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PRECLINICAL STUDY

# Bystander effects induced by electron beam-irradiated MCF-7 cells: a potential mechanism of therapy resistance

**Maryam Feghhi[1] · Jafar Rezaie[2] · Kamal Mostafanezhad[3] · Nasrollah Jabbari[4]**

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## Abstract

**Purpose** The distinct direct and non-targeting effects of electron beam radiation on MCF-7 cells remain obscure. We aimed to investigate the effect of electron beam irradiation (EBI) and conditioned media (CM) of the irradiated MCF-7 cells on MCF-7 cells. The cytotoxic effects of CM from irradiated MCF-7 cells on the mesenchymal stem cells and human umbilical vein endothelial cells (HUVECs) were also examined.

**Methods** Cell viability and apoptosis were assayed via MTT and flow cytometry analysis, respectively. The production of ROS (reactive oxygen species) was evaluated by the chemical fluorometric method, while the amount of extracellular vesicles was detected via acetylcholinesterase activity assay. Expression of genes involved in apoptosis, including caspase-3, -8, -9, and stemness such as Sox-2 and Oct-4, were calculated through *q*PCR. The wound healing rate of cells was monitored via in vitro scratch assay.

**Results** Compared to the control group, EBI groups showed decreased cell viability but increased apoptosis and ROS as well as acetylcholinesterase activity dose-dependently ($P < 0.05$). Concurrently with increasing the dose of the electron beam, the transcript levels of apoptotic genes (caspase-3, -8, -9) and stemness-related genes (Sox-2 and Oct-4) were up-regulated following EBI. The wound healing rate of irradiated MCF-7 cells increased dose-dependently ($P < 0.05$). Similar results were observed after treatment with CM from irradiated MCF-7 cells. Additionally, CM from irradiated MCF-7 cells decreased the viability of MCF-7 cells, mesenchymal stem cells, and HUVECs ($P < 0.05$).

**Conclusion** MCF-7 cells treated with an electron beam and CMs from irradiated MCF-7 cells exhibit an up-regulation in both genes involved in the apoptosis pathway and stemness. As a result, EBI can affect apoptosis and stemness in MCF-7 cells in direct and bystander manners. However, specific signaling pathways require careful evaluation to provide an understanding of the mechanisms involved in the EBI-induced alternation in tumor cell dynamics.

**Keywords** Electron beam · Breast cancer · MCF-7 · Bystander effects · Conditioned media

## Abbreviations

| | |
|---|---|
| AChE | Acetylcholinesterase |
| CM | Conditioned media |
| DCFH-DA | Dichlorofluoresceindiacetate |
| DMEM | Dulbecco's Modified Eagle Medium |
| DMSO | Dimethyl sulfoxide |
| EBI | Electron beam irradiation |
| EVs | Extracellular vesicles |
| FBS | Fetal bovine serum |
| HUVECs | Human umbilical vein endothelial cells |
| IOERT | Intraoperative electron radiotherapy |
| IORT | Intraoperative radiation therapy |
| IR | Ionizing radiation |
| MSCs | Mesenchymal stem cells |
| MU | Monitor unit |
| NTEs | Non-targeting effects |
| OD | Optical density |
| PBS | Phosphate-buffered saline |
| ROS | Reactive oxygen species |

✉ Nasrollah Jabbari
njabbarimp@gmail.com

[1] Department of Medical Physics, Urmia University of Medical Sciences, Urmia, Iran

[2] Solid Tumor Research Center, Cellular and Molecular Medicine Institute, Urmia University of Medical Sciences, Urmia, Iran

[3] Omid Research and Treatment Center, Urmia, Iran

[4] Department of Medical Physics and Imaging, Solid Tumor Research Center, Cellular and Molecular Medicine Institute, Urmia University of Medical Sciences, Urmia, Iran

## Introduction

Breast malignancy originates from the breast tissue and has caused great concern due to its high rate of incidence and mortality worldwide [1]. This cancer remains challenging due to resistance to different treatments [2]. Traditionally, different methods such as surgery, chemotherapy, hormonal therapy, and radiation therapy have been exploited by physicians to eliminate tumor cells [3, 4]. A well-known treatment for breast cancer is radiation therapy in which ionizing radiation (IR) directly destroys cancer cells [5]. Electron beam therapy is a kind of radiation treatment whereby electrons, instead of photons, are directed to a tumor site. Intra-operative radiation therapy (IORT) is a more effective method in reducing local recurrence than the standard external beam radiotherapy of the whole breast. This method is a direct irradiation of the operated area during surgery which delivers an increasing dose of radiation on the operated area [6]. It is an alternative to six weeks of external beam radiotherapy, which is equal to the deliverance of electron beam, called intra-operative electron radiation therapy (IOERT) or 50-kV X-ray to tumor region during the breast-conserving surgery [7, 8].

IOERT has been considered to improve the treatment of breast cancer and prevent its local recurrences after surgery in breast-conserving therapy [8]. It is well established that the electron beam damages the biomolecules and organelles of tumor cells, contributing to the elimination of tumor cells [9]. Growing evidence has demonstrated that cells dwelled in the tumor mass could be indirectly affected by IR, which was previously known as the non-targeting effects (NTEs) of radiation [10]. Bystander effects is a class of NTEs in which directly irradiated cells influence non-irradiated cells in an irradiated volume [11]. Irradiated cells mediate the bystander effects of IR by releasing various soluble factors, including cytokines, reactive oxygen species (ROS), and even exosomes into the extracellular matrix that eventually affect the fate and function of neighboring non-irradiated cells known as bystander cells [12]. In this regard, signals received from directly irradiated cells may induce mutations, DNA damage, apoptosis, chromosomal instability, migration, and resistance in non-irradiated cells [11, 13]. In recent years, most studies have only assessed different responses of tumor cells to IR and to signals from directly irradiated cells [12, 13].

Electron beam therapy is especially useful for the treatment of superficial tumors. However, the exact biological and molecular effects of conventional electron beam therapy and IOERT on tumor cells and its environment are not reported exactly up to now [7]. In line with these explanations, the underlying mechanisms involved in tumor cells' responses to direct and bystander effects of electron beam exposure need to be evaluated. Here, we aimed to highlight the direct and bystander effects of electron beam treatment on the MCF-7 human breast cancer cell line.

## Methods

### Cell culture

Human breast cancer MCF-7 cells (Royan, Iran), mesenchymal stem cells (MSCs) (Pastor, Iran), and human umbilical vein endothelial cells (HUVECs) (Pastor, Iran) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) enriched with 10% fetal bovine serum (FBS, Gibco) and 1% penicillin–streptomycin (Gibco) and were kept at 37 °C in a humid atmosphere of 5% $CO_2$.

### Electron beam Irradiation

Following a dosimetry calibration, a clinical 5-MeV electron beam of a Siemens PRIMUS medical linear accelerator (Siemens AG, Germany) was used for irradiation. The output of the machine is calibrated such that the 1.0 monitor unit (MU) is equal to 1.0 cGy. MCF-7 cells were seeded in 96- and 6-well plates 24 h prior to irradiation to measure cell viability using MTT assay and other tests, respectively. The medium level was adjusted to 11 mm over the cell monolayer to place the MCF-7 cells at the depth of maximum dose ($d_{max}$). A standard electron applicator ($25 \times 25$ $cm^2$) was placed on the accelerator head for irradiation. The cells in the plates were positioned at a 100-cm distance from the electron source, and irradiation was performed at an output rate of 300 MU/min. The cell culture plates were placed at the center of the radiation field to ensure that all the cells were irradiated with a uniform radiation dose. MCF-7 cells were divided into five groups and exposed to 2, 4, 6, 8, 10 Gy of the electron beam. The non-irradiated cells were kept as the control group and subjected to the same condition as that experienced by the electron beam-irradiated cells. The MU values were calculated by taking into account that 1 cGy = 1 MU. After irradiation, the cells were returned to the incubator and maintained under culture condition for 48 h post-irradiation, prior to evaluation. All the biological experiments were repeated in three different series.

### Conditioned medium preparation

For this purpose, 48 h after irradiation, the conditioned media (CMs) of all groups were collected and centrifuged (15,000*g* for 20 min at 4 °C), and 0.2-μm syringe filters were utilized to remove the remaining cells and debris. The

filtrated cells' CMs were saved in the freezer at − 80 °C for the next experiments.

