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Quantitative evaluation of abscopal effect based on biological effective dose in breast cancer tumors in mice

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ABSTRACT

Background: Due to abscopal effect, cell damage may occur outside of the radiation field and the quantification of this effect is one of the most challenging debates in radiation therapy. The aim of this study was to estimate the abscopal effect induced in non-irradiated tumors quantitatively by means of biological effective dose (BED). ***Materials and Methods***: Breast tumors using 4T1 and MC4-L2 cells, were induced into the flank region of Balb/c mice. When palpable, the tumor on one side of the body was irradiated with dose of 28Gy in 14 fractions and 2 Gy per fraction, 5 fractions per week. The tumor on the other side of the body was shielded with a lead plate. BED was estimated based on tumor volume. H&E staining and TUNEL assay were performed to assess histological changes and apoptosis in irradiated and non-irradiated tumors. ***Results:*** The effect of radiation on non-irradiated tumors was more than that on irradiated ones. The BED was 4.49 and 6.74 in 4T1 and MC4-L2 tumors, respectively. The ratio of the tumor volume in the last fraction to that in the first fraction for irradiated 4T1 tumors was 2.32 and in non-irradiated was 1.50. This ratio in irradiated and non- irradiated MC4-L2 tumors was 2.64 and 1.98, respectively. The number of apoptotic cells was higher in non-irradiated tissues. ***Conclusion:*** Results indicate that the occurrence of abscopal effect is highly depends on the type of tumor. By means of the abscopal effect, more radiation dose can be delivered to the tumor and metastatic sites.

Keywords: *BED, abscopal effect, breast cancer, radiation therapy, fractionation.*

INTRODUCTION

There is a new dogma that has been declared long time ago stating cell damage can be occurred outside of the radiation field [(1)]. The clinical response to irradiation that causes tumor regression at remote sites, is commonly called abscopal effect. The term abscopal is also interchangeably used with distant bystander effect [(2)]. Abscopal effect, that has a Latin origin; the prefix 'ab' means 'position away from' and 'scopus' as a 'target for shooting at', has been defined as an effect at a distance from the irradiated volume but within the same organ [(2, 3)].

There are so many case reports confirming the abscopal effect [(4-6)]; For example, a 67-year-old man presented with pigmented lesions stage IIIC malignant melanoma without response to chemotherapy, was a candidate to receive radiation therapy in 3 fractions. Six weeks after radiotherapy, the primary lesions

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showed some variations but all non-irradiated lesions were disappeared eighth months after irradiation [7].

Generally, there are some suggestions and hypotheses describing the mechanism of the abscopal effect, for example releasing of cytokines, inflammatory factors or tumor necrosis factors (TNF) [8]. The mechanisms of action in the abscopal effect have remained unknown and sophisticated but some fundamental biological events can be hypothesized. Releasing of cytokine(s), inflammatory factors or TNF(s) by on-site tumors irradiation and existing the membrane receptors in similar remote tumor nodes can cause the abscopal effect [9-13]. Results of some studies have shown that radiation therapy can induce tumor cell death and produce inflammatory signals [14]. Outcomes of another study showed that P_{53} acts as a transcription factor to express cytokines, probably because of inflammation responses after radiotherapy. The author noticed that these factors possibly produced inside the irradiated organ locally and then released systemically producing a systemic antitumor effect on the remote tumors directly and indirectly [13] .

Different efforts has been made to elucidate mechanisms of abscopal effect; For example, different doses and consecutive fractionation radiation [13, 15-18]. Emphasizing to above, Mancuso *et al.* applied different whole-body single doses, including 1, 2, 3 or 10 Gy of X-ray irradiation in mice [19]. Other investigations were based on a combination treatment such as administrating drugs and irradiation [12, 15]. Formenti *et al*. combined different irradiation doses with CTLA-4 blockade, including 20 Gy in a single fraction, 8 Gy in 3 fractions, or 6 Gy in five 5 fractions, on consecutive days [17]. Camphausen *et al*. applied an intense radiotherapy schedule, 2 Gy twice a day for 6 days or 10 Gy every day for 5 days, to provoke an abscopal effect with radiation therapy alone. In this study, radiation was delivered to normal tissue instead of tumor site and the results shown that immune mechanisms were not involved in mediating the abscopal effect [13].

