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Comparison of radiosensitizing effect of Resveratrol on monolayer and spheroid culture of DU145 prostatic cell line

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Background: Radiotherapy is an established therapeutic modality for prostate cancer. Resveratrol, a natural antioxidant, has been shown to inhibit carcinogenesis and to block the process of tumor initiation and progression. No data is available on the response of cellular spheroid to Reseveratol. In this study we have examined the effect of Resveratol on the radiation response of human prostate cell line DU145 in monolayer and spheroid cultures. ***Materials and Methods:*** Radiosensitivity was assessed using viability and colony formation assay. Apoptosis and necrosis were assessed using acridine orange/ ethidium bromide double staining. ***Results:*** The colony formation assay did not show any significant radio-sensitizing effect, but apoptosis assay showed significant radio-sensitizing effect of Resveratol on DU145 cells grown as monolayer. In the spheroid cells the results of apoptosis test were not significant and corresponded closely to the result of survival curve. ***Conclusion:*** While Resveratol could sensitize DU145 cells in monolayer to ionizing radiation, it did not have any effect on sensitivity of cells cultured in spheroid cultures. **Iran. J. Radiat. Res., 2012; 10(3-4): 177-181**

Keywords: *Resveratrol, X-ray irradiation, multicellular spheroid, radioresistance, apoptosis.*

INTRODUCTION

Radiotherapy is broadly used for the therapy of cancer through induction of apoptosis in tumor cells [(1)]. Biological radiosensitizers can enhance the sensitivity of tumors to ionizing radiation [(2)]. Resveratrol, a potent anticancer and radiosensitizer compound, is a natural polyphenol derived from grapes, plums and peanuts [(3)]. A large number of *in vitro* studies have dealt with the potent antiproliferative/pro-apoptotic effects of Resveratrol on different human prostatic cancer cell lines [(4)]. Previous study by Scarlatti *et al.* has cleared the underling molecular mechanism of overcoming radioresistancy in DU145 cells treated with Resveratrol. It has been shown that Resveratrol through increasing *de novo* production of ceramide in DU145 cell line results in apoptotic cell death [(2)]. Furthermore, these cells can be self-assembled into morphologically large-size, spheroid-shaped and stable aggregates so-called multicellular tumor spheroids (MCTS) through intracellular communication networks. The microenvironment of MTCSs is closer to *in vivo* tumors than monolayer cultures [(5)]. In 1972 Durand *et al.* demonstrated that exposing spheroids of Chinese Hamster V79 -1716 cells to ionizing radiation made them more resistance than monolayer cultured cells [(6)]. So far the effect of Resveratrol on cancer cells cultured in MCTS has not been evaluated. In this work we have compared the radiosensitizing effect of Resveratrol on MCTS and monolayer cultures of DU145 prostatic cell line.

MATERIALS AND METHODS

Reagent

Resveratrol from sigma- Aldrich was

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dissolved in ethanol (96% Merck) before use. Trypan blue, metal green, Penicillin and MTT (3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a yellow tetrazole), were from Sigma–Aldrich. Trypsin and ethidium bromide (EB) was from Merck. Cell culture media (RPMI-1640) and fetal bovine serum were from Gibco. Streptomycin was from Jaberebn-Hayan. Agar was from Difco, Detroit, MI, USA (Bacto agar). Acridine orange (AO) was from Hopkin & Williams Ltd.

Irradiation procedure

Irradiation of monolayer cell culture: cells were cultured at 25×10^4 cells per flask in T-25 culture After 4 days, the cells were exposed to 2, 4, 6, 8 and 10 Gy X-rays with dose rate of 200 cGy/min using linear accelerator (primus, Siemens). Control cells were not exposed to X-ray. For treatment of Resveratrol, after 24 h, cells were treated with 25 µM Resveratrol or 0.05% of ethanol (as vehicle) for 72 h. Then, cells were irradiated and cell viability and apoptotic and necrotic cells were evaluated.