### CM treatment

To investigate the bystander effects of irradiated MCF-7 cells, the non-irradiated MCF-7 cells, HUVECs, and MSCs were co-cultured with CMs of irradiated and non-irradiated MCF-7 cells (control CM) for 48 h and kept as bystander cells. According to the irradiation protocol, bystander cells received the CMs of relevant groups and were named control-CM, 2 Gy-CM, 4 Gy-CM, 6 Gy-CM, 8 Gy-CM, and 10 Gy-CM. Additionally, one group was kept as the control group which was cultured in the DMEM/FBS medium in the same condition.

### Cell viability

Cell viability was determined 48 h after irradiation using the MTT assay. Prior to irradiation and CM treatment, $5 \times 10^3$ cells per well were seeded in the wells of 96-well plates for 24 h. Next, the MTT reagent was dissolved at a concentration of 5 mg/ml in sterile phosphate-buffered saline (PBS) in the dark. Forty-eight hours after irradiation, the cell media were removed completely and replaced with 100 µl/well of the MTT solution and then incubated for 4 h. After 4 h, the wells were depleted, and 100 µl of dimethyl sulfoxide (DMSO) was added to each well for 20 min at room temperature. Absorbance was measured using a microplate reader at a wavelength of 540 nm. The analysis was accomplished by the following formula: (optical density (OD) sample/OD control) × 100.

### Flow cytometry analysis of apoptosis

We used the annexin-V (eBioscience) marker to monitor the apoptosis rate in MCF-7 cells. For this purpose, 48 h after irradiation and incubation with CMs, the cells were subjected to the annexin-V kit protocol according to the manufacturer's recommendations. In brief, the cells were fixed with 100 µl of binding buffer at room temperature for 15 min. Next, annexin-V (1 µl/ml) was added to each sample at room temperature over 15 min in a dark room. Following twice washing with PBS, the cells were suspended in a 1-ml final volume of the binding buffer. The analysis was performed by a flow cytometric system (BioRad, Hercules, CA, USA) and FlowJo (version 7.6.1) software.

### ROS production

The chemical fluorometric method was adopted for the detection of ROS production using an ROS assay kit (E-BC-K138, Elabscience). According to the kit's protocol, 1 h after irradiation, 10 µM of dichlorofluorescein diacetate (DCFH-DA) reagent was added to the cell culture medium and incubated at 37 °C over 30 min. Then, cell suspensions were centrifuged at 1000*g* for 5 min and washed with PBS twice. Finally, fluorescence was measured using a microplate reader (Bio-Rad) at a wavelength of 485 nm excitation and 525 nm emission.

### Acetylcholinesterase activity assay

Acetylcholinesterase (AChE) activity was applied to evaluate extracellular vesicles in CMs of MCF-7 cells via a cholinesterase kit (Cat No. BXC080; Iran). In brief, solution A (potassium hexacyanoferrate and pyrophosphate) was mixed with CMs and incubated for 5 min at room temperature. After that, solution B (2-butyrylthio-n,n,n-trimethylethanaminium iodide) was added and the absorbance was detected at 405 nm by three time points using a plate reader system (BioTek). AChE activity was analyzed by the recommended formula: Activity (U/l) = 65,800 × ΔAbs/min.

### Real-time PCR analysis

To analyze the expressions of apoptosis genes, including caspase-3, 8, and 9, and Sox-2 and Oct-4 genes dealing with stemness, real-time PCR analysis was performed. Total RNA was isolated using an RNA extraction kit (Cat. No: FABRK001, Iran). Next, RNA quality and quantity were evaluated using a Nanodrop system (BioTek). cDNA synthesis was accomplished by a commercial cDNA synthesis kit (Cat: YT4500, Iran). Next, the mRNA levels of genes were measured using SYBER Green dye-based PCR Master Mix (YT2551, Yekta Tajhiz Azma, Iran) and real-time PCR system (ABI 7500, Applied Biosystems). Data were normalized against GAPDH gene and analyzed using the $2^{-\Delta\Delta Ct}$ method. Table 1 lists the sequences of primers.

**Table 1** List of primer sequences used

| Genes | Sequences | Tm (°C) |
|---|---|---|
| Sox2 | F:AACATGATGGAGACGGAGCTGA | 63 |
| | R: GTCCGGGCTGTTTTTCTGGTTG | |
| Oct4 | F: CAAGAACATGTGTAAGCTGCGG | 63 |
| | R:TGGTTCGCTTTCTCTTTCGGG | |
| Caspase3 | F: AAACACTAGAAAGGAGGAGATGG | 63 |
| | R:CATGTCATCATCCAGTTTGCATT | |
| Caspase8 | F: AAACACTAGAAAGGAGGAGATGG | 60 |
| | R:CATGTCATCATCCAGTTTGCATT | |
| Caspase9 | F:GACGCCATATCTAGTTTGCCC | 60 |
| | R: CACTGCTCAAAGATGTCGTCC | |
| GAPDH | F:TTGACCTCAACTACATGGTTTACA | 59 |
| | R:GCTCCTGGAAGATGGTGATG | |

### Wound healing assay

The MCF-7 cells were cultured in a 12-well plate until they reached 80% confluence. The cell monolayer was scratched using a 200-µl tip. Next, the supernatants were replaced by CMs for 48 h. The cell-free scratched area was monitored at 0 h and 48 h of incubation and measured using the ImageJ software ver. 1.44p. The wound healing rate was reported by applying a formula: [(first surface area − second surface area)/first surface area] × 100.

### Statistical analysis

Statistical analyses were accomplished using SPSS software version 25, and the results are presented as mean ± SD. Shapiro–Wilk's and QQ-plot methods were used to test the normality of the data. The statistical significances between groups were calculated using the one-way analysis of variance (ANOVA) with Tukey's honest significance test. The selection of mean ± SD for data presentation and the statistical analysis test was based on previous studies. In all the analyses, $P < 0.05$ was considered statistically significant. In graphs, brackets represent significance between groups as $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$, $*****P < 0.00001$.

## Results

### Cell survival was decreased by electron beam irradiation

To investigate the possible effects of electron beam irradiation (EBI) on MCF-7 cells' viability, the MTT assay was performed 48 h post-irradiation. The MTT assay showed irradiation significantly decreased cell viability compared to the control group in a dose-dependent manner ($P_{\text{Control vs. 4 Gy group}} < 0.05$; $P_{\text{Control vs. 6 Gy and 8 Gy groups}} < 0.001$; $P_{\text{Control vs. 10 Gy group}} < 0.0001$; Fig. 1A). The same results were obtained when the 2 Gy group was compared to 6, 8, and 10 Gy groups ($P_{\text{2 Gy group vs. 6 Gy and 8 Gy groups}} < 0.01$; $P_{\text{2 Gy group vs. 10 Gy group}} < 0.0001$). Our finding indicated that EBI dose-dependently reduced the viability of MCF-7 cells at the endpoint of 48 h post-radiation.

### Electron beam irradiation enhanced the apoptosis rate of MCF-7 cells

Annexin-V marker was used to monitor the apoptosis rate of MCF-7 cells. The data showed a significant increase in the apoptosis rate of MCF-7 cells subjected to the electron beam in a dose-dependent manner ($P_{\text{Control vs. 4 Gy group}} < 0.05$; $P_{\text{Control vs. 6 Gy group}} < 0.01$; $P_{\text{Control vs. 8 Gy group}} < 0.001$; $P_{\text{Control vs. 10 Gy group}} < 0.00001$; Fig. 1B, C). In comparison with the 2 Gy group, the percentage of apoptotic cells was elevated in other groups ($P_{\text{2 Gy group vs.6 Gy group}} < 0.05$; $P_{\text{2Gy group vs.8 Gy group}} < 0.01$; $P_{\text{2 Gy group vs. 8 Gy group}} < 0.0001$). Compared to the 4 Gy group, apoptosis was increased in 8 Gy and 10 Gy irradiated cells ($P < 0.05$ and $P < 0.001$, respectively). The 10 Gy beam electron caused a significant increase in the percentage of apoptotic cells compared to 6 Gy ($P < 0.01$) and 8 Gy irradiation ($P < 0.01$) (Fig. 1B, C).