Radiation therapy regimens might be a crucial factor to induce the abscopal effect. In clinical radiation therapy, a dose of 2Gy per fraction for 5 fractions weekly is commonly used [20]. We decided to apply the real condition of radiotherapy regime to assess the abscopal effect in breast cancer which is a deadly disease among women. Nevertheless, so many studies have been performed on abscopal effect but there is a noticeable lack of quantitative assessment in these studies. The aims of this study were to induce abscopal effect and quantify it by measuring the biologically equivalent dose (BED) in irradiated and non-irradiated breast tumors.

MATERIALS AND METHODS

Cell lines

Triple-negative breast cancer adenocarcinoma (4T1) (ATCC Number: CRL-2539) and Estrogen receptor-positive (ER^+) breast ductal carcinoma (MC4-L2) cell lines were purchased from cell bank of Shahid Beheshti University of Medical Sciences, Tehran, Iran. These cells were cultured in RPMI-1640 (Gibco, Germany) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Sigma, U.S.A) with 1% glutamine-penicillin-streptomycin (Merck, Germany). The cells were kept in 37°C incubator with 5% CO_2 and harvested by trypsin- EDTA 0.25% (Merck, Germany).

Animals

Four to six week-old female Balb/c mice were purchased from the animal lab of Iranian Pasteur Institute. Mice were kept in cages in groups of 3-5 mice, and fed with animal standard mouse pellet and water with free access to food and water, and were subject to 12 -hour dark–light cycle in room temperature. All animal experiments were carried out according to the National Institutes of Health guide for the care and use of Laboratory animals [21].

Tumor measurement

The 4T1 and MC4-L2 cell lines (1×10^6 cells) were injected in flank region, and when tumor

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became palpable, the mice were randomized and irradiated in one side tumor while the other side of the body was shielded with a lead plate. Randomization was performed on the day of implantation to eliminate any potential observer bias. The total dose of 28 Gy was delivered in 14 fractions (14×2Gy), similar to the conventional radiation therapy courses (5 fractions per week). The size of tumors was measured every day after irradiation, by means of a digital caliper, with 1mm accuracy. The tumor volume (mm^3) was measured with the use of equation 1, defined as follows [22]:

$$Tumor\ volume = (w_1 \times w_2) \times (\frac{\pi}{6}) \quad \text{Equation (1)}$$

W_1 and W_2 are the largest and smallest tumor diameters (mm).

Tumor irradiation

Gamma irradiation was delivered by a ^{60}Co teletherapy machine (Theratron 760 C, AECL Canada), at Shohada hospital (Tehran, Iran) with a dose rate of 54 cGy / min and field size of 5×5 cm and source to surface distance (SSD) of 80 cm. Only one side of each mouse was irradiated (right side of treatment group) and all mice were anesthetized with isoflurane during the irradiation to prevent any movement. The mice were in four groups (5 mice per group) including 4T1 tumor-bearing, MC4-L2 tumor-bearing and the control groups of each tumor. The control groups received sham treatment with the immobilization and anesthetization of mice on the coach of the cobalt-60 machine without any exposure.

Calculation of irradiation dose

Tumor-bearing Mice were immobilized in the prone position and radiation was delivered to the targeted tumor-side. The reference point for the prescription dose was set at 0.5 cm depth (d_m) from the skin. The irradiated field was determined using a computer tomography-based simulation. The absorbed dose of the dorsal spine was below 10%. The BED was calculated by equation (2) for dose per faction d (and for a total dose and when n is fractions are given) and α/β ratio assumed for the tumor tissue [23]:

