

Resveratrol and radiation biological effects

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ABSTRACT

Resveratrol is a phytoalexin, a phenolic compound present in wines and several plants. This compound is related to a broad spectrum of biological activities such as antioxidant and anticarcinogenic effects that are very important in prevention of cancer, cardiovascular diseases and other diseases caused by oxidative processes. Over the last years, biological effects of ionizing radiation in the presence of resveratrol have been studied in different cell cultures. The aim of this study was to verify the effect of gamma radiation on mouse connective tissue cells (NCTC clone 929) in culture in the presence of *trans*-resveratrol. Cell viabilities were analyzed by neutral red uptake assay. The results demonstrated *in vitro* the radioprotective effect of *trans*-resveratrol on cell culture and it was more pronounced when cell culture was irradiated at 500-800 Gy doses in the presence of resveratrol concentrations between 12.5 and 25 µM. These results provide evidence that *trans*-resveratrol alters the cellular response to ionizing radiation, expanding the knowledge of resveratrol biological properties in physiological and pathological processes, contributing to the development of future studies about the possibility of including resveratrol and its derivatives in dietary supplements given to cancer patients during radiotherapy.

Key words: Resveratrol; dietary supplement; Radioprotection; Lethal dose of gamma radiation; Cytotoxicity assay.

INTRODUCTION

Resveratrol (3,5,4'-trihydroxystilbene) is a phenolic phytoalexin that occurs naturally in a wide variety of plants, such as grapevines, in response to injury as fungal infections, exposure to ultraviolet light and some chemical agents (1-3). Resveratrol is found in nature as both *cis*- and *trans*- isomers; however, the *trans*-isomer is believed to be the most biologically active form than *cis*-isomer (4).

Studies about biological activity and molecular mechanisms of *trans*-resveratrol show highest antioxidant properties (5). These properties of *trans*-resveratrol confer protection against atherosclerosis; coronary heart diseases (6); inflammatory processes (7, 8); neurodegenerative processes such as Alzheimer's disease (9); and prevents cancer development (10-12). Antioxidant properties of *trans*-resveratrol are mediated by its free radicals scavenger ability (13).

Free-radicals are atoms or molecules that have a single unpaired electron in one orbit. They are considered toxic products formed by both several physiologic

processes and incidence of ionizing radiation in the body (14, 15), allowing the study of new scavenger compounds is of great interest to human health.

Radiotherapy is a modality of treatment against cancer utilizing ionizing radiation, which causes biologic damages by direct and indirect actions. By the direct action, the radiation ionizes directly a biological macromolecule such as DNA and proteins. In turn, the indirect ionizing causes a chemical reaction that forms free-radical (16). Free radicals were most commonly formed by interaction between ionizing radiation and water, which constitutes 70% to 80% of cell contents. This process forms the "primary products of water radiolysis", whose components are extremely reactive and able to trigger several toxic reactions within the cells (17, 18).

Therefore, properties of *trans*-resveratrol such as its low toxicity, antioxidant activity, chemical stability and ability to inhibit the carcinogenic processes of different tumor cell types make the study of its radioprotective effect in normal cells an important contribution to medical oncology. Currently, the possibility of including resveratrol

and its derivatives in the dietary supplements given to cancer patients during radiotherapy may be relevant, although it is necessary more studies about this subject (7, 8, 10-12, 19, 20).

The aim of this research is to verify *in vitro* the gamma radiation effect on cell culture in the presence of *trans*-resveratrol, by cell survival measurement. For this study, it was necessary to know the resveratrol toxicity level carried out by *in vitro* cytotoxicity test and also the gamma radiation lethal dose determination in cell culture by *in vitro* assay.

MATERIALS AND METHODS

The culture minimum Eagle medium (MEM) was produced by Center for Cell Culture from Instituto Adolfo Lutz (São Paulo, SP) and reagents were purchased from Sigma and Merck (São Paulo, SP).

The used resveratrol was a *trans*-resveratrol preparation characterized by its high purity (>99% *trans*-resveratrol) from Attivos Magistrais - Pharmacopeia Group (São Paulo, SP).

Cell Culture

The mouse connective tissue fibroblastic cell line, NCTC clone 929, from American Type Culture Collection (ATCC CCL1), was maintained in MEM supplemented with 10% fetal calf serum (FCS), 0.1 mM non-essential amino acids and 1.0 mM sodium pyruvate (work-MEM). No antibiotics were added to the cell culture medium. The cell culture flask was kept in an incubator at 37°C with humidified atmosphere and 5% CO₂ until cell monolayers attained confluence which occurred after 24 h. Thereafter, the cells monolayer was detached from the cell culture flasks by 0.20% trypsin and 0.02% EDTA solution. The cell suspension was adjusted for the tests to be performed.

