2011 International Nuclear Atlantic Conference - INAC 2011
Belo Horizonte,MG, Brazil, October 24-28, 2011
ASSOCIAÇÃO BRASILEIRA DE ENERGIA NUCLEAR - ABEN
***ISBN:* 978-85-99141-04-5**

DETERMINATION OF GAMMA RADIATION LETHAL DOSE (LD_{50}) AND RESVERATROL CYTOTOXICITY LEVEL IN TUMOR CELLS LINE

Vanessa D. Magalhães[1], Sizue O. Rogero[1], Áurea S. Cruz[2], José R. Rogero[1]

[1] Instituto de Pesquisas Energéticas e Nucleares (IPEN/CNEN-SP)
Av. Professor Lineu Prestes, 2242
05508-000 São Paulo, SP
(11) 3133-5193
van.biologa@gmail.com

[2] Instituto Adolfo Lutz (IAL-SP)
Seção de Culturas Celulares, 2º andar
Av. Doutor Arnaldo, 355
01246-000 São Paulo, SP
(11) 3068-2910
aurcruz@terra.com.br

ABSTRACT

Cancer is a disease with high incidence and it is considered a worldwide public health problem. Resveratrol is a polyphenol occurring naturally in a wide variety of plants according to response of ultraviolet radiation (UV) exposition or according to mechanical stress resulting of pathogens or chemical and physical agents. This polyphenol possesses a pharmacological activity of carcinogenesis inhibition in multiple levels. It also protects cells by scavenging the free radicals which are considered toxic products. These free radicals are formed of natural process of cell aging and also by incidence of ionizing radiation in the organism. Thus, resveratrol is considered as a cell radioprotector. On the other hand, in some elevated concentrations resveratrol may be considered as a radiosensityzing. The aim of this work was the determination of radiation lethal dose (LD_{50}) and also verifies the citotoxicity level of resveratrol in tumor cells line: mucoepidermoid pulmonary carcinoma cells (NCI-H292) and rhabdomyosarcoma cells (RD). The cytotoxicity test was performed by neutral red uptake assay. The results of resveratrol $IC_{50\%}$ in NCI-H292 cells was 192µM and in RD cells was 128µM; and RD cells gamma radiation LD_{50} was 435Gy.

1. INTRODUCTION

Cancer is a disease with high incidence and is considered a worldwide public health problem. According to Brazilian Cancer National Institute [1] are expected 489,270 new cases of cancer in 2011.

Resveratrol may inhibit carcinogenesis in multiple stages. Systemic administration of resveratrol has been shown to inhibit the initiation and growth of tumors in a wide variety.

The effectiveness of resveratrol suggests that even low concentrations obtained from dietary sources, as well as red wine and grape juice, may be therapeutic in some cases [2].

Resveratrol (3, 4`, 5-trihydroxiestilbeno) is a polyphenol belonging to the group of phytoalexins [3]. This defense polyphenol is synthesized by a variety of plants in response of ultraviolet radiation (UV) exposure or according to mechanical stress caused by pathogens, chemical and physical agents [4]. The vines are plants that are considered highly capable to produce resveratrol [5]. In grapes, the synthesis occurs in the peel of the fruit.

In wines, especially in red wine the resveratrol concentration is relatively high due to the maceration and fermentation of the fruit with peel. The industrialized grape juices are considered good sources of resveratrol to abstemious, although its concentration is lower than wines [6].

The protective effects performed by resveratrol during the process of cell damage, produced by oxidative effects of free radicals, promote anti-inflammatory [7], anti-platelet [5] and anti-carcinogenic activity [5], prevent or inhibit degenerative diseases [8], decrease incidence of cardiovascular diseases [9]. Moreover, resveratrol is considered as a cell radioprotector. On the other hand, in some elevated concentrations resveratrol may be considered as a radiosensitizing.

The ionizing radiation is considered a whole carcinogenic agent, because it can act as an initiator and promoter of tumor carcinogenesis [10].

The aim of this work was to verify the cytotoxicity level of resveratrol and the *in vitro* determination of gamma radiation lethal dose (LD_{50}) to tumor cells line.