Irradiation of spheroid culture: Cells were cultured at 5×10^5 cells per Petri dish in 100 mm dishes. After 11 days, the spheroids were divided into T25 flasks. The cells were then exposed to 2, 4, 6 and 8 Gy of X-rays with dose rate of 200 cGy/min. After 8 day, spheroids were treated with 50 µM Resveratrol or 0.05% of ethanol (as vehicle) for 72 h. Then, cells were irradiated and cell viability and apoptosis and necrosis were evaluated.

MTT assay

Exponentially growing cells plated into 96 well plates and incubated 24 h at 37 ˚C in 5% CO_2. For spheroids, after 11 days, spheroids were dispersed, plated into 96 well plates, and incubated 24 h at 37 ˚C in CO_2. Cells were subsequently exposed to incremental concentration of Resveratrol (10 -100µM for monolayer and 50-200 µM for spheroid) in 200 µL RPMI and incubated for 72 h. Then, 20 µL MTT (5 mg/ml) was add to each well protected from light and incubated at 37 ˚C for 4 h. Formosan crystals were dissolved by adding 100 µL of DMSO (99% HPLC- Merck) for 30 min. An ELISA plate reader (Lab systems multiskan MS) was used to read the absorbance with a wavelength of 570nm.

Cell survival clonogenic assay

Briefly, 2×105 cells were incubated into T -25 Flask. After 24 h, the cells were incubated for 72 h at 37 ºC with or without Resveratrol (25µM). For spheroids, the cells were treated with Resveratrol (50 µM) at 8 days after spheroid formation and were incubated with or without Resveratrol for 72h. Next, the cells were irradiated with X-rays (2Gy). Cells were seeded in 60-mm dishes at various cell densities. After 9 days, the resulting colonies were stained with crystal violent dissolved in PBS. Colonies containing more than 50 cells were scored as survivors. Cell survival curve was analyzed with multi-target single-hit model (MTSH).

Assay for apoptosis and necrosis

Briefly, 2×10^5 cells were incubated into T-25 flasks. After 24 h, the cells were incubated for 72h at 37 ºC with or without Resveratrol (for monolayer 25 µM and for spheroid 50 µM). Next, the cells were irradiated with X-rays (2 Gy). Then, the cells were stained with AO (100 mg/ml) and EB (100 mg/ml). Viable, apoptotic and necrotic cells were counted under a fluorescence microscope.

Statistical analysis

Differences between groups were analyzed by using repeated measures analysis of variance (ANOVA) and P-value < 0.05 was considered to be significant.

RESULTS

MTT Assay

The effect of Resveratrol on the viability of DU145 cells determined by MTT assay is

shown in figure 1a. The half maximal inhibitory concentration (IC50) for Resveratrol was calculated as 43 µM. Cytotoxity of Resveratrol on spheroids was measured in the same way as monolayer cultures and IC50 was calculated 163 µM (figure 1b). There was significant difference in IC50 for monolayer and spheroid cells.