$$BED = nd(1 + d/[\frac{\alpha}{\beta}]) \quad \text{Equation (2)}$$

Hematoxylin and eosin (H&E) staining

Twenty four hours after the last radiation fraction, the mice were sacrificed by cervical dislocation. The tumor tissues were then isolated by incision immediately after necropsy and then fixed in a 10% formalin solution containing neutral phosphate-buffered saline and stored at 4 °C. The fixed tumor tissues were sectioned into 5 µM thin sections (three sections from each sample) and stained with Hematoxylin and Eosin (H&E) using a standard protocol for Irradiated and non-irradiated tumor tissues in both cell line originated tumors [24]. Briefly, sections were put in distilled water and stained nuclei with alum Hematoxylin and after rinsing in running tap water, differentiated with 0.3% acid alcohol. Subsequently, they were rinsed in Scott's tap water substitute. Finally, tissue sections were stained with eosin for 2 min and after Rinsing, they were cleared and mounted. After that, Samples were coded and the observations performed by a pathologist in a blind fashion to evade bias in the evaluation process. Sections with the same size were used for measurements and the frequency of detected pathologies was analyzed in SPSS 16 software.

TUNEL assay

Apoptotic cells in sections were detected by the TUNEL assay. Sections were probed using a Roche kit according to the manufacturer's instructions. Briefly, sections were dewaxed, dehydrated and permeabilized by 15 µg/ml proteinase-K for 20 minutes at 37˚C (Roche, Germany). Then, TUNEL reaction mixture was added to the sections and incubated for 1 hour at 37˚C. After several items of washing with PBS, the sections were incubated with Converter-POD for 30 minutes at 37˚C. DAB, as a chromogenic substrate of horseradish peroxidase (HRP), was applied to distinguish TUNEL positive cells. The counter staining was performed with hematoxylin and the cells were mounted with Entellan (Merck, Germany). The slides were observed through a light microscope (BX41, Olympus, Japan). The number of TUNEL positive cells was counted in six adjacent x100

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microscopic fields among the myocytes and the ratio of apoptotic cells to normal cells was obtained.

Statistical analysis

SPSS 16.0 software and One-way ANOVA test and repeated measures regression were used for data analysis ($p<0.005$ was considered as a significant value).

RESULTS

In both groups, the volume remissions of irradiated tumors were more than that in non-irradiated ones ($p=0.001$). All tumors showed volume changes in the initial fractions. The ratio of tumor volume in the last fraction to that in the first fraction in 4T1 originated tumor (group A) was 2.32 for irradiated tumors and 1.50 for non-irradiated tumors. In this group, BED elevated gradually and after a while reached a constant value but in group B, BED values were constant in both irradiated and non -irradiated tumors (figure 1).

In MC4-L2 originated tumors (group B), tumor volume ratios were 2.64 and 1.98 for irradiated and non-irradiated tumors, respectively. At the initiation of the abscopal effect, the mean tumor volume in group A for irradiated tumors was 1305.6 ± 303.00 mm^3, equivalent to the BED of 26.4 Gy, and for non-irradiated tumors was 1083 ± 125.50 mm^3.

The difference of tumor volume in these two sites was 222.2 mm^3, equivalent to the BED of 4.49 Gy. In group B, the irradiated tumor volume was 1951.2 ± 492.5 mm^3 and 1452.8 ± 438.5 mm^3 for non-irradiated ones at the BED of 26.4 Gy, with 498.4 mm^3 tumor volume difference, which was equivalent to the BED of 6.74 Gy (table 1).

Irradiated and non-irradiated tumor volumes versus radiation fractions in both cell lines tumors were shown in figures 2. The volume of non-irradiated tumors in group A, was more than that in irradiated ones until the 9^{th} fraction, while after that the volume of irradiated tumors became higher (figure 2A). It must be mentioned that the tumor volume in both irradiated and non-irradiated remained in a steady state from fraction 9 to 14.

In group B, the volume of irradiated tumors was more than that in non-irradiated ones in all fractions. The increase of tumor volume in non-irradiated groups was less than that in irradiated ones, as the final tumor volume (log) in non-irradiated tumors was 3.16 versus 3.29 in irradiated groups (figure 2B).

Histological assessments

Hematoxylin and Eosin Staining was applied to assess the histological changes in irradiated and non-irradiated tumor tissues in groups A and B. High polymorphism, moderate tissue distortion and severe cell proliferation were seen in group A tumors (fig. 3A); while in non-irradiated tumors, cell proliferation was moderate and many fiber strands, polymorphism and RBC extravasation were observed in this tumor tissues (figure 3B).