### Electron beam irradiation increased ROS production in MCF-7 cells

The fluorometric method was used to measure ROS production in the irradiated cells. Our findings indicated that EBI increased ROS

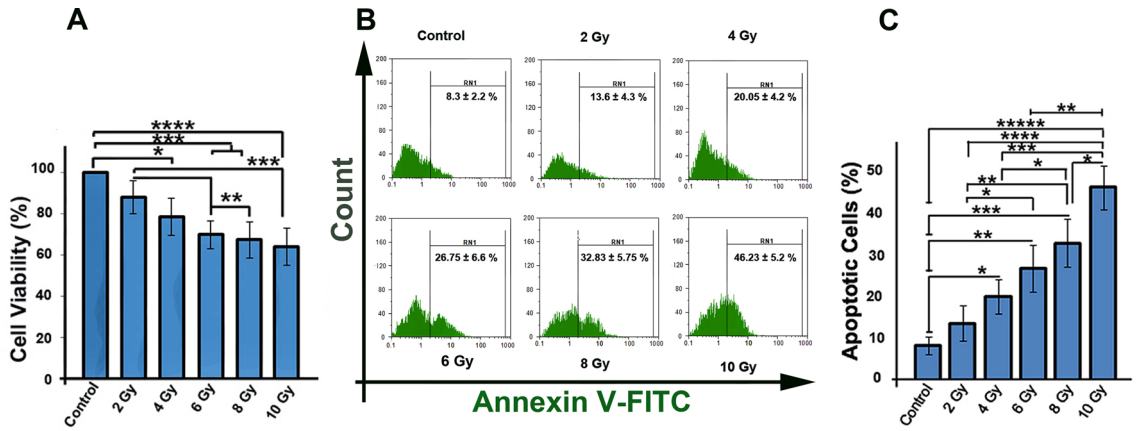


**Fig. 1** MTT assay and flow cytometry analysis. Viability of MCF-7 cell decreased after irradiation with beam electron over 48 h (**A**). Flow cytometric data showed a significant increase in percentage of apoptotic cells (**B** and **C**). Data are presented as means ± SD; $n = 3$. One-way ANOVA with Tukey post hoc test. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.00001$, $*****P < 0.00001$

production in MCF-7 cells in a dose-dependent manner ($P_{\text{Control vs. 2 Gy and 4 Gy groups}} < 0.05$; $P_{\text{Control vs. 6 Gy group}} < 0.001$; $P_{\text{Control vs. 8 Gy and 10 Gy group}} < 0.000001$; Fig. 2A). Compared to 2 Gy-CM, ROS production was amplified in 6 Gy, 8 Gy, and 10 Gy irradiated cells ($P_{\text{2 Gy group vs. 6 Gy group}} < 0.05$; $P_{\text{2 Gy group vs. 8 Gy group}} < 0.001$; $P_{\text{2 Gy group vs. 10 Gy group}} < 0.0001$). We found an increased level of ROS production in the 8 Gy and 10 Gy groups as compared to the 4 Gy group ($P_{\text{4 Gy group vs. 8 Gy and 10 Gy groups}} < 0.001$). In comparison with 6 Gy irradiation, 10 Gy irradiation caused a significant increase in ROS production in MCF-7 cells ($P < 0.01$; Fig. 2A).

## Electron beam irradiation increased AChE activity in CMs of MCF-7 cells

AChE activity, an enzyme associated with extracellular vesicles, was used to measure the extracellular vesicles' content in CMs. According to Fig. 2B, the high intensity of EBI increased AChE activity compared to the control group ($P_{\text{Control vs.6 Gy group}} < 0.05$; $P_{\text{Control vs.8 and 10 Gy groups}} < 0.01$). Compared to both 2 Gy and 4 Gy groups, the AChE activity was elevated in 8 Gy and 10 Gy groups ($P_{\text{2 Gy and 4 Gy groups vs.8 Gy and 10 Gy groups}} < 0.05$; Fig. 2B).

## Electron beam irradiation up-regulated the expression of caspase-9, caspase-8, and caspase-3 genes

To provide further insight into apoptosis, we measured the mRNA level of apoptotic genes by real-time PCR. The application of higher doses of EBI (8 Gy and 10 Gy) showed an increase in the level of caspase-9 transcript ($P_{\text{Control and 2 Gy groups vs. 8 Gy group}} < 0.05$; $P_{\text{Control and 2 Gy groups vs. 10 Gy group}} < 0.01$; Fig. 3). Compared to the 4 Gy and 6 Gy groups, the mRNA level of caspase-9 was augmented in 10 Gy group ($P_{\text{4 Gy and 6 Gy groups vs. 10 Gy group}} < 0.05$).

As shown in Fig. 3, compared to the control group, 10 Gy and 8 Gy EBI markedly induced the expression of caspase-8 in MCF-7 cells ($P < 0.05$ and $P < 0.01$, respectively). In addition, 10 Gy EBI caused a significant increase in the mRNA level of caspase-8 as compared to other doses of EBI ($P_{\text{2 Gy and 4 Gy groups vs. 10 Gy group}} < 0.01$; $P_{\text{6 Gy group vs.10 Gy group}} < 0.05$; Fig. 3).

Correspondingly, the transcript level of caspase-3, an apoptotic executer, increased in irradiated cells compared to the control group ($P_{\text{Control vs. 4 Gy, 6 Gy and 8 Gy groups}} < 0.05$; $P_{\text{Control vs.10 Gy group}} < 0.01$; Fig. 3). Compared to the 2 Gy group ($1.06 \pm 0.14$-fold change), the mRNA level of caspase-3 was amplified in 8 Gy ($1.57 \pm 0.31$-fold change) and 10 Gy groups ($2.17 \pm 0.41$-fold change) ($P_{\text{2 Gy group vs. 8 Gy group}} < 0.05$; $P_{\text{2 Gy group vs.10 Gy group}} < 0.01$). Furthermore, we showed that 10 Gy irradiation caused a significant upturn in the expression of caspase-3 compared to 4 Gy and 6 Gy irradiation ($P < 0.05$; Fig. 3). Calculating the caspase-9/caspase-8 ratio may signify the intrinsic/extrinsic apoptosis pathway switch. We found that this ratio had a > onefold change in all groups and upon an increase in the dose of irradiation, a slight but not significant increase was observed ($P > 0.05$; Fig. 3). The data indicated that apoptotic genes increased in a dose-dependent manner.

## Electron beam irradiation amplified the expression of Sox-2 and Oct-4 genes

Comparative quantities of transcripts contributing to the stemness and self-renewal of MCF-7 cells were monitored

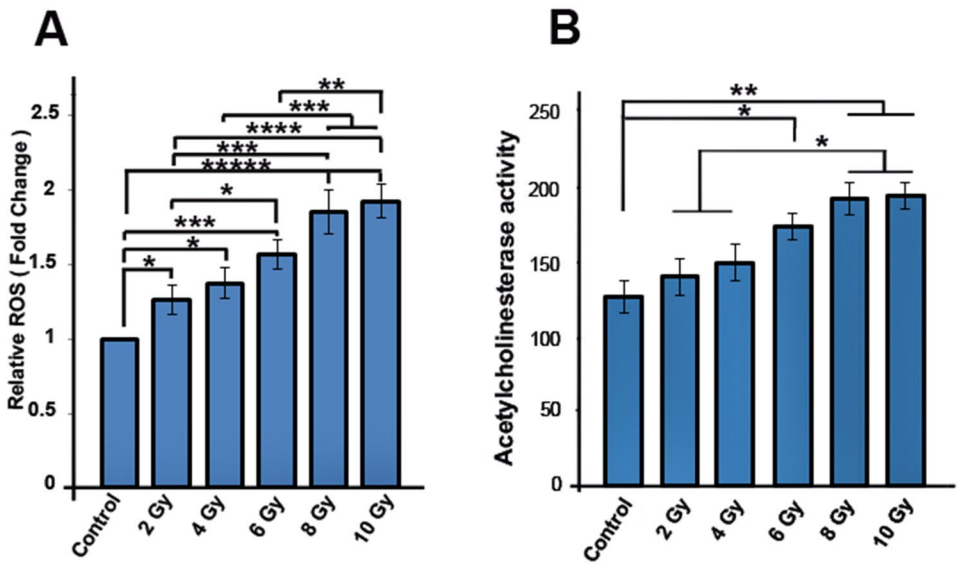


**Fig. 2** ROS production in MCF-7 cells was determined by chemical fluorometric assay (**A**). Acetylcholinesterase (AChE) activity in conditioned media of irradiated MCF-cells (**B**). Data are presented as means ± SD; $n = 3$. One-way ANOVA with Tukey post hoc test. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.00001$, *****$P < 0.00001$

