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RESEARCH ARTICLE

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Effect of resveratrol on the radiosensitivity of 5-FU in human breast cancer MCF-7 cells

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Abstract

The aim of this study was to assess the efficacy of resveratrol (Res) on radiosensitivity of 5-fluorouracil (5-FU) in the spheroid culture of MCF-7 breast cancer cell line using colony formation examination. Spheroids on day 9 with 300 μm diameters were treated with 20 μM resveratrol and/or 1 μM 5-FU for one volume doubling time (VDT) (42 hours) and then irradiated with 2 Gy gamma radiation (^{60}Co) in various groups. Then the viability of the cells and clonogenic ability were acquired by blue dye exclusion and colony formation assay, respectively. The population doubling time in the monolayer culture and the VDT of spheroid culture was 22.48 ± 0.23 hours and 42 ± 0.63 hours respectively. None of the drugs and combination of them had any effect on the viability of cells. The combination treatment of 5-FU+Res+ radiation significantly reduced the colony formation ability of spheroid cells in comparison with each treatment alone. Our results indicated that resveratrol can significantly decrease colony number of breast cancer spheroid cells treated with 5-FU in combination with gamma-rays. Thus, resveratrol as a hypoxia-inducible factor-1-alpha inhibitor increased the radiosensitization of breast cancer spheroid cells.

KEYWORDS

5-fluorouracil, breast cancer, radiation, radiosensitivity, resveratrol, spheroid

1 | INTRODUCTION

Breast cancer is the most typical malignancy among women and affects about 1.96 million women yearly worldwide.[1] Breast cancer is the most usual invasive cancer in women,[2] and the second cause of cancer death in women, after lung cancer.[3] In spite of progression in surgery, radiation therapy, and chemotherapy, the chance of any woman dying from breast cancer is around 1 in 37.[4] Hence, developing new therapeutic strategies are essential.[5] So there remains a need for more beneficial and less cytotoxic treatments.[6] However, the dose needs to control these tumors is high.[7] These undesirable results have incited the search for procedures to sensitize tumor cells to ionizing radiation as a choice.[8] Hence, more and more attention of researchers has been attracted to radiosensitizers,[9] which lower the radiation dose-response threshold for cancer cells without increasing the normal tissue radiosensitivity.[10] To obtain this result the most effective approach is to use halogenated pyrimidines, 5-fluorouracil (5-FU).[11] 5-FU is commonly used as an anticancer drug for cancer cells,[12] which can be used with X-ray. 5-FU is a metabolic analog of uracil and

Aghamiri and Jafarpour have contributed equally to this study.

thymine in RNA and DNA synthesis respectively.[13] Displacement of this analog impedes the synthesis of DNA in cells that actively divide.[14] Because of thymidylate synthase inhibiting mechanism, 5-FU can block the synthesis of the pyrimidine thymidine, which is a nucleoside needed for cell DNA replication.[15] 5-FU can increase the cytotoxicity of X-ray.[16,17] So an enhanced proliferation rate should lead to increased radiosensitization of 5-FU, and the degree of that directly reflected the extent of thymidine displacement in the replicating DNA.[18] It has been revealed that the absorption of 5-FU is decreased in the spheroid model.[19] Hypoxia, which can activate the hypoxia-inducible factor-1 alpha (HIF1-α) signaling pathway, is a usual characteristic in tumors[20] and decrease the radiation (Rad) and anticancer drugs sensitivity of cancer cells.[21] Therefore, inhibition of HIF1-α can sensitize resistant breast cancer cells to the cytotoxic effect of Rad and chemotherapeutic agents.[22] The inhibition of HIF1-α protein expression by resveratrol (trans-3, 4′, 5-trihydroxystilbene) (Res) in the hypoxia status has been demonstrated by numerous studies.[23] Res, a polyphenolic phytoalexin, can significantly prevent hypoxia-induced HIF1-α protein accumulation in tumor cells.[24] Therefore, hypoxia cells can be sensitized to Rad by uptake the analogs (5-FU) and move out of G0.[25] This plan may be a new innovative idea to increase the efficacy of cancer chemotherapy.[26] The success of many combination anticancer therapies and combined modality treatments is dependent on their potential to kill both the hypoxic and non-hypoxic cells of tumors.[27] Also, therapeutic efficacy can be increased by the combination of radiation therapy with anticancer drugs.[28] In this study, the effect of Res on the radiosensitivity of 5-FU in the spheroid culture of MCF-7 breast cancer with 300 μm diameter have investigated. Important in vivo relationship between cancer cells including individual hypoxic cells contact together, was revealed by the spheroid cell culture.[29] Briefly, spheroid cultures increase the accuracy of in vitro methods with the relevance of organized tissues.[30]