In group B, severe RBC extravasation was detected in irradiated tumors with few polymorphism, mitosis, and macrophages (figure 3C). In non-irradiated tumor tissues, extreme polymorphism, mitosis, and RBC extravasation were seen as well as severe tissue necrotic spots and also, the initial tissues were completely destroyed (figure 3D). The mammary secretion lobes were destroyed in all tumor tissues. Generally, the destruction effects were more severe in MC4-L2 originated tumors, especially in non-irradiated tumor tissues.

TUNEL assay

The results of TUNEL assay shown that the frequency of apoptotic cells in non- irradiated tumors was higher than that in irradiated tumors in both cell lines ($p<0.005$). The number of apoptotic cells in group A were more than that in another group (figure 4), as it was 49.66 in non-irradiated and 22.33 in irradiated 4T1 tumors and 55.66 and 26 in non-irradiated and irradiated MC4-L2 tumors, respectively (figure 5).

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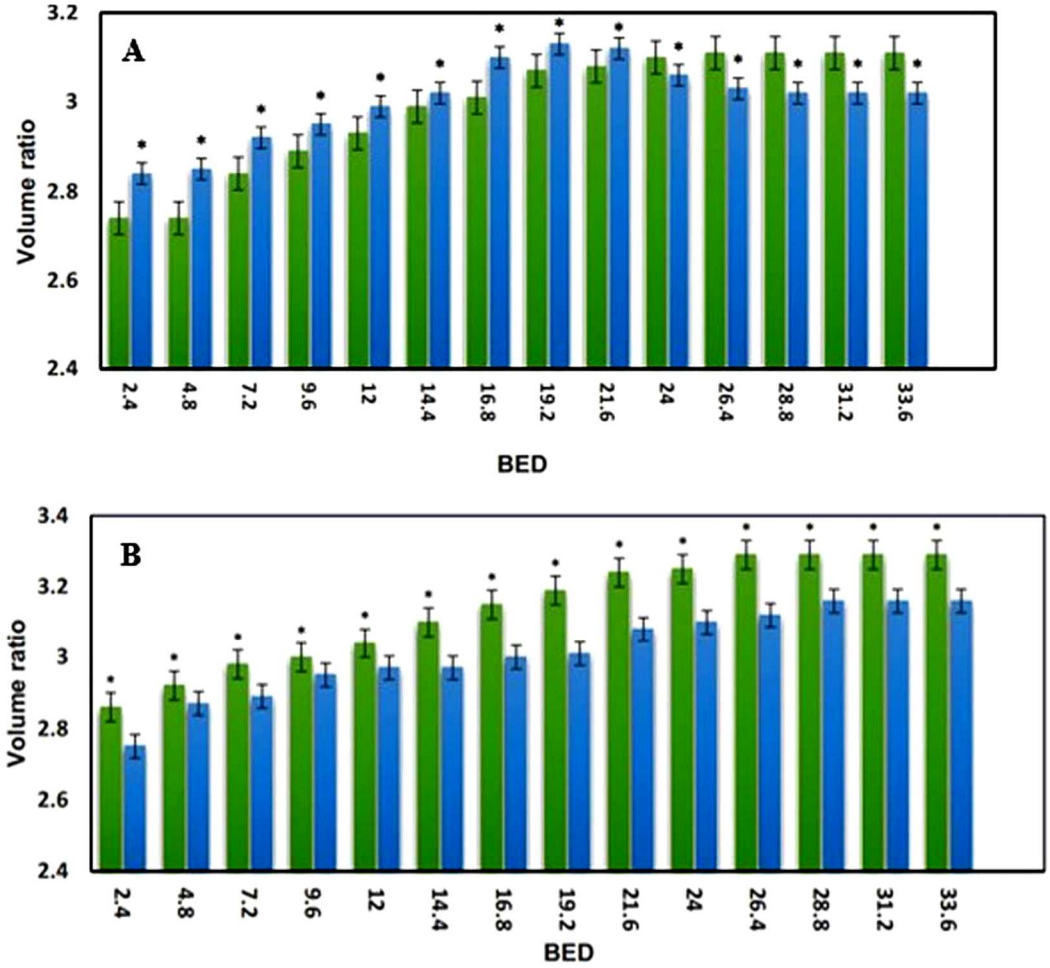

Figure 1. Ratio of irradiated and non-irradiated in A) 4T1, B) MC4-L2 tumor volumes is shown according to BED of each fraction ($p<0.005$*). All tumors showed volume changes in initial fractions.

