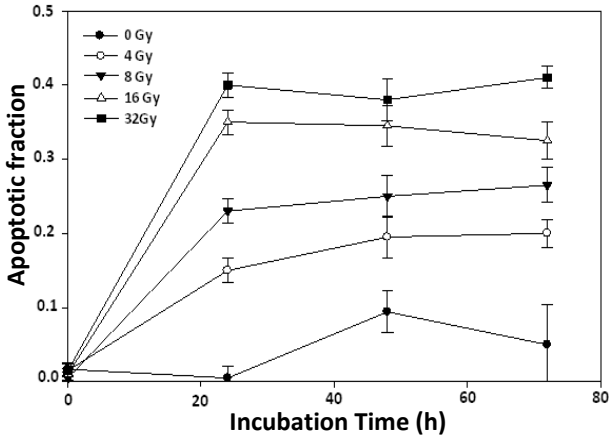

Figure 2. Apoptotic fraction curves induced by different gamma radiation (4-32 Gy) doses at 24, 48, 72 hours incubation.

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Comparison of baseline and induced apoptosis in controls and breast cancer patients

The results are summarized in table 1. Three parameters were analyzed and compared here: background apoptosis, Ap_0; eight Gy gamma rays induced apoptosis, Ap_8; subtraction of background value of apoptosis from gamma rays induced apoptosis, Ap_8-Ap_0. Results revealed Ap_0 in the breast cancer patients was more than 1.5 fold compared to the normal one (table 1). This difference was statistically significant ($p<0.01$). After 8 Gy irradiation, Ap_8 increased clearly in both groups. There were statistically significant differences among Ap_0 and Ap_8 in all incubation times ($p<0.03$). To eliminate possible effects of age and other confounding factors on the results of DNA damage after exposure, the net induced apoptosis (Ap_8-Ap_0) was also evaluated and confirmed the initial results ($p<0.05$).

Results are shown in figure 3. The figure presents the range of assay parameters observed in the study subjects, as well as the medians and the 10th, 25th, 75th, and 90th percentiles.

The net induced apoptosis of patients exhibited significantly higher median (solid lines) and mean values (dotted lines) than control (figure 3). In addition, a very similar range of distribution was found for the results of the controls and of the patients and is marked by the boundaries of the boxes that represent the 25th and 75th percentiles. Patients with results lying within the 25-75% range of the healthy controls were considered to show a "normal" cellular reaction to gamma irradiation. In addition, the patients exhibiting less apoptosis in non-irradiated or irradiated cells than marked by the 25-75% range were also classified as normal. However, the data of some of the patients differed considerably from this normal range. All patients who exhibited results lying outside the 90 th percentile of the healthy individuals were considered to have abnormal experimental markers or to show a "highly sensitive" cellular reaction to gamma rays.

After 24, 48 and 72 hours incubation time, 2 (6.66%), 3 (10%), 2 (6.66%) of the 30 controls and 10 (33.3%), 14 (46.66%), 10 (33.3%) of 30 breast cancer patients respectively showed basal apoptosis (Ap_0) higher than the basal cut off point.

Induced apoptosis (Ap_8) in irradiated cells respectively yielded about 3 (10%), 3 (10%), 3 (10%) of the 30 controls and 12 (40%), 9 (30%), 12 (40%) of 30 patients showed values higher than the induced cut off point.

The net induced (Ap_8-Ap_0) apoptosis after 24, 48, 72 hours respectively in 4 (13.3%), 3 (10%), 4 (13.3%) of the 30 controls and 11 (36.66%), 10 (30%), 11(36.66%) of 30 patients showed values higher than the net induced cut off point.

Correlation between age and the baseline, induced and the net induced apoptosis

Mean age of normal individuals and patients were 52.86 and 57.06 years with a

Table 1. Mean values of baseline apoptosis, induced apoptosis, net induced apoptosis from patients and controls, processed 24, 48, 72 hours after in vitro exposure to 8 Gy gamma rays using neutral comet assay.

