

Effects of gamma radiation on adipose-derived mesenchymal stem cells of human breast tissue

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ABSTRACT

► Original article

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Background: During radiation therapy, stromal cells surrounding the tumor (e.g. mesenchymal stem cells) may affect the treatment outcomes. We aimed to investigate the effects of gamma radiation on the mRNA expression of cytokines, DNA damage and population doubling time (PDT) of adipose-derived mesenchymal stem cells (ASCs). **Material and methods:** ASCs were enzymatically extracted from breast tissue and exposed to different doses of 5, 10, and 30 Gy of gamma radiation. The capability of ASC proliferation, the level of DNA damage, and the expression of angiogenic and immunosuppressive factors were evaluated using PDT, comet assay, and qRT-PCR, respectively. **Results:** Gamma irradiation of ASCs with different doses of 5, 10, and 30 Gy significantly increased PDT and DNA damage in a dose-dependent manner ($P < 0.05$). A dose of 5 Gy caused a significant increase in SDF-1 whereas a 10 Gy dose significantly increased the expression of both SDF-1 and IL-10 ($P < 0.05$) in ASCs. **Conclusion:** Results indicate that different doses of gamma irradiation may induce angiogenesis and immunosuppression in ASCs via different cytokines which may affect the radiotherapy outcome.

Keywords: Radiation, stem cell, tumor microenvironment, angiogenesis, immunosuppression.

INTRODUCTION

Radiotherapy, which includes 4 to 5 weeks of daily radiation treatment courses, is a standard method for the treatment of most breast cancer patients ⁽¹⁾. Despite the significant role of radiotherapy in cancer treatment, some side-effects associated with breast cancer irradiation have been reported ^(2, 3). The first response to irradiation in the tumor micro environment is inflammation caused by oxidative stress affecting stem cells in the tumor microenvironment which are very sensitive to radiation ^(4, 5). Studies have shown

that cancer cells are capable of interacting with their surrounding stroma ⁽⁶⁾. Mesenchymal stem cell (MSC) recruited to the tumor microenvironment causes tumor progression and metastasis of tumor cells due to their immunomodulatory and angiogenic properties ^(7, 8). Indeed, MSCs are pluripotent and progenitor cells with the capability of unlimited division and differentiation to various cell types ⁽⁹⁾. MSCs induce angiogenesis through the release of proangiogenic factors such as VEGF ⁽¹⁰⁾. However, exposing MSCs to distinct doses of irradiation led to the emergence of anti-angiogenic properties but did not change the

immunomodulatory features of these cells ^(11,12). Irradiated adipose-derived mesenchymal stem showed decreased proliferation and senescence, however, they maintained their osteogenic but not adipogenic and chondrogenic differentiation ability ⁽¹³⁾. ASCs from breast tissue were assigned with an intermediate radiation sensitivity and high repair capacity of DNA through increased expression of cyclin-dependent kinase (CDK) inhibitor p21 ⁽¹⁴⁾.

On the other hand, the role of stem cells in tumor cell development and possible modification of radiation treatment has been reported ^(15,16). Due to the presence of MSCs in the vicinity of tumor cells and their important role in modulation of the tumor environment, the impacts they receive from different doses of radiation may affect their relationship with the tumor cells and the fate of the tumor. Accordingly, we aimed to investigate the effect of gamma radiation on the mRNA expression of angiogenic and immunosuppressive factors, DNA damages and on population doubling time of ASCs from breast tissue. The results may provide new insights into the design of cancer treatment.

MATERIALS AND METHODS

Sampling of adipose tissue

The present study was approved by the ethics committee of Shiraz University of Medical Sciences (Ethics Committee Code No.IR.SUMS.REC.1396.S.290). The samples were randomly collected from the women referred to the MRI and Frahmand far Hospitals for mammoplasty surgical services of hospitals in Shiraz, Iran. All donors provided written and signed informed consent to have the possibility of testing on their samples. Medical information of all participants will be kept private. Participants with a history of chemotherapy, radiotherapy, and medical conditions (e.g. autoimmunity) were not included in this study.