2 | MATERIALS AND METHODS

2.1 | Cell line

Human breast cancer cell line, MCF-7, was procured from Pasteur Institute of Iran. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) (PAA), 100 mg/mL of streptomycin (PAA) and 100 U/mL of penicillin and they were maintained at 37°C in a humidified atmosphere (90%) containing 5% CO2.

2.2 | Monolayer culture and doubling time calculation

Cells were grown in T-25 tissue culture flasks as a monolayer at a density of 4×10^4 cells/cm^2. Cells were incubated at 37°C under a humidified atmosphere of 5% CO_2. After their proliferation, cells were harvested by trypsinizing cultures with 0.25% trypsin (w/v; Sigma, St Louis, MO) and 1 mM ethylene diamine tetraacetic acid (EDTA; Sigma) in phosphate buffer saline (PBS; Sigma). Three passages after thawing, MCF-7 cells were cultured at a density of 10^5 per well in multiwell plates (24 wells/plate) (SPL) overnight. The MCF-7 cells from triplicate wells were removed by 1 mM EDTA/0.25% trypsin (w/v) treatment and counted in a hemocytometer. An average of nine counts was used to determine each point. By using the slope of the growth curve logarithmic phase, the doubling time was assessed.

2.3 | Spheroid culture

Spheroids were generated using the liquid overlay method. Viable cells (5×10^5) were seeded into 100 mm Petri dishes (SPL), which covered by a thin layer of 1% agar (Merck, Darmstadt, Germany) comprising 10 mL of DMEM containing 10% FBS. The plates were incubated at 37°C under a humidified atmosphere of 5% CO_2. Half of the culture medium was recovered with fresh culture medium two times a week.

2.4 | Irradiation procedure

For gamma radiation, the medium was replaced with a new medium, and the spheroids were irradiated using ^{60}Co source (Theratron-780c; MDS Nordion, Ottawa, ON, Canada) at a dose rate of 1.34 cGy/s for 2 Gy. For Rad treatment, culture flasks were put under collimator of equipment at 85 cm distance between the head of the machine and the floor of flasks, and the period of irradiation and the field size were 2.49 minutes and 20×20 cm^2, respectively.

2.5 | Spheroid treatment

On day 9, spheroids with 300 μm diameter were treated with 5-FU and/or Res and/or gamma radiation. 5-FU and/or Res treatment was done for one volume doubling time (VDT) (42 hours) at 37°C in a humidified atmosphere and 5% CO_2. As a control, the spheroids of one plate were not treated. The treatments were carried out as stated in the following groups:

1. Control without treatment
2. Treated with Res (IC10: 20 μM) for 42 hours.

3. Treated with 5-FU (1 μM) for 42 hours.
4. Irradiated with ^{60}Co (2 Gy).
5. Treated simultaneously with Res (20 μM) and 5-FU (1 μM) for 42 hours.
6. Treated with 5-FU (1 μM) for 42 hours and then irradiated with ^{60}Co (2 Gy).
7. Treated simultaneously with 5-FU (1 μM) and Res (20 μM) for 42 hours, then irradiated with ^{60}Co (2 Gy).

To determine the viability of treated and control spheroids trypan blue dye exclusion assay was used. Then, the cell damages were assessed using colony formation.