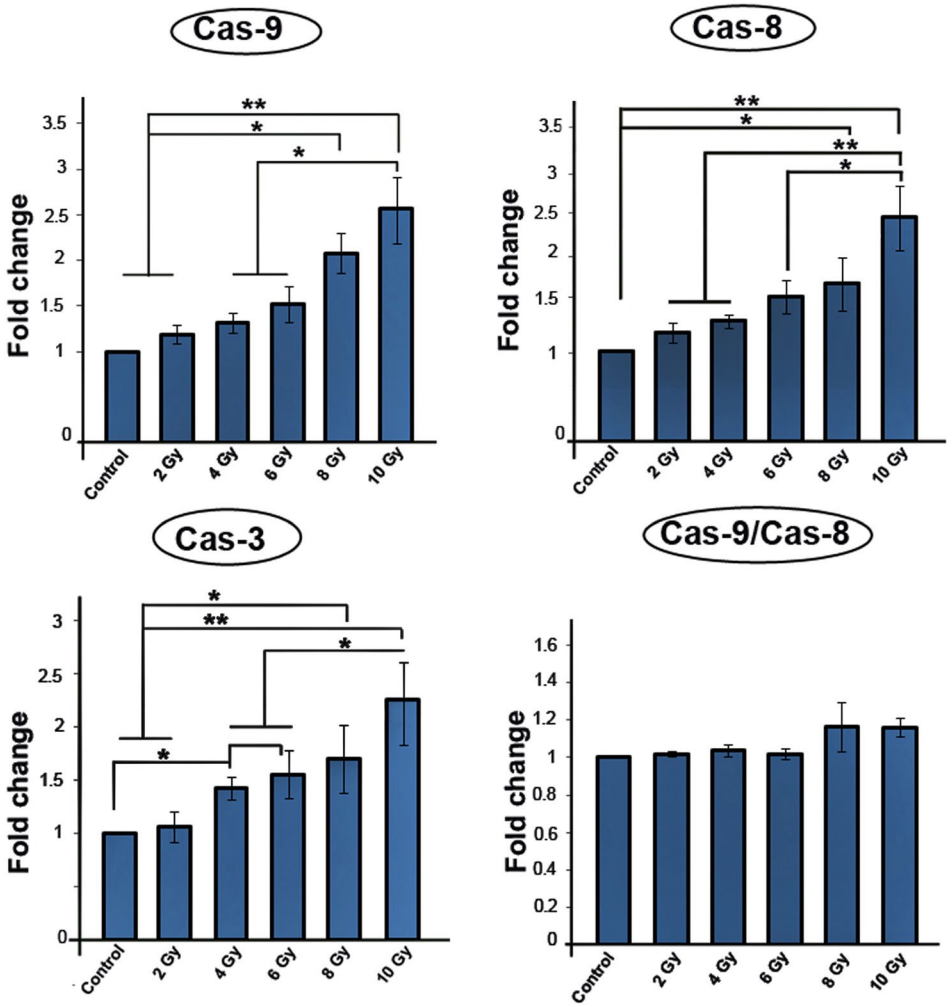


**Fig. 3** Real-time PCR analysis of caspase-9, caspase-8, and caspase-3 genes involved in apoptosis pathway. Data are presented as means ± SD; $n = 3$. One-way ANOVA with Tukey post hoc test. *$P < 0.05$, **$P < 0.01$. Cas means caspase

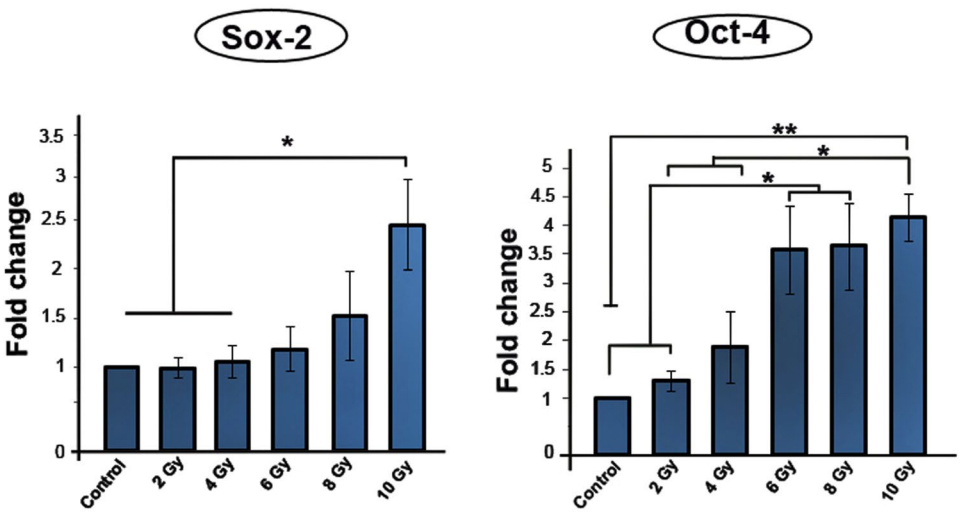


**Fig. 4** Real-time PCR analysis showed that the transcript level of Sox-2 and Oct-4 increased in MCF-7 cells upon exposure to electron beam irradiation. Data are presented as means ± SD; $n = 3$. One-way ANOVA with Tukey post hoc test. *$P < 0.05$, **$P < 0.01$

by real-time PCR. The results showed that 10 Gy irradiation elevated the mRNA distribution of Sox-2 in MCF-7 cells ($P_{\text{Control,2 Gy, and 4 Gy groups vs.10 Gy group}} < 0.05$; Fig. 4). Additionally, the transcript level of Oct-4 increased in high doses of EBI ($P_{\text{Control vs.6 and 8 Gy groups}} < 0.05$; $P_{\text{Control vs. 10 Gy group}} < 0.01$; Fig. 4). Compared to the 2 Gy group, the expression level of Oct-4 increased in 6 Gy, 8 Gy, and 10 Gy groups ($P_{\text{2 Gy vs. 6 Gy and 8 Gy groups}} < 0.05$; $P_{\text{2 Gy vs. 10 Gy group}} < 0.01$). There was a significant difference between 4 Gy ($1.88 \pm 0.62$-fold change) and 10 Gy ($4.15 \pm 0.41$-fold change) groups ($P < 0.05$) (Fig. 4). The data showed that exposure to the electron beam up-regulated the expression of stemness genes such as Sox-2 and Oct-4 in MCF-7 cells.

## Wound healing rate of MCF-7 cells was enhanced following exposure to the electron beam

An in vitro scratch assay was established to monitor the effect of EBI on the wound healing rate and migration potential of MCF-7 cells. The results obtained in this panel revealed that EBI dose-dependently enhanced the wound healing rate of MCF-7 cells within 48 h ($P_{\text{Control vs.6 Gy group}} < 0.05$;