ASCs: isolation and culture

Mesenchymal stem cells (MSCs) were isolated from normal adipose tissue of the breast. In this

study, all participants were healthy women whose mean age $\pm$ SD was 38 ± 4 years. All donors were selected randomly and adipose tissue of breast was isolated during mammoplasty surgery and straightway transported to the Cancer and Stem Cell Laboratory, Institute for Cancer Research, SUMS and further processed for isolation of mesenchymal stem cells based on established protocol ⁽¹⁷⁾. In brief, the breast adipose tissue was washed twice with Phosphate Buffered Saline (PBS). Then, it was processed by briefly mincing and incubating with collagenase type 1 enzyme (Gibco, USA) in Hank's balanced salt solution buffer (Gibco, USA) at 37°C for 40 minutes. Afterward, the cell suspension was centrifuged at 1000 g for 5 min and the resulted cell pellet was cultured in DMEM medium supplemented with 1% penicillin/streptomycin and 10% fetal bovine serum (FBS, Gibco, USA). The cell culture medium was changed every three days and the third passage of the cells was used for the experiments. Before any experiment, ASCs were characterized for the expression of MSC specific markers (positive for CD44, CD105 and CD166 and negative for CD14, CD34 and CD45) using flow cytometry method (FACS Calibur, BD biosciences, USA). Briefly, ASCs were harvested using dissociation solution (Sigma, USA). Then, 5×10^6 ASCs were washed twice with PBS and stained with phycoerythrin (PE)-conjugated mouse anti-human CD44, CD105 and CD166 (BD Biosciences, USA) and fluorescein isothiocyanate (FITC)-conjugated mouse anti-human CD14, CD34 and CD45 (BD Biosciences, USA). Corresponding isotype antibodies were used as negative controls (BD Bioscience, USA).

Irradiation geometry

When the confluency of ASCs reached 80-90%, they were exposed to radiation with doses of 5, 10 or 30Gy of gamma radiation (dose rate of 0.28Gy/min) ⁽¹²⁾. Our control group was ASCs which was kept in the same condition without exposing to gamma irradiation. Dosimetry was performed with an ionization chamber (Dosemeter PTW Unidos 1001/PTW TM 313, RPD Inc) in the Radiotherapy and

Radiation Oncology Department of Namazi Hospital, SUMS. ASCs were irradiated with gamma rays emitted from a Theratron cobalt-60 therapy unit (MDS Nordion, Canada). We tried to have enough build-up and uniform irradiation of gamma rays on the cells. For this purpose, the exposure time was calculated for a distance of 80 cm and 35×35 cm² field of view and a depth of 4cm.

Comet assay

In this study, neutral comet assay was used for the detection of DNA double-strand breaks in ASCs after exposure to 5, 10 and 30Gy of gamma radiation. To fix the possible DNA breaks following irradiation, the samples were placed in an icebox and straightway returned to the incubator. The comet assay was performed as described by Wang et al. but with a slight modification ⁽¹⁸⁾. Briefly, irradiated cells were washed with PBS and centrifuged at 300g for 5 minutes. The supernatant was removed and the remaining cell suspension was combined with %1 low melting agarose (Type VIIA; Sigma-Aldrich, Poole, UK) at a concentration of 1×10⁶ cells/ml. Afterward, 100 µl of this suspension was loaded onto microscope slides coated by high-melting agarose (Sigma-Aldrich, Poole, UK) to form a microgel. To achieve reliable results, we prepared two slides for each sample. The comet slides were immersed in cold fresh lysis solution, 2.5 M NaCl, 1% N-sodium lauryl sarcosinate, 30 mM Na₂EDTA, 10 mM Tris, 1% Triton X-100, 10% DMSO, for 1.5 hours at 4 °C. After lysis, they were located in buffer for 20 minutes in a horizontal electrophoresis tank filled with cold fresh Tris-borate-EDTA buffer to release the tight double-helical structure of DNA for electrophoresis. Electrophoresis was performed for 30 minutes at room temperature (25 V, 300 mA) and the samples were neutralized with a neutralization solution for 5 minutes and fixed in ice-cold methanol. Finally, DNA was stained with propidium iodide (20µg/ml) and imaged with an Atomic Force Microscope (Nano wizardly-JPK, Germany). The comet tail regions of ASCs were measured using Comet Score software (about 50 cells in each slide).