2.6 | Dissociation of the cells from 3D culture

First spheroids were centrifuged (10 minutes at 1200 rpm) and 1000 μL of buffer phosphate was added and centrifuged again. Then 300 μL trypsin/EDTA solution was added. So after 5 minutes, 700 μL culture medium plus 10% of FBS were added and pipetted them several times to ensure separation of the cells. Then, we carry out the number of living cells and counting.

2.7 | Trypan blue dye exclusion assay

The single cells from treated and control spheroid cultures were combined with trypan blue at a ratio of 9:1. After 2 to 3 minute, the combination was checked under an inverted microscope (Bell). The percentage of viable cells with clear cytoplasm out of the total cells was the viability of each group of cells.

2.8 | Colony formation assay

Control and treated single cells suspensions from spheroid culture were seeded in 60 mm Petri dishes (SPL) with DMEM supplemented with 10% FBS for colony formation test. Tumor cells in culture were incubated at 37°C under a humidified atmosphere of 5% CO_2. After 10 days, cultures fixed with 2% formaldehyde in PBS, stained with crystal violet, then an inverted phase microscope (Bell) was used for colonies counting that included a minimum of 50 cells. To estimate the ability of cells to form colonies, the various concentration of single cells from spheroid (500, 1000, 2000, 3000, 4000, 5000, 6000 cells) were seeded into 60 mm plates with 5 mL DMEM containing 10% FBS. Plating efficiency (PE) was assessed, using the following equation

$$PE\ (\%) = (\text{number of colonies}/\text{number of seeded cells}) \times 100$$

2.9 | Statistical analysis

Data were expressed as mean values ±SEM with "n" denoting the number of examination. Statistical analysis was conducted using One-way analysis of variance followed by the Tukey's test as the post hoc analysis using SPSS version16. A P value of less than 0.05 was considered significant.

3 | RESULTS

3.1 | Cell characteristics

3.1.1 | Monolayer culture

Monolayer culture of the MCF-7 cell line was performed on tissue culture flasks. The population doubling time determined from the logarithmic phase of the growth curve in the monolayer culture was roughly 22.48 ± 0.23 hours.

3.1.2 | Spheroid culture

The liquid overlay technique[31] was used to culture spheroids. The VDT (the period of time required for a spheroid to double in volume) estimated from the spheroid growth curve was almost 42 ± 0.63 hours, which was used as the drug treatment time. Figure 1 shows the picture of the plates after treatment.

3.1.3 | Viability assay

Rapidly after cell treatment with Res, 5-FU, and Rad, cells were counted, and viability was determined using the trypan blue dye exclusion assay. As can be seen in Figure 2, Res, 5-FU, Rad, and combination of them did

FIGURE 1 MCF-7 Tumor cells spheroid plates following treatment

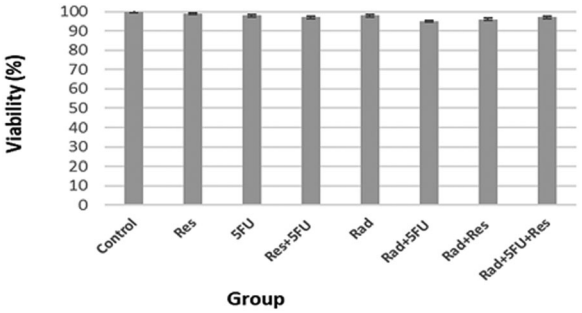

FIGURE 2 Effect of resveratrol (Res), fluorouracil (5-FU), and gamma radiation (Rad) and the combination of them on the viability of MCF-7 spheroid cells with 300 μm in diameter, using the trypan blue dye exclusion assay. The values are the mean ± SEM of three experiments

not have any effect on the viability of MCF-7 spheroid cells ($P > 0.05$).

3.2 | Effects of Res, 5-FU and gamma-ray radiation on colony forming ability

Colony formation assay was applied by 20 μM Res, 1 μM 5-FU, and 2 Gy of gamma-ray radiation on the seven groups as mentioned in section 2 for studying cell response to treatment. Figure 3 shows the effect of treatments on the PE of MCF-7 breast cancer cell line in spheroid culture. Twenty micromolar Res alone had no significant effect on the PE of spheroid cells in comparison with control ($P > 0.05$) but 1 μM 5-FU and 2 Gy of gamma-ray radiation separately reduced the PE of spheroid cells ($P < 0.01$). Figure 3 shows the role of 5-FU in the radiosensitivity of MCF-7 breast cancer cell line.