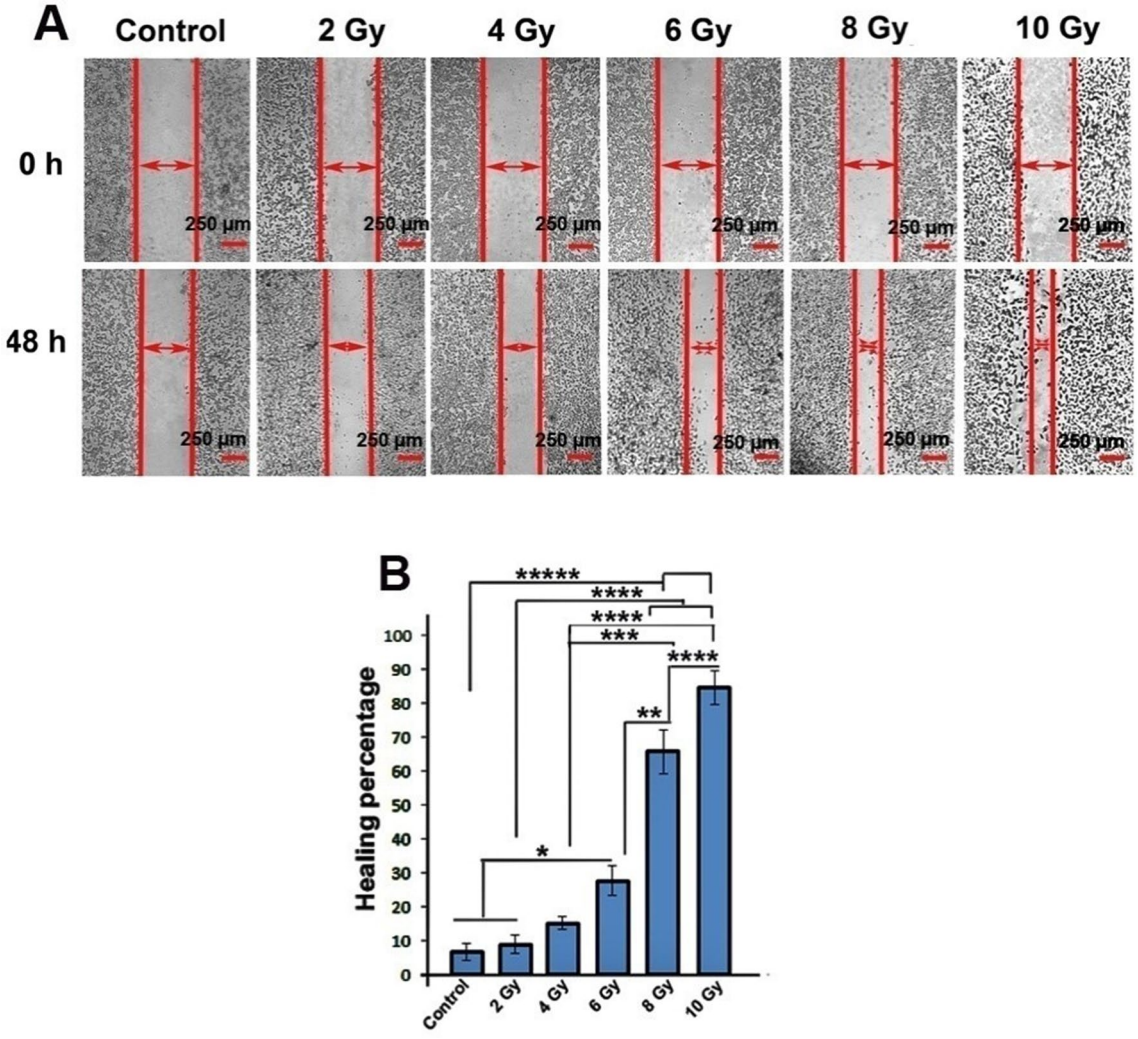


**Fig. 5** Representative in vitro scratch analysis of wound healing rate of irradiated MCF-7 cells after 48 h of irradiation (**A**, **B**). Data are presented as means ± SD; $n = 3$. One-way ANOVA with Tukey post hoc test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.00001$, $^{*****}P < 0.00001$. (Scale bar: 250 µm)

$P_{\text{Control vs.8 Gy and10 Gy groups}} < 0.00001$; Fig. 5A, B). Compared to the 2 Gy group, the wound healing rate was significantly elevated in 6 Gy, 8 Gy, and 10 Gy groups ($P_{\text{2 Gy vs. 6 Gy group}} < 0.05$; $P_{\text{2 Gy vs. 8 Gy and 10 Gy groups}} < 0.0001$). The same results were obtained as either the 4 Gy group or 6 Gy group compared with both 8 Gy and 10 Gy groups ($P_{\text{4 Gy group vs. 8 Gy group}} < 0.001$; $P_{\text{4 Gy group vs. 10 Gy group}} < 0.0001$; $P_{\text{6 Gy group vs. 8 Gy group}} < 0.01$; $P_{\text{6 Gy group vs. 10 Gy group}} < 0.001$; Fig. 5A, B).

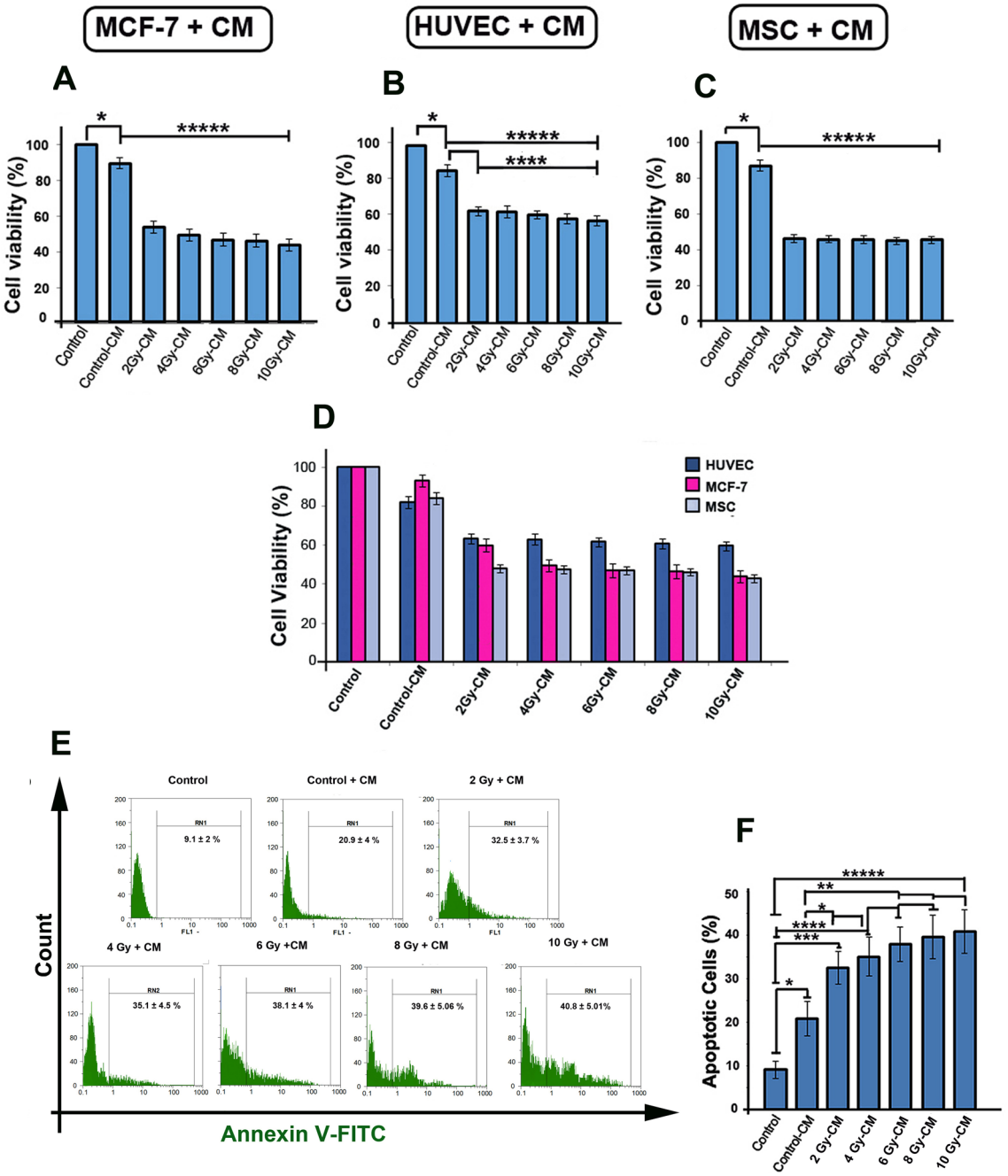


**Fig. 6** MTT viability showed that conditioned media from irradiated MCF-7 cells decreased viability of MCF-7 cells (**A**), HUVECs (**B**), and MSCs (**C**). Representative comparison of viability of different cell lines used to evaluate effect of conditioned media from MCF-7 cells on (**D**). Flow cytometry analysis indicated that irradiated MCF-7 cell-derived conditioned media increased apoptosis rate of non-irradiated MCF-7 cells. Data are presented as means ± SD; $n = 3$. One-way ANOVA with Tukey post hoc test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.00001$, $^{*****}P < 0.00001$

### Condition media from irradiated MCF-7 cells decreased the survival rate of MCF-7 cells, MSCs, and HUVECs

The cell survival rate of non-irradiated MCF-7 cells, HUVECs, and MSCs co-cultured with CMs of MCF-7 cells was investigated using the MTT assay. Based on Fig. 6, irradiated CMs decreased the survival rate of MCF-7 cells ($P_{\text{Control and control-CM vs. 2, 4, 6, 8, and 10 Gy-CM groups}} < 0.00001$; Fig. 6A). Compared to the control group, cell viability was reduced in the control-CM group ($P < 0.05$). Incubation of HUVECs with CMs from MCF-7 cells represented a substantial reduction in cell viability compared to the control group ($P_{\text{Control vs. control-CM}} < 0.05$; $P_{\text{Control vs. 2, 4, 6, 8, and 10 Gy-CM groups}} < 0.00001$; Fig. 6B). Compared to the control-CM group, the cell viability rate was declined in other groups ($P_{\text{Control-CM vs. 2, 4, 6, 8, and10 Gy-CM groups}} < 0.0001$).

In the case of MSCs, irradiated CMs also profoundly decreased the viability of MSCs ($P_{\text{Control vs. control-CM, 2, 4, 6, 8, and 10 Gy-CM groups}} < 0.00001$; Fig. 6C). Furthermore, there was a significant difference between the control and control-CM groups ($P < 0.05$), which may have resulted from the control cells' culture in the fresh medium. The comparison of the viability of MCF-7 cells, HUVECs, and MSCs is presented in Fig. 6D.