Population Doubling Time (PDT)

In order to compare the growth rate of ASCs following exposure to gamma radiation emitted from the Co-60 source, the PDT was measured in the third passage ASCs irradiated with either 0, 5, 10 or 30Gy and then were harvested and 5×10³ cells were seeded in 96 multi-well plates. The cells were incubated again and counted 24, 48, 72 and 168 hours after irradiation by trypan blue staining. The experiment was repeated five times for each cell in each condition. Finally, the doubling time of ASCs was calculated using online doubling time calculator (<http://www.doubling-time.com/compute.php>).

RNA isolation and cDNA synthesis

Seventy two hours after gamma radiation ASCs cell culture supernatant was removed and the adhered ASCs were washed twice with PBS. Next, total RNA was extracted using 1ml of cold RNX-Plus (Cinnagene, Iran). To check the accuracy of the RNA samples, optical densities of isolated RNA were read at 260 and 280 nm using a NanoDrop spectrophotometer (Wilmington, DE, USA). In the next step, cDNA was produced from the extracted RNAs using the cDNA synthesis kit following the manufacturer's instruction (Fermentas, Canada).

Quantitative real-time PCR (qRT-PCR)

To assess the effect of gamma radiation on angiogenic factors of vascular endothelial growth factor (VEGF), stromal cell-derived factor-1 (SDF-1), immunosuppressive factors of human leukocyte antigen-G5 (HLA-G5), and interleukin 10 (IL-10) in ASCs, the qRT-PCR method was used. All qPCR reactions were measured at least two times using 2XSYBR Green Master Mix (Applied Biosystems, USA) based on the hot-start Jumpstart Taq DNA Polymerase enzyme (Sigma, USA). The amplification was done for 40 cycles (95°C 20 sec, 60 °C 20 sec, 72 °C 40 sec) and the qRT-PCR amplification products were verified by melting curve analysis. The β-actin gene was used as the housekeeping gene. To verify reaction efficiencies for each primer set, standard curves were prepared using data from serially diluted samples. Melting curve analyses were also

performed for each primer set. Prior to data analysis, the melting curve for each gene was examined, and by examining this curve, the specificity of the target gene and the lack of a primer dimer was verified. Each experiment was conducted twice independently, with each sample measured in duplicate. Finally, considering the expression of control samples (β -actin), the $\Delta\Delta C_t$ method was used to determine the fold change of mRNA compared to the control group. Table 1 shows the forward and reverse primers for β -actin, VEGF, SDF-1, IL-10, and HLA-G5. All the primers used in this study were designed by AllelID software (Oligo Perfect Designer, Invitrogen, USA).

Statistical analysis

Data were analyzed by SPSS software version

Table 1. Forward and reverse primers of β -actin, SDF-1, VEGF, IL-10 and HLA-G5 genes for real-time PCR amplification.

Primers	Sequence	Product length
β -actin forward	ACAGAGCCTCGCCTTTGCCG	190
β -actin reverse	CACCATCACGCCCTGGTGCC	190
SDF-1 forward	TGCCAGAGCCAACGTCAAG	73
SDF-1 reverse	CAGTTGGGTACAATCTGAA	73
VEGF forward	CCCACTGAGGAGTCCAACAT	186
VEGF reverse	TTTCTTGCGCTTTCGTTTTT	186
IL-10 forward	TGGTGAAACCCGTCTCTAC	96
IL-10 reverse	CTGGAGTACAGGGGAGTAT	96
HLA-G5 forward	CTGGTTGTCTTGCAGCTGTAG	80
HLA-G5 reverse	CCTTTTCAATCTGAGCTCTTCTT	80

21. For the normal distribution of data, parametric tests and for the non-uniform distribution of data, nonparametric statistical methods (Mann-Whitney and Wilcoxon) were used. $P < 0.05$ was considered significant.