Microplate Preparation

For cytotoxicity and lethal dose assays, the cell suspension was adjusted to 3.5 x 10⁵ cell/mL and 0.2 mL of this cell suspension was distributed to each well of a flat-bottomed 96 wells microplate. To verify the effect of gamma radiation on cell survival in presence of resveratrol, the cell suspension was adjusted to 1.0 x 10⁵ cell/mL and 0.2 mL/well was seeded in microplates. Prepared microplates were incubated during 24 h at 37°C in a humidified 5% CO₂ atmosphere.

Cytotoxicity Assay

The cytotoxicity of resveratrol was assessed by neutral red uptake assay. Stock solution of resveratrol (1.0 x 10⁴ µM) was prepared in pure ethanol. The NCTC clone 929 cells line was exposed to 0.2 mL of resveratrol solutions serially diluted with work-MEM. The resveratrol concentrations were: 100, 50, 25, 12.5, and 6.25% which

correspond to 250, 125, 62.5, 31.25, and 15.63 µM. The positive-control (0.02% phenol solution) and negative-control (polyvinyl chloride extract) received the same dilution. Volumes of 0.2 mL of diluted controls and resveratrol solutions were added in each well. It was added 0.2 mL/well of fresh work-MEM to cell control wells. All the samples were tested in triplicate. The microplate was incubated during 24 h at 37°C in a humidified incubator with 5% CO₂ atmosphere. After incubation, the culture medium was replaced by culture medium containing neutral red and the microplate was incubated again during 3 h at 37°C. Afterwards, the dye-containing medium was discarded; microplate was washed twice with phosphate buffered saline solution (PBS) and once with 1% of 10% calcium chloride in 0.5% formaldehyde solution. Volume of 0.2 mL of extracting solution (50% ethanol in 1% acetic acid) was added in each well to promote cell rupture and neutral red release. The dye absorbance was measured at 540 nm using an automated spectrophotometer ELISA reader type RC Sunrise - Tecan.

Lethal Dose of Gamma Radiation Method

To determine the gamma radiation dose that induces the death of 50% of cell population in culture (LD₅₀), microplates containing NCTC clone 929 line cells were irradiated by gamma rays. The irradiation process was performed in a ⁶⁰Co gamma source (GammaCell 200, Atomic Energy of Canada, Ltd., CAN) at a dose rate of 2.64 kGy/h, with different doses (1000, 750, 500, and 250 Gy), in a single fraction at room temperature. Non-irradiated cells, used as control, were kept under protection from light at room temperature during the irradiation process of the others microplates. After irradiation, culture medium was replaced by work-MEM and afterwards the microplates were incubated at 37°C in a humidified 5% CO₂ atmosphere, during 24 h. The following procedures of neutral red dye incorporation, microplates washing and the optical densities reading were performed as described above in the cytotoxicity assay.

Cell Culture Exposures to Gamma Radiation in the Resveratrol Presence

This assay evaluates the effect of gamma radiation on cell culture in a presence of resveratrol. The resveratrol concentration range used was lower than IC₅₀ and the gamma radiation dose range close to LD₅₀. Microplates containing NCTC clone 929 line cells received serially diluted resveratrol with work-MEM in a concentration of 30, 25, 12.5, 6.25, and 0 µM. These microplates were incubated during 24 h at 37°C with humidified atmosphere and 5% CO₂. Following incubation, microplates were exposed to a single fraction of gamma radiation doses of 800, 500, 300, and 0 Gy in a ⁶⁰Co gamma rays source, GammaCell 200, Atomic Energy of Canada, Ltd., CAN, at 2.28 kGy/h dose rate and room temperature. Control

microplate, with non-irradiated cells, was kept under protection from light at room temperature during the irradiation process of the others microplates. Just after irradiation, microplates culture medium was replaced by work-MEM and afterwards these microplates returned to incubator at 37°C in a humidified 5% CO₂ atmosphere during 24 h. After incubation time, procedures of neutral red dye incorporation, microplates washing and the optical densities reading were performed as described above in the cytotoxicity and lethal dose of gamma radiation assays.

RESULTS AND DISCUSSION

In vitro cytotoxicity of resveratrol and the lethal dose of gamma radiation assays were performed to assist in the cell culture exposures to gamma radiation in resveratrol presence assay. All experiments were performed in mouse connective tissue cells, NCTC clone 929 cell line. This cell line was used due to its easy to cultivate and favorable cellular doubling time of about 24 h.