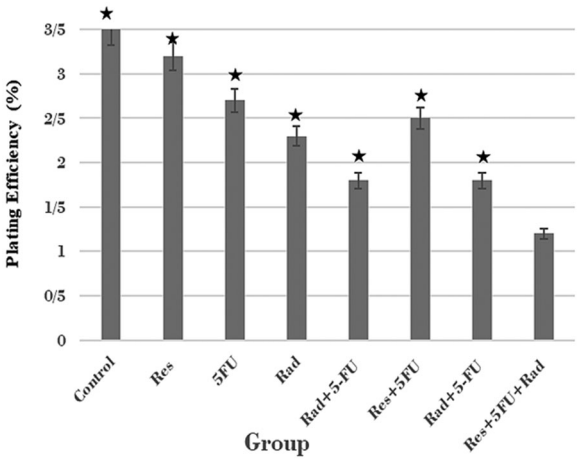

FIGURE 3 Effect of 1 μM 5-FU and/or 20 mM resveratrol (Res) and/or 2 Gy of gamma-ray radiation on the plating efficiency of MCF-7 breast cancer cell line in spheroid culture with 300 μm in diameter. ($^{*}P < 0.001$ vs Res+5-FU+Rad). The values are the mean ± SEM of three experiments. 5-FU, 5-fluorouracil

Reduction of PE due to the combination treatment of 5-FU and radiation is significantly more than each one alone ($P < 0.01$). After that, the role of Res as HIF inhibitor was studied on the radiosensitivity of 5-FU. Figure 3 also shows the effect of Res, 5-FU and gamma radiation alone or in combination with each other on MCF-7 breast cancer spheroids. As can be seen, the combination treatment of 5-FU+Res+gamma radiation significantly reduced the colony formation ability of spheroid cells in comparison with each treatment alone or the combination of 5-FU+Rad or 5-FU+Res ($P < 0.001$).

4 | DISCUSSION

Breast cancer is the most ordinary invasive cancer in women.[32] Nowadays, combined treatments using chemical/biological agents and radiotherapy have been engaged to either enhance tumor radiosensitivity or reduce radiation side-effects.[2,33] Because the irradiation efficient enough to control the tumors far exceeds the tolerance of normal tissues. Bartelink et al[34] showed that there is cell death in MCF-7 cells treated with 2 Gy of X-ray radiation. On the other hand, 5-FU is one of the most commonly used chemotherapeutic agents.[35] Also, it has been used extensively with radiation.[36] 5-FU is a metabolic analog of thymine in DNA synthesis or uracil in RNA synthesis.[37] Replacement of this analog inhibits DNA synthesis in cells that actively divide.[14,38] Also, 5-FU can increase the cytotoxicity of ionizing radiation.[39] 5-FU, a chemotherapeutic agent has been using as a treatment for a variety of tumor types nowadays,[40-43] both alone and in combination with other drugs. Researchers confirmed that 5-FU can sensitize the cells to ionizing radiation that is depended directly with the level of halogenated pyrimidine that incorporated into DNA[11,44] (in vitro and in vivo studies). Although the molecular mechanisms of 5-FU radiosensitization are not clear it is assumed that 5-FU sensitizes cells through enhancing the formation of double-strand break.[45] Deveci et al[16] and Wu et al[46] were shown the 5-FU prevented the proliferation of MCF-7 breast cancer cells. Stephan et al[47] indicated an extremely remarkable relationship between the incorporation of 5-FU in RNA and loss of clonogenic survival. Nivethaa et al[48] showed the effectiveness of the 5-FU in inhibiting the growth of the carcinogenic MCF-7 cells. Wang et al[49] revealed the expression of breast cancer resistance protein was upregulated by the overexpression of survivin in the anticancer drug-resistant cell line MCF-7/5-FU. Rad treatment requires free radicals from oxygen to kill target cells,[50] and about two to three times radiation dose is required for hypoxic cells compared