Table 1. The BEDs in each fraction and logarithm of 4T1 and MC4-L2 tumor volume are listed in this table. The difference of tumor volume in these two sites was 222.2 mm3, equivalent to the BED of 4.49 Gy. In group B, the irradiated tumor volume was 1951.2 ± 492.5 mm3 and 1452.8 ± 438.5 mm3 for non-irradiated ones at BED of 26.4 Gy, with 498.4 mm3 tumor volume difference, which was equivalent to BED of 6.74 Gy

Fractions	1 st		2 nd	3rd	4th	5 th	6 th	7 th	8 th	9 th	10th	11 th	12 th	3 th	14 th
BED	**2.4**		**4.8**	**7.2**	**9.6**	**12**	**14.4**	**16.8**	**19.2**	**21.6**	**24**	**26.4**	**28.8**	**31.2**	**33.6**
Log of volumes 4T1 cells tumor	Irradiated	2.74	2.74	2.84	2.89	2.93	2.99	3.01	3.07	3.08	3.1	3.11	3.11	3.11	3.11
	Non irradi-ated	2.84	2.85	2.92	2.95	2.99	3.02	3.10	3.13	3.12	3.06	3.03	3.02	3.02	3.02
Log of volumes MC4-L2 cells tumor	irradiated	2.86	2.92	2.98	3.0	3.04	3.10	3.15	3.19	3.24	3.25	3.29	3.29	3.29	3.29
	Non-irradiated	2.75	2.87	2.89	2.95	2.97	2.97	3.00	3.01	3.08	3.10	3.12	3.16	3.16	3.16

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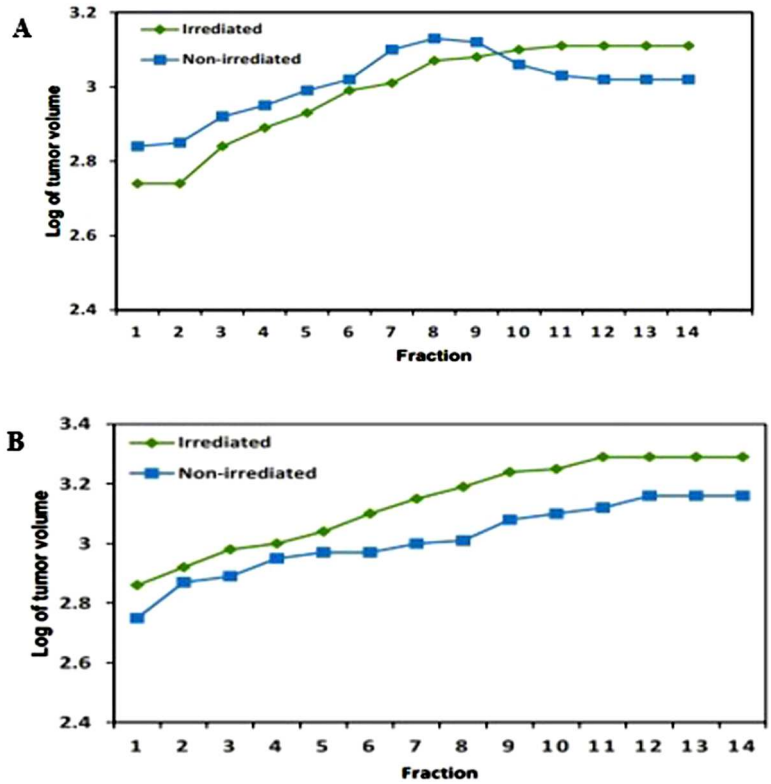

Figure 2. Log of irradiated and non-irradiated in A) 4T1, B) MC4-L2 tumor volume versus fractions are presented. The volume of non-irradiated tumors in group A, was more than that in irradiated ones until the 9th fraction, and after that the volume of irradiated tumors became higher, while in group B, volume of irradiated tumors was more than that in non-irradiated ones in all fractions.