The cytotoxicity of *trans*-resveratrol assay was carried out according to the International Standardization Organization and the earlier publication (21, 22). In this assay, the cell survival percentage of each extract concentration of the sample and controls was determined by the Equation 1:

$$\text{Cell Viability (\%)} = \frac{[A]_{\text{test}}}{[A]_{\text{control}}} \times 100 \quad (1)$$

Where, *A* is the medium of corresponding absorbance of each triplicate.

Positive-control and negative-control are used to check the assay performance and to validate the test results.

Table 1 shows the cell viability percentage results calculated from cytotoxicity assay.

Survival curves are obtained by cell viability percentage plotted as a function of the resveratrol extract concentration, positive and negative controls, as shown in Figure 1.

The cytotoxicity index (IC₅₀) was estimated by graphical analysis in the Figure 1. It was estimated at the intersection between the IC₅₀ line and the cell viability curve. In this analysis, resveratrol exhibited 50% cell viability at a concentration of about 20%. The correspondent resveratrol in the extract concentrations were: 100% = 250 µM, 50% = 125 µM, 25% = 62.5 µM, 12.5% = 31.25 µM, and 6.25% = 15.63 µM. Therefore, IC₅₀ of about 20% corresponds to the value of 50 µM. It means that *trans*-resveratrol in a concentration of 50 µM induces injury or death of 50% of the cell population used in this assay.

This obtained result is in accordance with the Sgambato et al. study, which showed a dose-dependent decrease in cell viability in all cell lines tested and their IC₅₀ achieved were 22 to 109 µM. These values depended of cell line sensitivity (19).

In the lethal dose assay (LD₅₀), the cell viability percentage was calculated for each radiation dose microplates in relation to microplate control by Equation 2:

$$\text{Cell Viability (\%)} = \frac{[A]_{\text{irradiated}}}{[A]_{\text{control}}} \times 100 \quad (2)$$

Where, *A* is the medium of corresponding absorbance of each microplate.

The cell viability percentage results are presented in Table 2. For graphical analysis, the cell viability percentage was plotted in function of the irradiated dose. The survival curve obtained was presented in Figure 2.

LD₅₀ is estimated in the intersection of 50% viability line with the survival curve. Thus, LD₅₀ value was about 354 Gy; this is the radiation dose which induces the death of 50% of cell population in the assay.

The evaluation of gamma radiation effect on cell culture, in a presence of resveratrol, was by calculation of the cell viability percentage (it was calculated in relation to control cells=100%) for each microplate, by Equation 3. Non-irradiated microplates were standardized as cell viability control, considered 100%.

$$\text{Cell Viability (\%)} = \frac{[A]_{\text{Resveratro + Gamma Radiation}}}{[A]_{\text{control}}} \times 100 \quad (3)$$

Where, *A* is the medium of corresponding absorbance of each microplate, considering resveratrol concentration and irradiation dose.

Table 3 shows cell viability percentage results of gamma radiation effect on cell culture in the presence of resveratrol assay.

Cell viability percentage was plotted in relation to resveratrol concentration for different gamma radiation doses. Cell viability curves obtained were presented in Figure 3.

Table 3 and Figure 3 show that cell viability decreases with increasing gamma radiation dose. When the irradiation dose is close to gamma radiation lethal dose, there is no change of cell viability in relation to resveratrol concentration. However, when gamma radiation is within 500 and 800 Gy it is observed a cell viability increase in the presence of 25 and 30 µM *trans*-resveratrol concentrations. These results suggest that *trans*-resveratrol has radioprotective effect in normal cells culture at 25 µM and

30 μM *trans*-resveratrol concentrations when irradiated at 500 Gy and 800 Gy doses. This radiation dose is higher than lethal dose of gamma radiation ($\text{LD}_{50} = 354 \text{ Gy}$).

Similar results were identified in the *in vivo* study conducted by Carsten et al. (23). In this study the authors showed that resveratrol antioxidant property contributes to the reduction of the mean total chromosome aberration frequency in mouse bone marrow cells and it suggests a radioprotective action of resveratrol.

In contrast to the radioprotective effect of resveratrol shown in normal cells exposed to the damaging effects of ionizing radiation observed in our work, the *in vitro* studies conducted in tumor cells line by Liao et al. (24) and Zoberi et al. (25) showed that resveratrol is a potent radiosensitizer. This property is correlated to genetic alterations shown by different tumor cells lines and also by its ability to inhibit cell cycle progression. However, specifics biochemical pathways of resveratrol determining the radiosensitization of tumors cells and radioprotection of normal cells to ionizing radiation are less clearly understood.

All these studies are important for understanding that resveratrol plays an important role in the cell response to ionization radiation, both in the radioresistance and radiosensitivity of normal and tumor cells lines.