with oxic cells.[51] Also, 5-FU absorption is significantly decreased when the tumor size is increased, and the cells in the median layers suffer from hypoxia due to oxygen deficiency.[52] A resultant component of the hypoxic response is the activation of the HIF1 transcription factor.[20] Under hypoxic conditions, HIF1-α is transferred from cytoplasm to nucleus and by attaching to HIF1-β, forms the HIF1 complex.[53] It activates more than 60 genes with several functions, leading to an increase in O_2 delivery. Also, hypoxic cells arrest in the G0 phase and their number is reduced in the S phase.[54] The implication of HIF1 in tumor resistance to treatments led us to assume that inhibition of HIF1 may sensitize hypoxic cells to the cytotoxic effect of Rad by 5-FU absorption. If hypoxic cells exit from G0 to S phase, can absorb 5-FU and become sensitive to Rad. To achieve this situation in the in vitro cell culture, we used 300 μm spheroids to ensure the existence of hypoxia cells.[55] Res (trans-3,4′,5-trihydroxystilbene) is a naturally occurring polyphenolic compound highly enriched in grapes, peanuts, red wine and a wide variety of food sources.[56] The recent document suggests that Res in combination with drugs, ionizing radiation or cytokines can be effectively used for the sensitization to apoptosis.[57] It appears that Res can sensitize the cells to various cytotoxic agents such as cyclosporine, paclitaxel, 5-FU, and IUdR.[26] Recent data have shown that Res inhibits activation of HIF1-α in the hypoxia conditions.[58] Zhang et al[59] showed the different doses of Res (5, 10, 50, and 100 mM) decreased HIF1 and VEGF mRNA in SCC-9 human tongue squamous cell carcinoma and HepG2 hepatoma monolayer cells in hypoxia condition. Also, Res inhibits HIF1-α protein expression via regulating protein translation. After inhibition, the HIF1-α, cells progress from G0 to cell cycle and could absorb 5-FU in the S phase. So the radiosensitivity of cells increased. Firouzi et al[60] demonstrated treatment of 350 mm U87MG glioblastoma spheroids with 20 mM Res can increase the radiosensitivity of 1 mM IUdR. Liao et al[61] showed that 25 mM Res decreased the percentage of G0/G1 lung cancer cells (NCI-H838), and the number of them has increased in the S phase. In this study, we used this effect of Res to increase the 5-FU absorption in hypoxia cells (Figure 3). Khoei et al[62] showed 2ME2 can inhibit HIF1-α activity and increased IUdR radiosensitivity and cytotoxic damages. They also showed that Res like 2ME2 has been acted as HIF1-α inhibitor but is safer because of its herbal nature and it can increase the cytotoxic damages of IUdR +Rad.[58] They observed a significant decrease in PE percentage in combination treatment of Res+IUdR+Rad. Finally, in this study, the effect of Res as HIF inhibitor on the radiosensitivity of 5-FU was evaluated. Figure 3 shows that the combination of 5-FU+Rad+Res was efficient to highly reduce the clonogenic ability of MCF-7 cells in comparison with 5-FU+Rad. It appears Res could inhibit HIF1α expression and induce to progress the hypoxia spheroid cells in the cell cycle to the S phase. In this condition, these cells could uptake 5-FU and sensitize to Rad.

5 | CONCLUSION

Our data suggest a new strategy for anticancer therapy for breast cancer because the combination of Res with 5-FU can decrease the colony formation ability of gamma-ray radiation in 300 μm spheroids. So the cytotoxicity effect of Rad and therapeutic ratio increases.

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CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest.

AUTHOR CONTRIBUTIONS

MS conceived and designed the experiments. AJ and SA performed the experiments and wrote the manuscript. FZ and MA analyzed the data. SA checked the grammar and plagiarism of the manuscript. SA also submitted the manuscript.

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