RESULTS

Comet assay

We used the comet assay to assess double-strand breaks of DNA in ASCs following various doses of 5, 10 and 30 Gy irradiation. Accordingly, we found that the percentage of DNA in the tail of the non-irradiated ASCs was $9.53 \pm 2.1\%$. However, for ASCs receiving 5 Gy of gamma radiation, the percentage of DNA in the

tail was $25.41 \pm 2.15\%$, which was significant in comparison to the non-irradiated cells ($P = 0.003$). Also, for ASCs receiving 10 and 30 Gy of irradiation, the percentage of DNA in the tail was $31.66 \pm 1.48\%$ and $61.47 \pm 4.84\%$, respectively, which were significant in comparison to non-irradiated ASCs ($P = 0.0002$ and $P < 0.0001$, respectively) (figure 1).

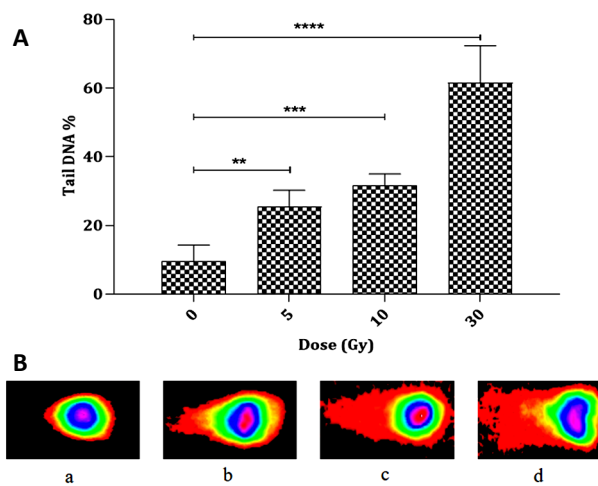


Figure 1. (A) The percentage of DNA in the tail of ASCs irradiated samples; the experiment was conducted twice for each sample. Error bars represent standard deviation of the mean and statistical significance was set at $P < 0.01$ (**), $P < 0.001$ (***) and $P < 0.0001$ (****). **(B)** DNA damage, detected with alkaline comet assay (refer to the Methods), after the exposure of ASCs to a) 0 Gy, b) 5 Gy, c) 10 Gy and d) 30 Gy of gamma radiation.

Effect of irradiation on PDT of ASCs

First, the proliferation pattern of ASCs at passage three was evaluated at different time points of 24, 48, 72 and 168 hours and the mean $\pm$ SD of the number of ASCs were 3220 ± 319 , 4870 ± 497 , 10440 ± 1050 and 20400 ± 1710 , respectively (figure 2).

Evaluating the PDT showed that non-irradiated ASCs had the fastest doubling time recorded as 63.11 ± 5.35 hours. In irradiated ASCs, a relationship between the radiation intensity and increase in doubling time has been observed. The PDT of ASCs was 91.06 ± 6.81 hours ($P = 0.022$), 138 ± 12.35 hours ($P < 0.001$) and 327 ± 31.44 hours ($P < 0.001$) after exposing to 5 Gy irradiation, 10 Gy irradiation, and 30 Gy of gamma radiation, respectively (figure 3).