### Irradiated CMs from MCF-7 cells increased the apoptosis rate of MCF-7 cells

We also monitored the apoptosis rate of MCF-7 cells following incubation with CMs from irradiated MCF-7 cells. The flow cytometric analysis indicated that CMs increased the percentage of apoptotic cells in a dose-dependent manner ($P_{\text{Control vs. control-CM group}} < 0.05$; $P_{\text{Control vs. 2 Gy-CM group}} < 0.001$; $P_{\text{Control vs. 4, 6, and 10 Gy-CM groups}} < 0.0001$; Fig. 6E, F).

Compared to the control-CM, a significant increase in the apoptosis rate of cells incubated with irradiated CMs was found ($P_{\text{Control-CM vs. 2 and 4 Gy-CM groups}} < 0.05$; $P_{\text{CM-control vs. 6, 8, and 10 Gy-CM groups}} < 0.01$).

### Irradiated CMs from MCF-7 cells induced the expression of caspase-9, caspase-8, and caspase-3 genes in MCF-7 cells

Based on Fig. 7, the mRNA level of caspase-9 was dose-dependently amplified in 8 Gy-CM and 10 Gy-CM groups when compared with both control and control-CM groups ($P_{\text{Control vs. 6 Gy-CM group}} < 0.05$; $P_{\text{Control and control-CM vs. 8 Gy-CM group}} < 0.01$; $P_{\text{Control and control-CM vs. 10 Gy-CM group}} < 0.001$). Compared with fold changes in caspase-9 of both 2 Gy-CM ($1.28 \pm 0.15$-fold change) and 4 Gy-CM ($1.45 \pm 0.09$-fold change) groups, these values reached $1.80 \pm 0.14$ and $2.36 \pm 0.32$-fold changes in 8 Gy-CM and 10 Gy-CM groups, respectively ($P < 0.01$; Fig. 7). A similar result was achieved when the 6 Gy-CM group was compared with the 10 Gy-CM group ($P < 0.01$).

Similar to the expression pattern of caspase-9, CMs from high-dose-irradiated cells had potential for the profound induction of caspase-8 expression ($P_{\text{Control vs. 8 Gy-CM group}} < 0.05$; $P_{\text{Control vs. 10 Gy-CM group}} < 0.01$; $P_{\text{Control-CM and 2 Gy-CM groups vs. 10 Gy-CM group}} < 0.01$; Fig. 7). In comparison with 4 Gy-CM and 6 Gy-CM groups, the mRNA level of caspase-8 was significantly elevated in the 10 Gy-CM group ($1.40 \pm 0.1$ and $1.43 \pm 0.13$ vs. $2.32 \pm 0.31$-fold change; $P < 0.01$).

We also found that CMs from electron beam-irradiated cells significantly induced the expression of caspase-3 in MCF-7 cells in a dose-dependent manner ($P_{\text{Control vs. control-CM and 2 Gy-CM group}} < 0.05$; $P_{\text{Control vs. 4 Gy-CM and 6 Gy-CM group}} < 0.01$; $P_{\text{Control vs. 8 Gy-CM group}} < 0.01$; $P_{\text{Control vs. 10 Gy-CM group}} < 0.001$; Fig. 7). Data from Fig. 7 also demonstrated the same trend for the control-CM group compared with other groups ($P_{\text{Control-CM vs. 6 Gy-CM}} < 0.05$; $P_{\text{Control-CM vs. 8 Gy-CM group}} < 0.01$; $P_{\text{Control-CM vs. 10 Gy-CM group}} < 0.001$). The transcript level of caspase-3 increased in 8 Gy-CM and 10 Gy-CM groups when compared either with the 2 Gy-CM group or 4 Gy-CM group ($P < 0.01$). Furthermore, the mRNA form of caspase-3 significantly increased in the 8 Gy-CM group and 10 Gy-CM group as compared with the 6 Gy-CM group ($P_{\text{6 Gy-CM vs. 8 Gy-CM group}} < 0.05$; $P_{\text{6 Gy-CM group vs. 10 Gy-CM group}} < 0.01$; Fig. 7).

### Irradiated CMs from MCF-7 cells induced the expression of Sox-2 and Oct-4 genes in MCF-7 cells

Gene expression assessment by real-time PCR showed that CMs from 8 to 10 Gy irradiated MCF-7 cells induced the expression of Sox-2 gene ($P_{\text{Control, control-CM, 2 Gy-CM, and 4 Gy-CM groups vs. 8 Gy-CM}} < 0.05$; $P_{\text{Control, control-CM, 2 Gy-CM, and 4 Gy-CM groups vs. 10 Gy-CM}} < 0.01$; Fig. 8). The mRNA level of Sox-2 increased in 8 Gy-CM and 10 Gy-CM groups compared to the 6 Gy-CM group ($P < 0.05$). Similar to the Sox-2 gene, we also found that the other self-renewal gene, Oct-4, up-regulated in 8 Gy-CM and 10 Gy-CM groups compared with the other groups ($P_{\text{Control, control-CM, 2 Gy-CM, and 4 Gy-CM groups vs. 8 Gy-CM}} < 0.01$; $P_{\text{Control, control-CM, 2 Gy-CM, and 4 Gy-CM groups vs. 10 Gy-CM}} < 0.001$; Fig. 8). Additionally, in comparison with the 6 Gy-CM group ($1.38 \pm 0.26$-fold change), the mRNA levels of Oct-4 were increased in 8 Gy-CM ($2.48 \pm 0.16$) and 10 Gy-CM ($3.35 \pm 0.29$-fold change) groups ($P < 0.05$ and $P < 0.01$; respectively). The analysis of the caspase-9/caspase-8 ratio

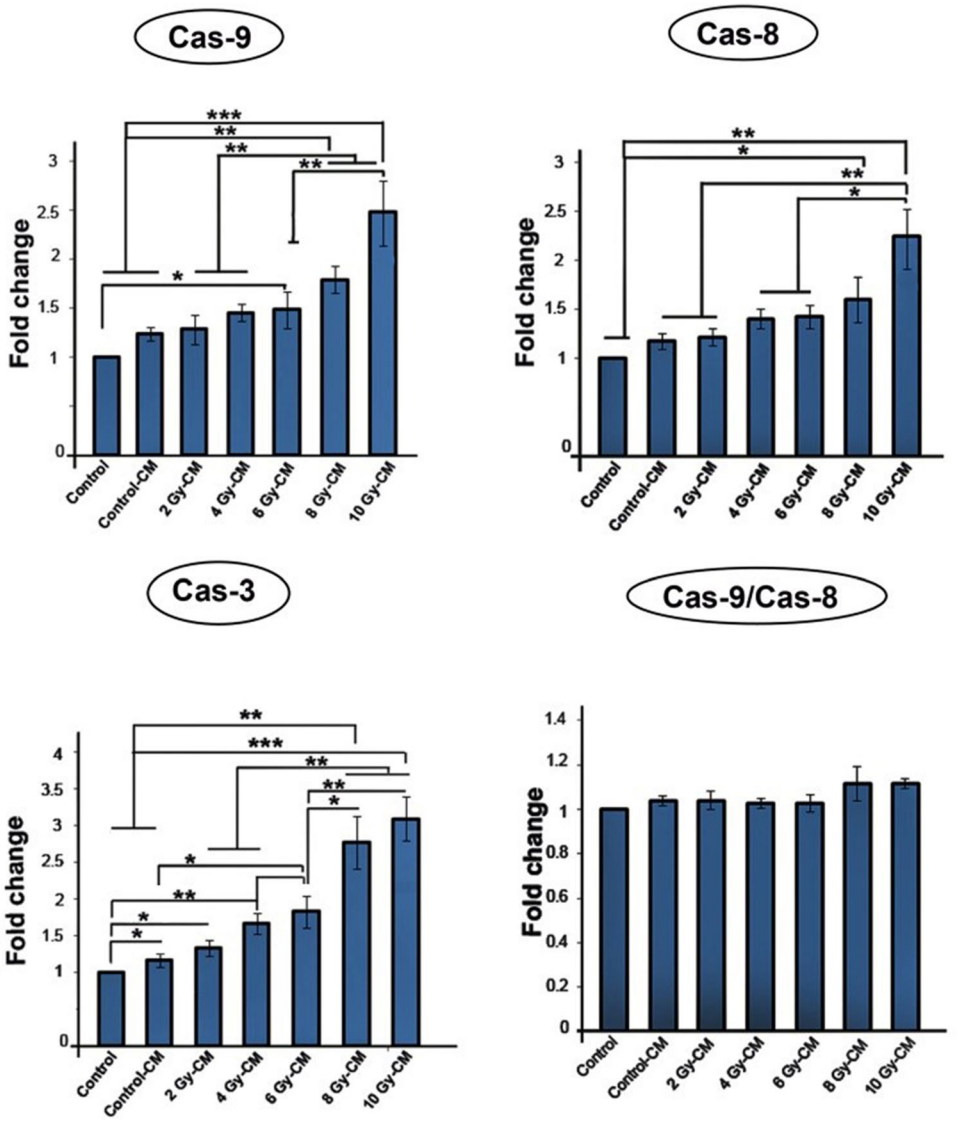


**Fig. 7** Expression of caspase-9, caspase-8, and caspase-3 genes, involved in apoptosis flux, relatively quantified by real-time PCR in MCF-7 cells. Data are presented as means ± SD; $n = 3$. One-way ANOVA with Tukey post hoc test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$