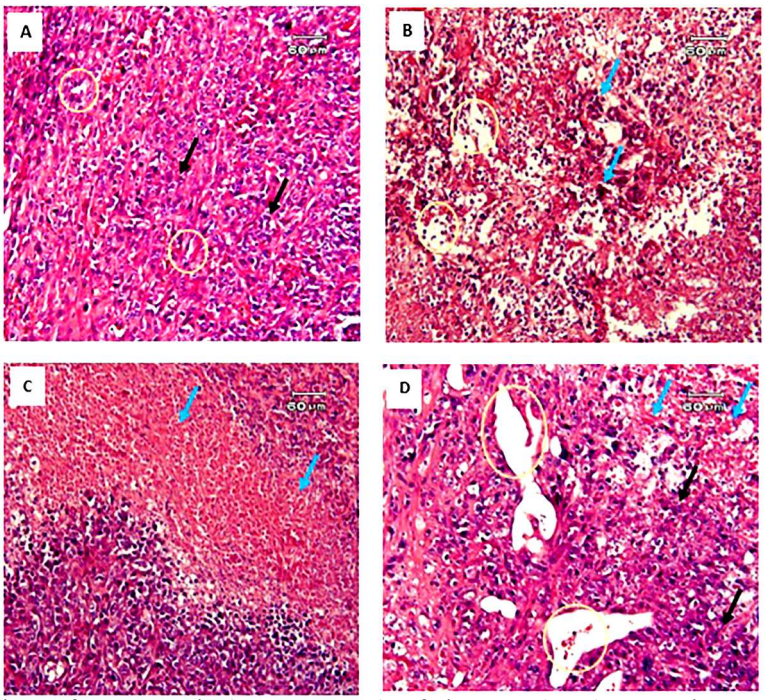

Figure 3. H&E staining (magnification x200), Photomicrographs of A) 4T1 irradiated tumor, B) 4T1 non-irradiated tumor, C) MC4-L2 irradiated tumor, D) MC4-L2 non-irradiated tumor are shown. High polymorphism, moderate tissue distortion and severe cell proliferation were seen in group A tumors, while in non-irradiated tumors, cell proliferation was moderate. The destruction effects were more severe in MC4-L2 originated tumors especially in non-irradiated tumor tissues. In this figure, RBC extravasation (blue arrow), polymorphism (yellow circle), mitosis (black arrow) are marked.

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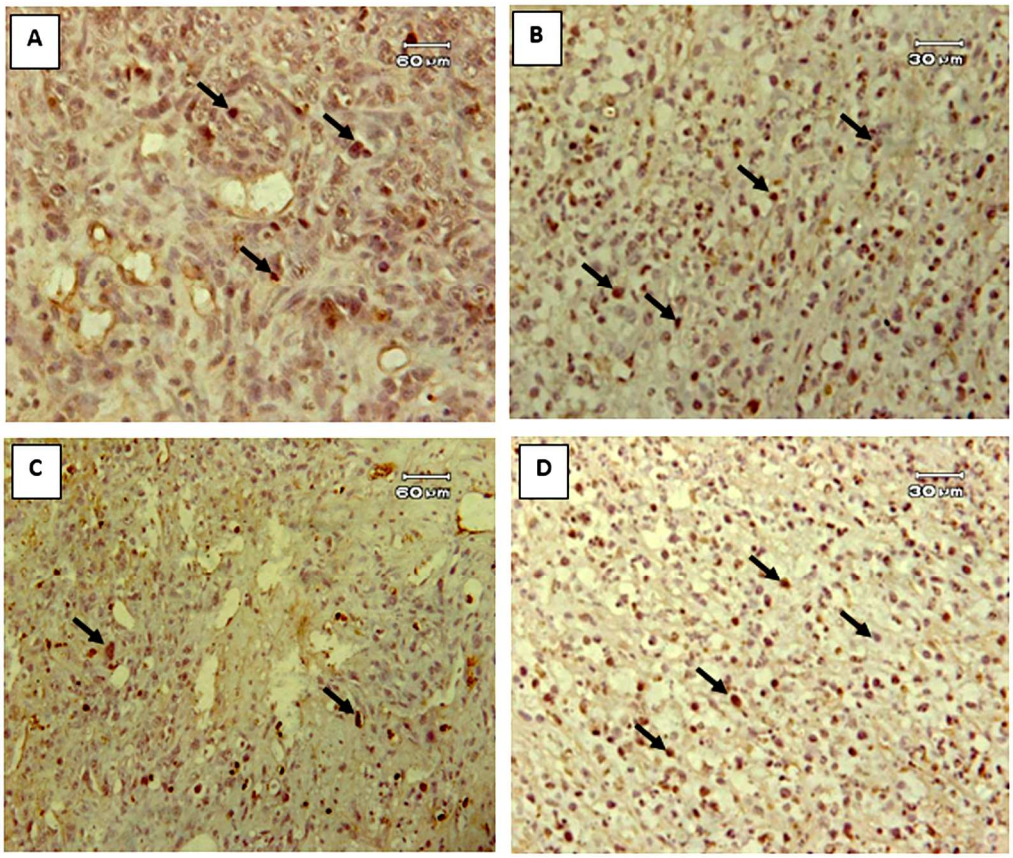