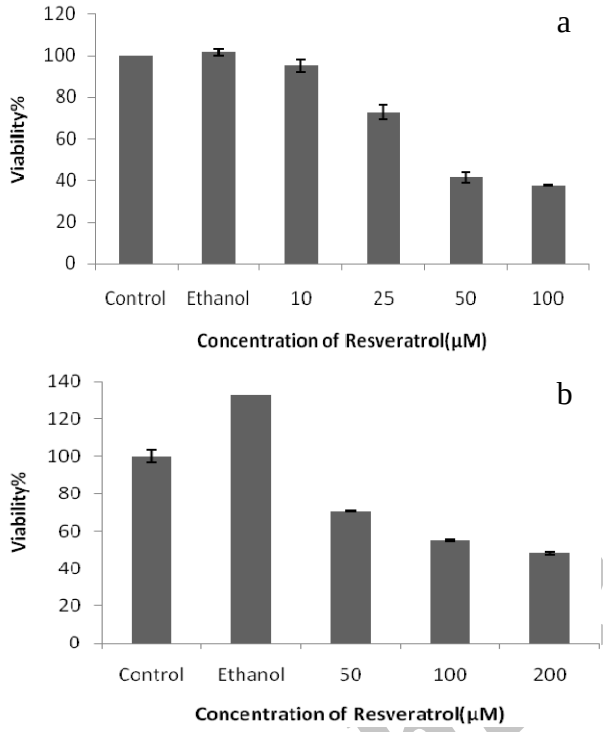

Figure 1. a) Cell viability measured with MTT in DU145 Cells. The values of optical density measured at λ = 570 nm are reported as percentage with respect to the optical density registered for untreated control, the latter considered as 100% of cell viability. The values are the mean ± SEM of three experiments performed in triplicate (*n* = 3). b) Cell viability measured with MTT in DU145 cells grown as spheroids. The values are the mean ± SEM of three experiments performed in triplicate (*n* = 3).

Effect of Resveratrol on radiosensitivity of monolayers

Figure 2A shows the cell survival curves of actively growing DU145 cells in mono layer cultures exposed to X-rays with and without Resveratrol treatment. Fitting parameters for MTSH model is shown in table 1a. In survival curve of DU145 cells the value of n, decreased in the combined treatment of Reseveratrol and radiation as compared to radiation alone. Based on D_0 values, Resveratrol, slightly but not significantly, increased the radiosensitivity of Du145 cells cultured in monolayer condition.

Table 1. a) The fitting parameters for the MTSH model in DU1145 cells. b) The fitting parameters for the MTSH model DU145 cells grown as spheroids.

Groups	Dq	D0	n
X-ray and Resveratrol	1.16±0.087	0.75±0.004	4.63±0.49
X-ray	2.04±0.283	0.79±0.025	13.16±6.44

Groups	Dq	D0	n
X-ray and Resveratrol	1.94±0.075	0.92±0.05	8.29±1.67
X-ray	2.11±0.755	1.16±0.26	6.18±9.35

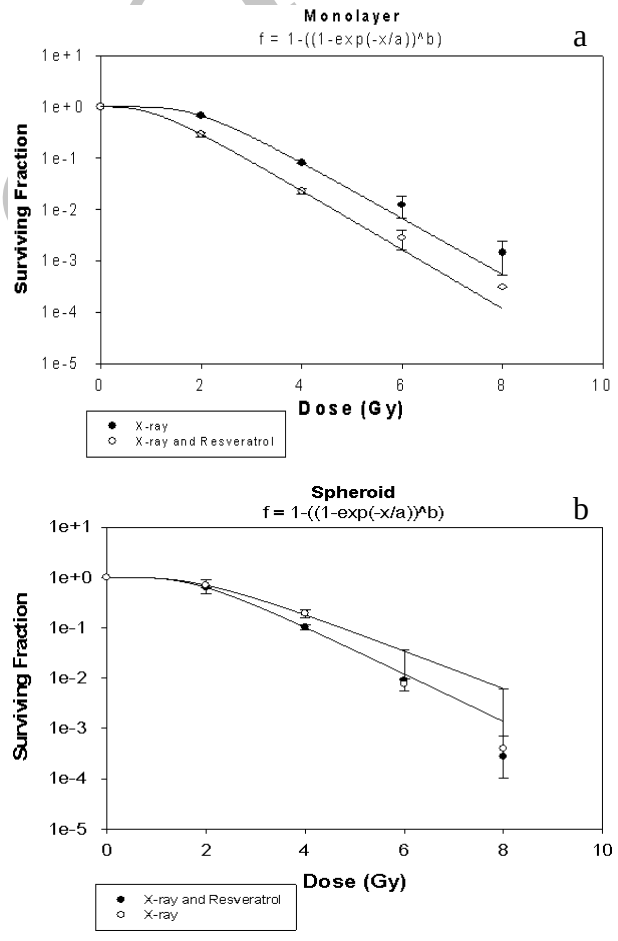

Figure 2. a) Survival curve of DU145 cells following X-ray irradiation, with or without resveratrol treatment. Data points and bars represent the average and standard error of 2 separate experiments. b) Survival curve of DU145 cells grown as spheroids and irradiated with or without resveratrol treatment. Data points and bars represent the average and standard error of 2 separate experiments.