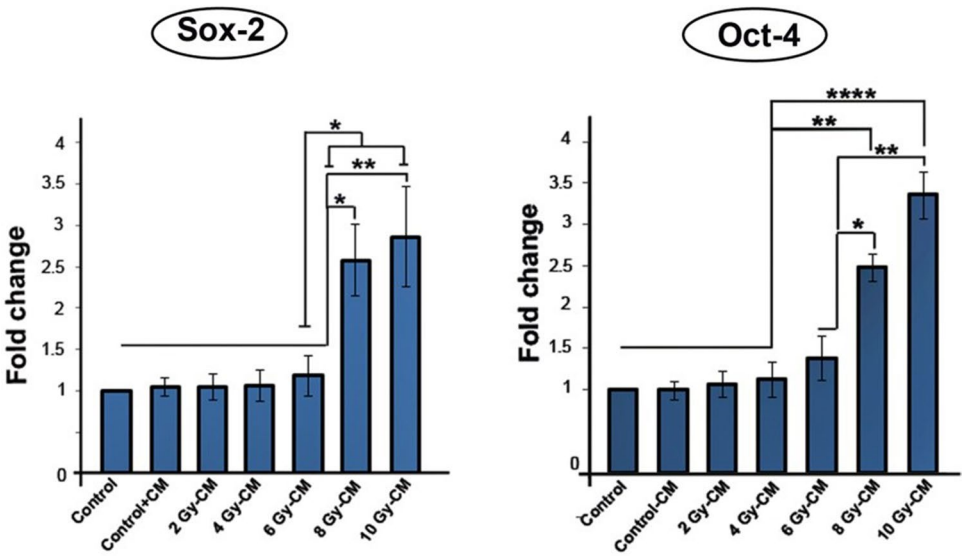


**Fig. 8** Data obtained from real-time PCR analysis showed that conditioned media of irradiated MCF-7 cells induced the expression of Sox-2 and Oct-4 genes in MCF-7 cells. Data are presented as means ± SD; $n = 3$. One-way ANOVA with Tukey post hoc test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{****}P < 0.0001$

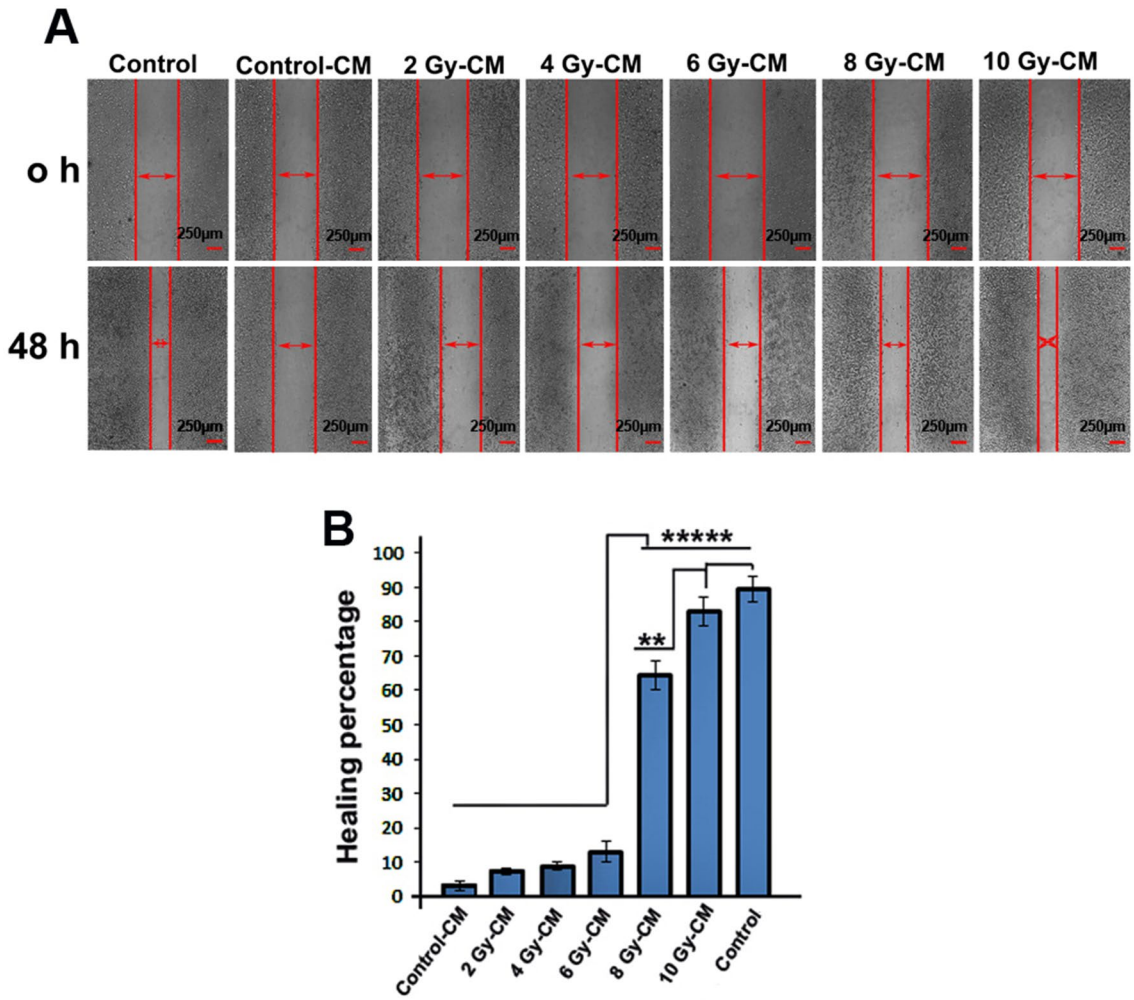


**Fig. 9** Conditioned media obtained from irradiated MCF-7 cells induced MCF-7 cells migration in a wound healing model (**A**, **B**). Data are presented as means ± SD; $n = 3$. One-way ANOVA with Tukey post hoc test. $^{**}P < 0.01$, $^{*****}P < 0.00001$

showed that this ratio only slightly exceeded onefold in all groups ($P > 0.05$; Fig. 7).

### CMs from MCF-7 cells increased the wound healing rate of MCF-7 cells

To investigate the effect of irradiated CMs on the wound healing rate and migration of MCF-7 cells, we established an in vitro scratch assay. Our results showed that 8 Gy and 10 Gy irradiated CMs along with the control raised the wound healing rate of MCF-7 cells over 48 h of incubation ($P_{\text{Control-CM, 2 Gy-CM, 4 Gy-CM, 6 Gy-CM groups vs. 8 Gy-CM, 10 Gy-CM, and control groups}} < 0.00001$; Fig. 9A, B). In addition, the wound healing rate increased in 10 Gy-CM and control groups as compared to the 8 Gy-CM group ($P_{\text{8 Gy-CM group vs. control and 10 Gy-CM groups}} < 0.01$). Interestingly, we found that the wound healing rate of the control group increased, which was supposedly due to incubation in fresh DMEM containing FBS.