Figure 4. TUNEL staining shows the TUNEL positive cells which are shown by their deep brown color (magnification x200) in A: 4T1 irradiated tumor and B: 4T1 non-irradiated tumor, C: MC4-L2 irradiated tumor, D: MC4-L2 non-irradiated tumor. Black arrows show TUNEL positive cells.

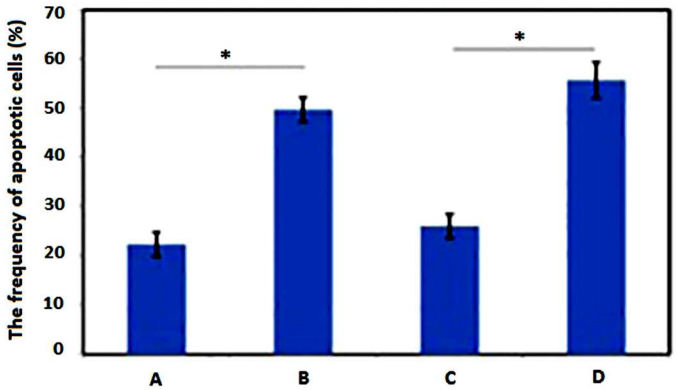

Figure 5. The frequency of apoptotic cells. A: 4T1 irradiated tumor, B: 4T1 non-irradiated tumor, C: MC4-L2 irradiated tumor and D: MC4-L2 non-irradiated tumor. The number of apoptotic cells in group A were more than that in group B (P<0.005*).

DISCUSSION

In this study, the abscopal effect was assessed quantitatively in breast cancer tumors by means of BED. According to the LQ model, Barendsen [25] has proposed one of the most important concepts in radiobiology, i.e. BED factor. The BED is applied to quantify the biological effects and even to compare between various clinical trials using different fractionation schemes. Indeed, this concept is the connective factor between the physical phase of absorbed dose

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and its biological influences on the tissues [26].

Our results showed a controlled tumor growth in non-irradiated tumor sites that was occurred after 10 or 11 fractions of irradiation, but results of Camphausen *et al*. [13] indicated a constant growth in tumor volumes during the irradiation. Abscopal effect was observed in non -irradiated tumors by means of the selected radiotherapy regime, as that is a valuable finding because the abscopal effect was missed in many prior studies such as study of Formenti *et al*. that their results shown all radiotherapy regimens caused a growth delay in the initial tumors but it had no impact on secondary tumors outside the radiation field. In their study, the abscopal effect was only induced in mice treated with the combination of 9H10 (monoclonal antibody against CTLA-4) and fractionated radiotherapy [17].

The immune-modulating impacts of radiation on the abscopal effect induction depend on radiation dose and signals generated by irradiated and non-irradiated cells [16]. It is possible that different cell line originated tumors have different responses to the abscopal effect occurrence; Based on our results, the abscopal effect detected in 4T1 tumors was equivalent to 4.49 Gy BED, while it was 6.74 Gy in MC4-L2 tumors.