Effect of Resveratrol on radiosensitivity of spheroids

The results of clonogenic survival assay are shown in figure 2B. Fitting parameters for MTSH model is shown in table 1b. Resveratrol, slightly but not significantly, increased the radiosensitivity of Du145 cells cultured in spheroid condition.

In the survival curve, Dq, which is a measure of the width of the shoulder of survival curve, showed a significant decrease in the combination of the two treatments (Resveratrol and radiation) in monolayer compared to spheroid in the same condition. D_0 value represents the measure of sensitivity of the target. In the present study Resveratrol decreased D_0 in both monolayer and spheroid cultures irradiated with 2 Gy of X-Ray.

Effect of Resveratrol on radiation-induced apoptosis and necrosis in DU145 cells

Figure 4 A shows the percentage of apoptotic and necrotic cells after X-irradiation with and without Resveratrol treatment. For cells irradiated without Resveratrol treatment, the percentages of apoptosis and necrosis were approximately 18.7 and 0.7 respectively. But in these cells, early apoptotic cells percentage was more than late apoptotic cells percentage. For cells irradiated with Resveratrol treatment, the percentage of apoptotic cells markedly increased, to approximately 59.5. These data were significantly more than the percentage of apoptotic cells induced by both Resveratrol and radiation treatment alone.

Effect of Resveratrol on radiation-induced apoptosis and necrosis on DU145 Cells grown as spheroid

Figure 3 shows images of apoptotic, necrotic, as well as control DU145 cells as monolayer and spheroid after AO/EB staining. Late apoptotic cells have an orange nucleus showing condensation of chromatin and necrotic cells displayed an orange nucleus with intact structure. Figure 4B shows the percentage of apoptotic and necrotic cells after X-irradiation with and without Resveratrol treatment in spheroid cells. For cells irradiated without Resveratrol treatment, the percentages of apoptosis and necrosis were approximately 24.8 and 6 respectively. For cells irradiated with Resveratrol treatment, the percentage of apoptotic cells increased but these data were not significant and corresponded closely to the result of survival curve.

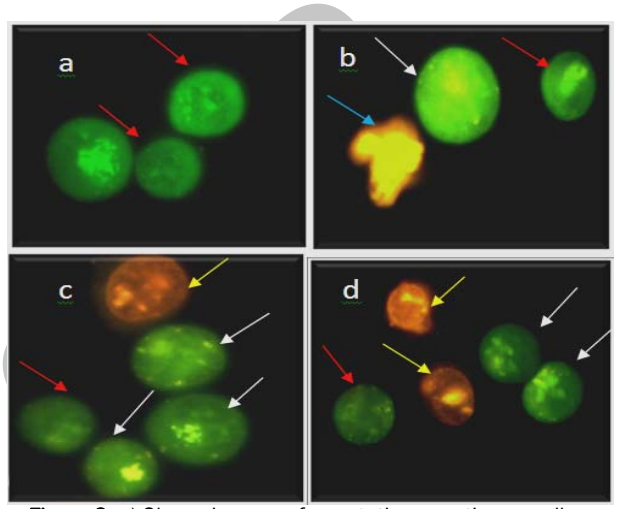

Figure 3. a) Shows images of apoptotic, necrotic, as well as control Du145 cells after acridine orange/ethidium bromide staining. b) Shows images of apoptotic, necrotic, as well as control Du145 spheroids after acridine orange/ethidium bromide staining. c) shows Du145 cells after treatment with Resveratrol and radiation. d) shows Du145 spheroids after treatment with Resveratrol and radiation. The red arrows show viable cells. The yellow arrows show late apoptotic cells (dead=orange) with condensation and chromatin clumping. The white arrows show early apoptotic live cells (green) with chromatin super-aggregation i.e. highly condensed chromatin. The blue arrows show necrotic cells.