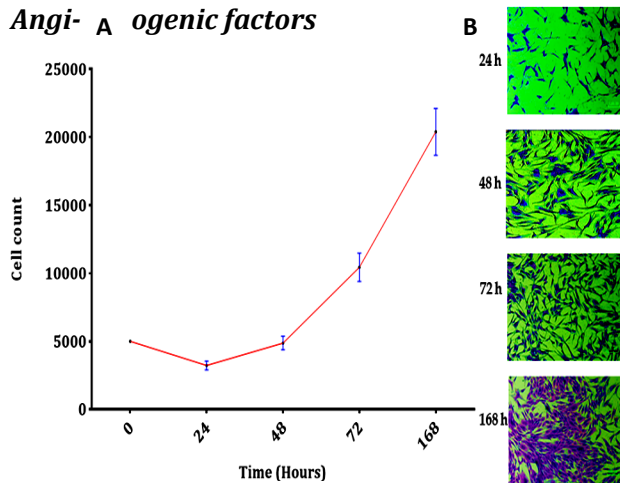
Angiogenic factors

Figure 1. The growth curve (A) and the morphologic patterns (B) of ASCs. The cells were counted 24, 48, 72 and 168 hours by trypan blue staining. Error bars represent standard deviation of the mean.

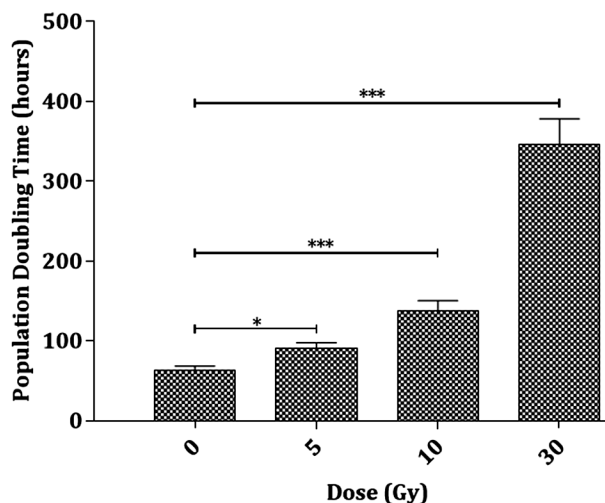
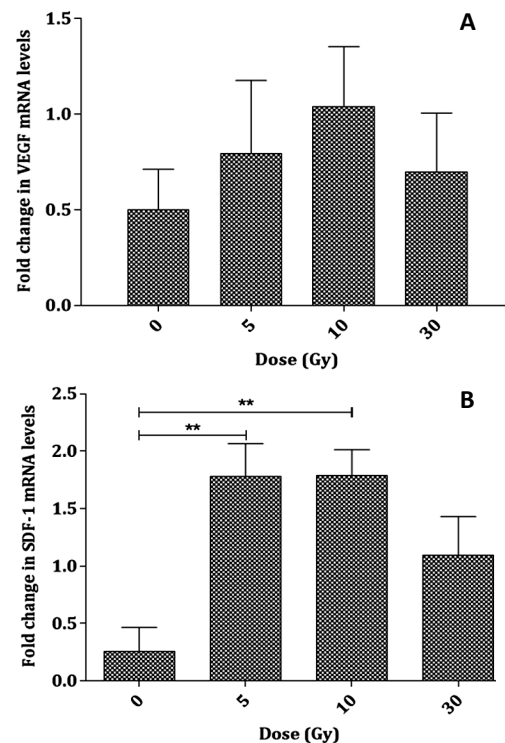


Figure 3. The effect of irradiation on the Population Doubling Time of ASCs. Error bars represent standard deviation of the mean and statistical significance was set at $P < 0.05$ (*) and $P < 0.001$ (***)).

To understand the effect of gamma radiation on the angiogenic capability of ASCs, the expression of VEGF and SDF-1 was investigated, 72 hours after irradiation. As shown in figure 4A, irradiation to ASCs did not statistically change the expression of VEGF in doses of 5Gy, 10Gy, and 30Gy compared to non-irradiated ASCs ($P > 0.05$). However, the irradiation seems to upregulate VEGF mRNA in all states.

Figure 4B shows that the irradiation increased the mRNA expression of SDF-1

in all groups. 5Gy and 10Gy gamma radiation significantly increased the expression of SDF-1 up to 6.84-fold ($P = 0.007$), and 6.85-fold ($P = 0.006$), respectively. This increase was not significant for 30Gy of irradiation ($P = 0.80$).