Sample	Time	Ap_0 Mean ± SD	*P*	Ap_8 Mean ± SD	*P*	Ap_8 - Ap0 Mean ± SD	*P*
Control 1	24h	0.013± 0.009	0.009	0.205±0.063	0.022	0.193±0.059	0.044
Breast Cancer		0.021 ±0.013		0.258±0.104		0.237±0.103	
Control 1	48h	0.0202 ±0.007	0.000	0.219±0.08	0.006	0.199±0.08	0.023
Breast Cancer		0.029 ±0.007		0.278±0.08		0.248±0.082	
Control 1	72h	0.023 ±0.008	0.000	0.261±0.089	0.006	0.237±0.087	0.048
Breast Cancer		0.034 ± 0.011		0.318±0.091		0.284±0.091	

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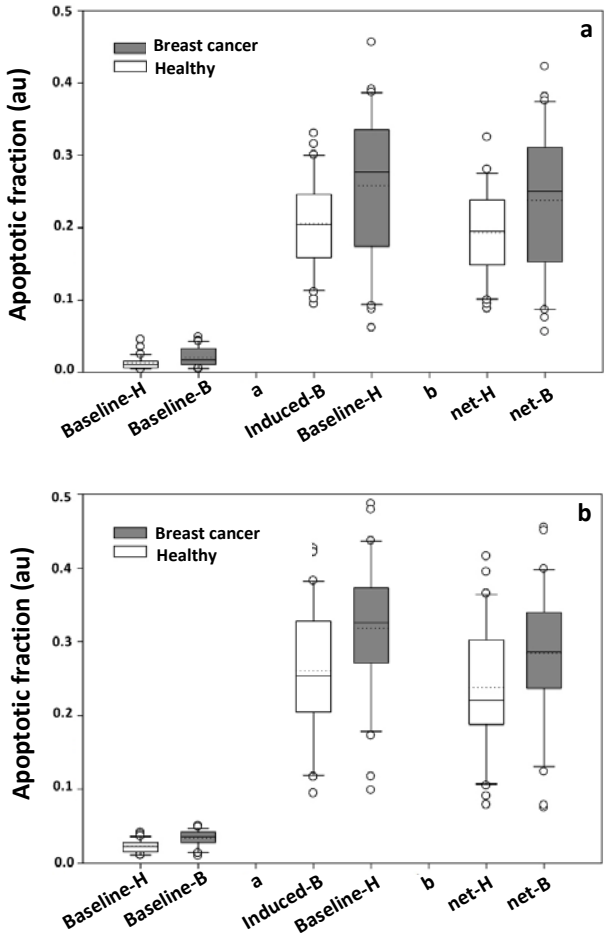
range of 25-89 and 26-81 years respectively. Based on our results the baseline, the induced and the net induced apoptosis were higher in young breast cancer patients. However, age did not have a significant effect on apoptosis values in either group. These results are summarized in table 2.

DISCUSSION

Breast cancer is one of the most common malignancies and the cause of death due to cancer in 40-45 years old women. Unfortunately the detection of breast cancers by mammography and physical touch in early stages of the disease is hardly possible. So most of the breast cancers show extensive spread prior to recognition [31]. Various studies on cancer predisposing syndromes using cytogenetic methods have shown that the chromosomal radiosensitivity in cancer cases is higher than the healthy ones. Hence chromosomal radiosensitivity could be an appropriate biomarker to determine the probability of malignancy [31-33]. Micronucleus assay, metaphase analysis and comet assay can assert higher radiosensitivity of breast cancer patients than healthy individuals [3, 14, 21, 34]. Performed investigations using alkaline comet assay have shown extensive differences in breast cancer patients. Some cases exhibited very high