The results of histological staining shown that the tissue in non-irradiated tumors was destroyed and the abscopal effect was clearly detected in both mice group A and B. The TUNEL assay results also revealed that the number of apoptotic cells in non-irradiated tumors was higher than that in irradiated ones.

The abscopal effect is not only beneficial to control the remote tumors but also helpful to reduce normal tissues toxicity, as this effect induces some BED without any radiation in non-irradiated tumors; so, it can be possible to deliver more radiation dose to these remote tumors without any radiotoxicity. There are two main theories explaining the mechanisms of abscopal effect. It is assumed that lymphocytes in irradiated volume during local radiotherapy induce the systematic antitumor effects [10]; the second hypothesis is that local radiation makes cytokines to release in circulation and to facilitate the systemic antitumor effect [13].

Demaria *et al*. studied the abscopal effect in Mice bearing a syngeneic mammary carcinoma, 67NR. They tested the hypothesis that the abscopal effect elicited by radiation is immune mediated, this was assessed by enhancing the number of available dendritic cells using the growth factor Flt3-Ligand (Flt3-L) [10]. The mice were treated with Flt3-L daily for 10 days after local radiation therapy at a single dose of 2 or 6 Gy and the second non-irradiated tumor was used as an indicator of the abscopal effect. The results of this study shown non-irradiated tumor was impaired by the combination of radiation therapy and Flt3-L. surprisingly, the outcomes indicated that the abscopal effect was tumor-specific and growth of a non-irradiated A20 lymphoma in the same mice containing a treated 67NR tumor was not affected; this is similar to our results which clarified the tumor type is a critical factor affected on abscopal effect.

In the latest study, a single fraction of radiation therapy was used which seems to not similar to the actual radiation regimens of radiation therapy. For detailed assessment, a fractionation radiation should be used in such studies. Dewan *et al*. applied several radiation therapy regimens to assess the effect of different fractionation on the abscopal effect [12]. TSA mouse breast carcinoma cells were injected into mice which were randomly assigned to eight groups receiving no radiotherapy or three distinct regimens of radiotherapy (20 Gy × 1, 8 Gy × 3, or 6 Gy × 5 fractions in consecutive days) in combination or not with 9H10 monoclonal antibody against CTLA-4 and tumor growth/ regression was followed in mice. The results showed that abscopal effect occurred only in mice treated with the combination of 9H10 and fractionated radiotherapy ($p<0.01$). Apparently, fractionated but not single-dose radiotherapy induced an abscopal effect in this study. In this regard, Postow *et al*. also reported a case of the abscopal effect in a patient with melanoma treated with ipilimumab and radiotherapy [27]. Actually, their hypothesis was that the abscopal effect may be mediated by activation of the immune system. The results of this study

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showed tumor shrinkage with antibody responses to cancer–testis antigen NY-ESO-1, changing in peripheral-blood immune cells, and elevating in antibody responses to other antigens after radiotherapy.

Indeed, the limitation of our study was to not assess the tumor inhibition molecular factors and also tumor suppress related genome. For a detailed assessment of mechanisms of abscopal effect in the non-irradiated tumor in both cancer cell line, there is a demand to investigate the factors such as P_{53}, inflammatory signals, Bcl-2/ Bax genome or other involved factors [2]. The other limitation was that hyperfractionation radiation therapy regimen was not used to compare the current outcomes which were based on common radiation therapy. Recently, there are many attentions focused on hyperfractionation radiotherapy which is believed an appropriate approach to treat many progressive cancers [28]. Undeniably, the assessment of abscopal effect and resulting BED in different radiotherapy regimen could clarify the best approach to apply that in this regard.

CONCLUSION

All in all, according to the BED values, non-irradiated tumors were demolished by irradiation of main tumors and more radiation fractions could be delivered via induction of the abscopal effect. Our results showed that local irradiation of one tumor could involve the response of another tumor at out of irradiated site in mice and the type of tumor (4T1 and MC4 -L2 cell line originated tumors) is a significant factor in inducing this effect.

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

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