Im-

Figure 4. The fold change of mRNA level in A. VEGF and B. SDF-1 in ASCs exposed to 5, 10 and 30Gy irradiation compared to non-irradiated ASCs. Each experiment was conducted twice independently, with each sample measured in duplicate. Error bars indicate the standard deviation of the mean and statistical significance was set at $P < 0.01$ (**) regarding non-irradiated ASCs.

munosuppressive factors

In the next step, we examined the effects of 5, 10 and 30Gy of gamma radiation on IL-10 and HLA-G5 expression. According to figure 5A, the irradiation increased the mRNA expression of IL-10 in all groups, but the exposure of ASCs to 10Gy of gamma radiation significantly increased the expression of IL-10 4.8-fold ($P = 0.002$), regarding non-irradiated ASCs. This increase was not significant for 5Gy ($P = 0.08$) and 30Gy radiation ($P = 0.28$).

As shown in figure 5B, irradiation to ASCs did not statistically change the expression of HLA-G5 after exposing to 5Gy, 10Gy and 30Gy

compared to non-irradiated ASCs ($P>0.05$).

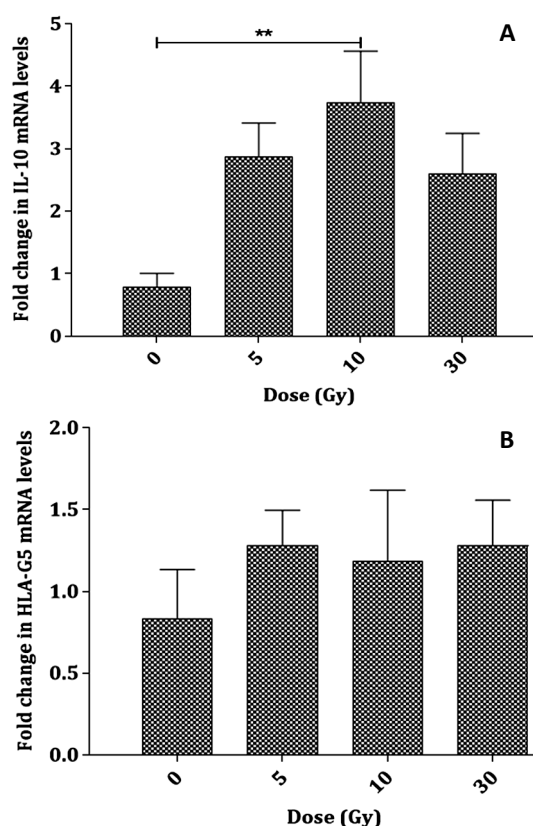


Figure 5. The fold change of mRNA level in **A:** IL-10 and **B:** HLA-G5 in the irradiated ASCs. Each experiment was conducted twice independently, with each sample measured in duplicate. Error bars indicate the standard deviation of the mean and the statistical significance was set at $P<0.01$ (**).

DISCUSSION

The present study was conducted to investigate the effect of gamma radiation on proliferation, DNA breaks, and expression of angiogenic and receive factors in ASCs from normal participants. Results showed that gamma radiation can increase DNA breaks and PDT in ASCs. Also, the irradiation of ASCs caused an increase in the expression of IL-10 and SDF-1.

We used the comet assay to investigate single-strand breaks in ASCs following exposure to various doses of gamma radiation. Venkatesh et al. reported that undifferentiated human embryonic stem cells have high sensitivity to ionizing radiation and the percentage of DNA breaks following gamma radiation in these cells

was higher than the differentiated cells ⁽¹⁹⁾. In contrast, Wu *et al.* reported that MSCs have less radiosensitivity to gamma radiation in the early stages of growth, due to their stronger repair systems ⁽²⁰⁾. Our results showed that by increasing the radiation doses from 5 to 30Gy, compared to the non-irradiated cells, the percentage of DNA breaks in ASCs increased. Since MSCs in the tumor microenvironment might positively effective in the growth and development of tumors, the possibility of controlling tumors by eliminating these cells using an appropriate dose of irradiation and inducing DNA breaks is not far away from the mind.