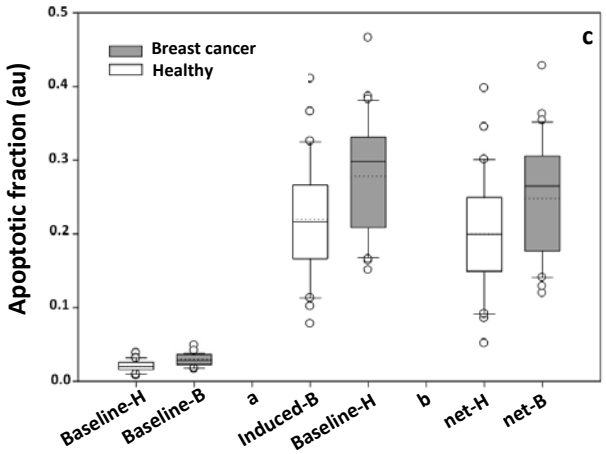


Figure 3. Box plots showing baseline (Ap_0), induced apoptosis (Ap_8) and net induced apoptosis (Ap_8-Ap_0) for controls (H) and breast cancer patients (P) measured by neutral comet assay after 8 Gy gamma rays irradiation at various time intervals: a) 24 hours; b) 48 hours; and c) 72 hours. The boxes extend from the 25th percentile to the 75th percentile, with a horizontal line at the median (50th) and a dotted line at the mean value. The whiskers present the 10th and the 90th of the data. In addition, all values that were located outside the borderlines marked by the whiskers are presented as points.

Table 2. results of correlation between apoptosis and groups age before and after 8 Gy gamma irradiation.

Sample	Number	Ap_0		Ap_8		Ap_8- Ap_0	
		r	p	r	p	r	p
Control	30	0.0004	0.8274	0.0009	0.4941	0.0008	0.4932
Breast Cancer	30	-0.0005	0.0873	-0.0035	0.1261	-0.0031	0.1816

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DNA damage and some revealed severely reduced DNA repair capacity after 15 and 30 minutes [35]. Other studies on breast cancer cases using of three methods such as cytokinesis-block micronucleus (CBMN) assay, 6-TG-resistant cells scored (TG) assay and comet assay have shown higher DNA-damage in patients after 3 Gy X-ray exposure than controls [36]. Because in some cases, the radiosensitivity of the same patient was different as detected by the different assays, it is suggested that multiple assays should be used to evaluate the patients radiosensitivity [36].

In the present study we investigated 30 breast cancer patients and 30 healthy volunteers as controls. The radiosensitivity of both groups was assessed by radiation induced-apoptosis at 24, 48, 72 hours after 8 Gy gamma irradiation under the neutral comet assay. Although there was a trend toward an increased basal and induced apoptosis level in the cells from young breast cancer patients, but our results failed to reach statistical significance which were also mentioned in some previous studies [19, 34]. However, the majority of previous studies assessing the role of age in the DNA damage and apoptosis of human sperm and mouse kidney cells confirms that, the DNA damage increases by increasing the age [34, 33, 37].

In our study, breast cancer patients showed more basal and induced apoptosis compared to the controls. Shahidi *et al.* have previously shown higher basal DNA damage in breast cancer patients than controls by using alkaline and neutral comet assay. In this study, the control group didn't reveal any residual DNA damage after 24 hours incubation following irradiation but cells from patients exhibited more than 20% residual DNA damage [21]. In contrast to this finding some investigators didn't confirm any significant difference in the radiation-induced DNA damage among cases and controls [38]. Studies on the effects of radiotherapy on the lymphocytes apoptosis revealed haplo insufficiency of either ATM or BRCA1 causing reduced apoptosis due to radiotherapy [39, 40]. Using flow cytometry to measure apoptosis in this study confirms that the level of apoptosis increases as the radiation dose increases but there was an inverse correlation between initial DNA damage and induced apoptosis [40]. Meaning low level of initial DNA damage can be related to a high radiation induced-apoptosis. The mechanism behind this is still unclear. The study done by Hernandez *et al.* on 26 consecutive breast cancer patients to analyze the initial DNA damage and radiation induced-apoptosis showed considerable difference in radiotherapy response. In contrast to our study, they showed individuals with high initial DNA damage have lower radiation induced-apoptosis [1, 3].

In summary the present study may indicate that the increased rate of apoptosis in leukocytes of breast cancer patients might be associated with deficient repair mechanisms in cellular DNA. Therefore, apoptosis might have potentials as a predictive assay; however, large interindividual variation exists. More studies are required to investigate the causes of interindividual variation and how it might be minimized.

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