## Discussion

In the present study, the effect of different doses of EBI on the dynamics of the MCF-7 cells was evaluated. Furthermore, we examined the bystander effects of irradiated MCF-7 cells on different cell lines, which are known to play pivotal roles in cancer treatment. Consistently with previous studies [14–16], we found that the viability and apoptosis rate of irradiated MCF-7 cells increased, respectively (Fig. 1). Irradiation elicits cytotoxic effects on the dynamics of cells through biomolecules and organelles damage that induces apoptosis [17–19]. The ROS production inside cells increased upon the increase in electron beam dose (Fig. 2A), which may be correlated to the induction of toxicity in the

cells and the elevated apoptosis rate [20]. Different caspases contribute to apoptosis flow both in intrinsic and extrinsic pathways [21], which we examined by the measurement of the mRNA levels of caspase-3, -8, -9 in irradiated MCF-7 cells over a period of 48 h post-irradiation. Caspase-9 and caspase-8, the cysteine proteases, are implicated in intrinsic and extrinsic apoptosis, respectively, that activate apoptosis through interaction with caspase-3 [21]. Our results showed an increase in the transcript levels of all caspase-3, -8, -9 genes (Fig. 3). Increased expression levels of these genes were concomitantly observed with a higher level of ROS production in MCF-7 cells that correlated with the result of the apoptosis assay. We also calculated the caspase-9/caspase-8 ratio to predict the relative superiority of intrinsic or extrinsic pathways in our study. The values of this ratio were slightly higher rather than 1 in all the irradiated groups, and no significant difference was observed between them (Fig. 3). These data support the idea that the majority of apoptosis may occur through the intrinsic pathway in electron beam-treated cells. Electron beam could harm cellular biomolecules and organelles [18] and produce ROS [22] that may activate the intrinsic pathway of apoptosis. In addition, we found that the AChE activity of CMs of irradiated cells was elevated in a dose-dependent manner (Fig. 2B), suggesting the increased secretion of extracellular vesicles from irradiated cells [23]. A recent study by Arscott et al. has confirmed that IR induced extracellular vesicles' biogenesis and secretion [24], which may explain the mechanism in which the intracellular cytotoxicity is expelled by these vesicles to maintain homeostasis and adaptation [25]. To the best of our knowledge, this is the first report of increased AChE activity of CMs of MCF-7 cells following exposure to the electron beam. It seems that EBI influenced the ROS/apoptosis/EVs secretion axis in MCF-7 cells. Besides these, we measured the effect of EBI on MCF-7 cells regarding stemness-related genes. In this regard, the data showed that the expression of Sox-2 and Oct-4 increased in irradiated cells (Fig. 4), indicating the induction of a stem cell-like phenotype in MCF-7 cells [26, 27]. This was in accordance with the work by Ghisolfi et al. which found that gamma irradiation of cancer cells increased the mRNA level of Sox-2 and Oct3/4 genes [28]. Similarly, it was demonstrated that IR induced cancer stem-like cells via dedifferentiation and increased the expression of stem cell markers such as SOX-2 in glioblastoma cells [29]. It is confirmed that the up-regulation of Sox2 and Oct-4 genes may play a pivotal role in supporting self-renewal, reprogramming, and plasticity capacity in cancer stem cells [30, 31]. Our findings seem to show that the mRNA level of these genes following EBI may represent alternation in the MCF-7 cells' population and enrichment of cancer stem cells [32]. Consistent with Imaizumi et al. [33], we also found that EBI was capable of inducing the migration response of MCF-7 cells by the scratch wound healing assay (Fig. 5). Different authorities have shown that irradiation not only induces apoptosis in tumor cells but also encourages the motility and invasion of tumor cells through several pathways [34, 35]. Further data collection is required to uncover underling mechanisms involved in EBI-mediated cell migration.

Furthermore, we aimed to investigate the bystander effect of EBI on non-irradiated cells. In this scenario, we noted that treatment of MCF-7 cells, MSCs, and HUVECs with CMs from irradiated MCF-7 cells decreased the viability of all cell lines (Fig. 6A–D). Jella et al. [36] proved the decrease in the viability of tumor cells co-cultured with CMs of irradiated cells. They also revealed a remarkable increase in the caspase activity and apoptosis rate of cells receiving irradiated CMs, which is similar to our results indicating that CMs from irradiated MCF-7 cells increased the apoptosis rate of MCF-7 cells. As shown in Fig. 6A–D, compared to the control group, the decreased cell viability value of the control-CM group may be due to the presence of FBS in fresh cell culture media of control groups. More recently, Lepleux et al. reported that CMs from chondrosarcoma cells irradiated with X-rays and C-ions at different doses (0.05 to 8 Gy) suppressed the proliferation of T/C-28a2 chondrocytes and decreased cell survival [37]. Consistently, Yang et al. found that the percentage of apoptotic A549 cells was increased when cultivated with CMs of irradiated cells, indicating a decrease in cell viability [38]. For further insight into apoptosis, we monitored the expression of apoptotic genes, including caspase-3, -8, and -9, in the CM-treated MCF-7 cells (Fig. 7). Our results confirmed the up-regulation of the genes in MCF-7 cells cultured with CMs from irradiated MCF-7 cells. Increased expression levels of these genes were correlated with an increased percentage of annexin-V positive cells (Fig. 6E, F). It seems that both intrinsic and extrinsic pathways were activated in our experiment; however, the caspase-9/caspase-8 ratio was slightly higher than 1 in all the CM groups (Fig. 7), indicating more activation of the intrinsic pathway. To the best of our knowledge, little is known about the impact of bystander effects of electron beam on cancer stem cell-related genes. Interestingly, we found that CMs from irradiated cells induced the expression of stemness- and self-renewal-related genes, Sox-2 and Oct-4, in MCF-7 cells (Fig. 8). According to previous studies, these genes are involved in resistance and adaption to stress conditions [39, 40]. Bystander effects may be mediated through different soluble factors, cytokines [7], and even EVs [41]. It was well established that the bystander effects of IR include the chromosomal inconsistency, DNA damage, alteration, resistance, proliferation, and apoptosis in non-irradiated cells [7]. Xu et al. have reported that X-ray irradiation increases exosome biogenesis and secretion [42]. In addition, several laboratories demonstrated that EVs from irradiated tumor cells promoted genomic instability and resistance in non-irradiated cells [43]. In our experiment,

increased EVs in CMs may partly explain the up-regulation of these genes in MCF-7 cells. An explanation may be that soluble factors enriched in the CMs of irradiated MCF-7 cells could induce the resistance and self-renewal property of MCF-7 cells via the up-regulation of Sox-2 and Oct-4 genes. In an attempt to highlight the possible effect of CMs on the migration capacity of non-irradiated MCF-7 cells, increased wound healing rate of MCF-7 cells treated by CMs from high-dose-irradiated cells was shown by the in vitro scratch assay. These values show a good relationship with the paper by Mutschelknaus et al. that further supports the idea that the content of cytokines and EVs of irradiated CMs promotes the migration ability of MCF-7 cells [44]. As presented in Fig. 9, CMs from the cells irradiated with the high-dose (8 Gy and 10 Gy) irradiated cells significantly induced migration, presumably due to increased cytokine levels in these cells. Moreover, we found that the wound healing rate of control cells increased, which may be because of being cultured in a fresh medium containing FBS that promotes cell proliferation. The results point to the probability that the population of MCF-7 cells under electron beam exposure release soluble factors including EVs, not only to expel cellular damage but also to induce resistance and compensatory responses against stress conditions.

In summary, by investigating irradiated MCF-7 cells with different doses of electron beam affecting the viability, apoptosis, EVs' release, the expression of genes involved in apoptosis and stemness, and migration capacity, we shed light on cellular responses of MCF-7 against EBI. We showed that cellular responses increased along with the increase in doses of irradiation. Although EBI showed cytotoxic effects, we also observed facilitated EVs' secretion, cell migration, and up-regulation of Sox2 and Oct-4 in MCF-7 cells, which may represent the side effects of EBI. Additionally, by the study of possible bystander effects of EBI, we discovered that irradiated MCF-7 cells send paracrine signals that influence the survival and apoptosis rate of non-irradiated cells. These signals from high-dose-irradiated cells also induce stemness and resistance in the tumor cell population, and they even promoted wound healing rate, indicating the elevated migration potential of MCF-7 cells.

## Conclusion

Although the present study analyzed the in vitro response of MCF-7 cells against EBI, our results showed that MCF-7 cells treated with an electron beam and CMs from irradiated MCF-7 cells exhibit an up-regulation in both genes involved in the apoptosis pathway and stemness. Therefore, EBI can affect apoptosis and stemness in MCF-7 cells in direct and bystander manners. However, further studies are essential to elucidate the exact underlying mechanisms involved in EBI-induced alternation of tumor cell dynamics.

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**Authors contributions** NJ and JR were responsible for the conceptualization and acquisition of the data. NJ and JR were responsible for the methodology. NJ and JR were responsible for the writing, review, and/or revision of the manuscript. MF, NJ, JR, and KM were responsible for the administrative, technical, or material support. NJ was responsible for the study supervision. All authors read and approved the final manuscript.

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**Data availability** Data are available on request.

## Declarations

**Conflict of interest** All authors declare that they have no conflict of interest or other disclosures.

**Ethical approval** The ethics committee of Urmia University of Medical Sciences approved the all procedures of this experiment (Ethical Approval No: IR.UMSU.REC.1398.101).

**Consent for publication** All authors declare that they have not sent for publication.

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