Also, we recorded the longest PDT in ASCs after 30Gy irradiation. Generally, our findings showed that when ASCs were exposed to 5, 10 and 30Gy of gamma radiation, PDT of the cells increased in a dose-dependent manner. In line with our results, Cmielova *et al.* reported a dose-dependent increase in PDT of bone marrow MSCs after gamma radiation, but no significant effect on cell viability following gamma radiation was observed. They showed that 20Gy irradiation reduces proliferation capacity in bone marrow MSCs ⁽²¹⁾. Also, some studies have revealed a correlation between prolonged PDT and a relative increase in radioresistance ^(22, 23). Accordingly, increased PDT in irradiated cells may provide more time to cope with irradiation-induced DNA damage. Hence, it seems that increasing the dose of irradiation is likely to increase DNA damage in the irradiated ASCs, activates repair mechanisms and finally increases PDT in irradiated cells.

Several investigations demonstrated that irradiation can increase the angiogenic capability of irradiated cells by up-regulating the expression of different cytokines, chemokines, and growth factors ^(24, 25). Our observation indicates that after exposure to 5 and 10Gy gamma radiation, SDF-1 was significantly upregulated in ASCs. In support of our work, Ponomaryov *et al.* reported an increased expression of SDF-1 in BM-derived osteoblasts after irradiation which can improve retention, homing, and repopulation of the cells damaged

by irradiation ⁽²⁶⁾. Moreover, Bastianutto *et al.* reported that 5Gy of gamma radiation increased the expression of SDF-1 that leads to the recruitment of hematopoietic stem cells to the radiated bone marrow of mice ⁽²⁷⁾. The irradiation reduces the vascularity leading to the expression of Hypoxic Inducible Factor-1 (HIF-1), activation of the SDF-1/CXCR4 pathway and finally repair the damage⁽²⁸⁾. Moreover, it has been shown that the expression of SDF-1 leads to an increase in the angiogenic ability of stem cells ⁽²⁹⁾. Based on the present study, we concluded that the exposure to low doses of gamma radiation (5 and 10Gy in our study) are more effective than a high dose of irradiation on the production of SDF-1 in ASCs. Since expression of SDF-1 by MSCs may eventuate the tumor development, SDF-1 inhibitors following tumors irradiation may guide the improvement of clinical strategies to treat cancer cells and prevent the tumor recurrences after irradiation.

It has been demonstrated that after irradiation stem cells begin to secrete cytokines such as IL-10 for the repair of the damage through suppressing the immune system and preventing inflammation ⁽³⁰⁾. In our study, we observed that irradiation of 10Gy to ASCs resulted in a significant increase in the expression of IL-10 in comparison to non-irradiated cells. Kang *et al.* reported that 9Gy irradiation increases the expression of IL-10 in hematopoietic stem cells of the mice; Suggesting that the secretion of IL-10 by hematopoietic stem cells may act as a contributory mechanism augmenting the self-renewal capacity of irradiated damaged cells ⁽³¹⁾. Thus, in comparison to our results, it seems that exposure to various doses of gamma radiation has different effects on the expression of IL-10 in different types of stem cells.

Based on the present study, with an increase in irradiation dose up to 10Gy, compensatory mechanisms (e.g. IL-10, and SDF-1) appear to be introduced to repair the damage while both may contribute to the development and growth of cancer. Obviously, assessment of two genes may not be enough to come to this conclusion and for better elucidation more studies on a broad range of molecules are undoubtedly required.

On the other hand, increasing the radiation dose to 30Gy augmented the DNA break and PDT while causing milder effects on the production of SDF-1 and IL-10. Therefore, during radiotherapy, the response of the stromal cells present in the tumor microenvironment may affect the treatment outcomes. Accordingly, it would be possible to obtain better results by choosing an optimal therapeutic dose of radiation affecting both tumor cells and stromal cells in the tumor microenvironment to treat tumors with radiotherapy.

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Conflicts of interest: Declared none.

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