DISCUSSION

Resveratrol is well-known as a potent radiosensitizer compound in a variety of cancer cell lines. Scarlatti *et al.* showed that resveratrol enhanced tumor cell killing and inhibited the clonogenic survival in resistant irradiated-DU145 cells. In the survival curve (Resveratrol and X-ray) the shoulder region declined rapidly. Regarding the shoulder region of the curve as a measure of the repair capacity of the cell for

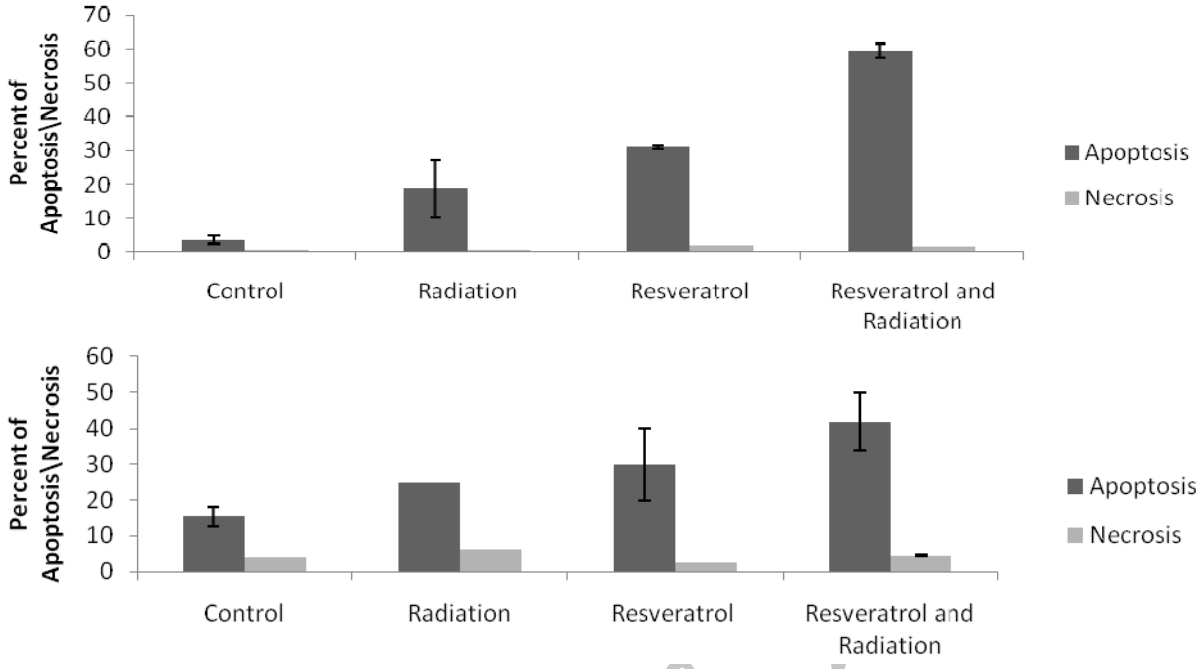

Figure 4. a) Extent of necrosis and apoptosis in DU145 cells irradiated with X-rays, with or without Resveratrol treatment. Values are the means ± SD of two independent experiments. b) Extent of necrosis and apoptosis in DU145 cells grown as spheroids irradiated with X-rays, with or without Resveratrol treatment. Values are the means ± SD of two independent experiments.

radiation damage after exposure to low doses, especially ~ 2 Gy [7], it may be suggested that Resveratrol sensitizes DU145 in 2Gy dose of radiation through inhibition of repair enzymes. This effect was not clear in the survival curves of spheroids. The clonogenic radiosensetization was more pronounced for spheroid cultures compared to monolayers. According to results presented in the previous section the most pronounced effect of Resveratrol was on the induction of apoptosis. As expected, the apoptotic shock was more sever in monolayer cultures as compared to spheroids. Our results indicate that Resveratrol can sensitize DU145 prostatic cells in monolayer and spheroid cultures to radiation but, under similar conditions, Resveratrol and 2Gy of radiation are more effective on monolayers then on spheroid cultures. The low efficacy of Resveratrol on the cell death in spheroid cells is likely due to the fact that the drug cannot reach all the cells, especially those located in the core of the aggregate.

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