CONCLUSION

This research provides data on the *in vitro* radioprotective potential of *trans*-resveratrol (cells culture irradiated at 500 Gy and 800 Gy doses in the presence of 25 μM and 30 μM *trans*-resveratrol concentrations), resveratrol low toxicity ($\text{IC}_{50} \sim 50 \mu\text{M}$) and *in vitro* lethal dose of gamma radiation on mouse connective tissue cell line culture ($\text{LD}_{50} \sim 354 \text{ Gy}$).

The data obtained in this work expand the knowledge about *trans*-resveratrol biological properties on physiological and pathological processes, it contributing to the development of future studies about the possibility of including resveratrol and its derivatives in dietary supplements given for cancer patients during radiotherapy to assist in the effectiveness of treatment.

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TABLE 1 - Cell viability percentage of the resveratrol cytotoxicity assay by neutral red uptake method

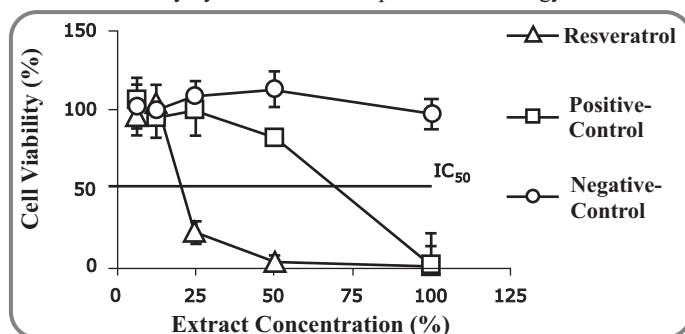
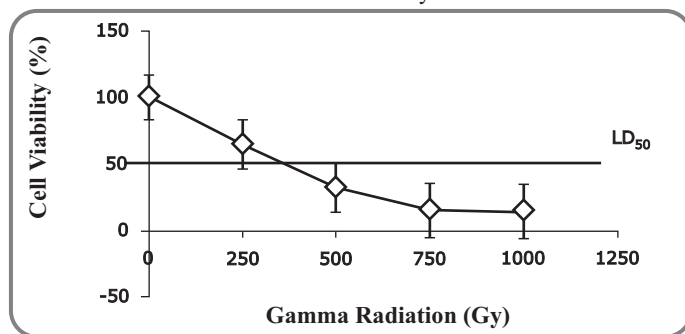
Extract Concentration (%)	Cell Viability $\pm$ vc (%)		
	Resveratrol	Positive-Control	Negative-Control
6.25	95.98 $\pm$ 9.13	106.55 $\pm$ 9.25	102.25 $\pm$ 14.29
12.5	102.91 $\pm$ 9.66	95.77 $\pm$ 4.20	99.27 $\pm$ 12.18
25	22.96 $\pm$ 8.98	100.34 $\pm$ 11.49	109.00 $\pm$ 7.23
50	3.31 $\pm$ 7.87	82.54 $\pm$ 10.31	112.99 $\pm$ 11.42
100	1.07 $\pm$ 11.14	1.93 $\pm$ 16.44	97.33 $\pm$ 6.31

TABLE 2 - Cell viability percentage of the gamma radiation lethal dose assay

	Gamma Radiation Dose (Gy)				
	0	250	500	750	1000
Cell Viability (%)	100.00 $\pm$ 16.99	64.85 $\pm$ 19.12	31.72 $\pm$ 17.70	16.22 $\pm$ 20.45	13.80 $\pm$ 19.99

TABLE 3 - Cell viability percentage of the cell culture exposed to gamma radiation in the presence of resveratrol assay

Gamma Radiation Dose (Gy)	Resveratrol Concentration (μ M)				
	0	6.25	12.5	25	30
0	100.00 $\pm$ 18.29	100.00 $\pm$ 15.32	100.00 $\pm$ 19.13	100.00 $\pm$ 19.94	100.00 $\pm$ 19.86
300	69.41 $\pm$ 12.12	58.08 $\pm$ 13.30	58.94 $\pm$ 19.48	69.59 $\pm$ 19.65	67.32 $\pm$ 15.07
500	46.39 $\pm$ 19.59	48.57 $\pm$ 15.02	41.97 $\pm$ 19.78	66.88 $\pm$ 20.49	64.48 $\pm$ 15.15
800	22.86 $\pm$ 19.60	28.84 $\pm$ 16.52	23.38 $\pm$ 20.26	52.94 $\pm$ 17.72	31.36 $\pm$ 19.80

FIGURE 1 - Cell viability curves of the resveratrol cytotoxicity assay by the neutral red uptake methodology.**FIGURE 2** - Cell survival curve of the gamma radiation lethal dose assay.**FIGURE 3** - Cell viability curves of gamma radiation effect on cell culture in the presence of resveratrol assay.