2. METHODOLOGY

The used tumors cell lines NCI-H292 (human mucoepidermoid pulmonary carcinoma) and RD (human rhabdomyosarcoma), provided by Seção de Culturas Celulares of Instituto Adolfo Lutz and obtained from ATCC (American Type Culture Collection). The NCI-H292 cells were grown and maintained in culture medium RPMI 1640, supplemented with 15% fetal bovine serum (FBS) (work medium), and the RD cells were grown and maintained in culture medium Eagle+L-15, supplemented with 15% FBS (work medium) at 37 ºC and humid atmosphere with 5% CO_2.

The cytotoxicity of resveratrol and gamma radiation lethal dose assays was carried out according to International Standard Organization (ISO 10993-5) and previous published paper [11, 12].

2.1 Determination of gamma radiation Lethal Dose (LD_{50})

Tumors cells were plated in 96-well microplates at concentration of $5x10^4$ cells in each well and maintained in their respective cell culture medium at 37ºC and humid atmosphere with 5% CO_2. It was used 5 microplates for gamma radiation LD_{50} determination assay and each microplate was submitted to different gamma radiation dose: 0 Gy (used as control), 250 Gy, 500 Gy, 750 Gy and 1000 Gy.

After irradiation the culture medium was discarded and received a fresh work medium and the microplates were incubated at 37°C and humid atmosphere with 5% CO_2 for 24 hours. The culture medium was changed to a neutral red dye solution in culture medium and once more the microplates were incubated for 3 hours, time for neutral red incorporation by living cells. Thereafter the dye solution was removed and the microplates were rinsed two times with phosphate buffer solution (PBS) pH 7.4 and once with rinsing solution (1% $CaCl_2$ 10% in 0.5% Formol). The extraction solution were added (2% acetic acid in ethanol 1:1), which causes cellular lyses, releasing the neutral red and the microplates were read in spectrophotometer ELISA reader Sunrise from Tecan, in 540nm.

The cell viability percentage of the each microplate was calculated with the optical density (OD) in relation to microplate control (0 Gy = 100%).

2.2 Determination of Resveratrol Cytotoxicity ($IC_{50\%}$)

Tumor cells were plated in 96-well microplates at the concentration of $5x10^4$ cells in each well of microplate and maintained in the respective cell culture medium at 37ºC and humid atmosphere with 5% CO_2. To resveratrol cytotoxicity index ($IC_{50\%}$) determination in tumor cells line assay, was used one microplate for each cell line.

As a positive control was used extract in culture medium of natural rubber latex (NRL), and as a negative control high-density polyethylene (HDPE) extract, after incubation for 24 hours at 37ºC. The control extract solutions were serially diluted (dilution 1:2) in five different concentrations in culture medium. The resveratrol was dissolved in pure ethanol and this solution was diluted with culture medium to obtain a corresponding solution of 250 µM and five serially dilutions (dilution 1:2) in culture medium were made. In each microplate well was distributed 200 µL of the controls and resveratrol solutions. It was added fresh culture medium on the corresponding cell control wells.

After the microplates were incubated at 37ºC and humid atmosphere with 5% CO_2 for 24 hours, the medium was discarded and replaced by neutral red solution. From this part, the methodology was the same as in the LD_{50} methodology, described in 2.1 item.

3. RESULTS AND DISCUSSION

3.1 Determination of gamma radiation Lethal Dose (LD_{50})

The cell viability percentage was calculated with the mean OD of each microplate in relation to control microplate (0Gy) and presented in Tab.1.

Table 1. Cell viability percentage of NCI-H292 and RD cells line irradiated by gamma radiation in different doses.

Dose (Gy)	Cell Viability (%)	
	NCI-H292	RD
0	100	100
250	97	74
500	100	42
750	103	11
1,000	77	10

The NCI-H292 (human mucoepidermoid pulmonary carcinoma) tumor cell line showed to be very resistant to gamma radiation in the LD_{50} assay. At the maximum dose of gamma radiation used (1000Gy – 1kGy), this cell line showed 77% of cellular viability. Projecting these results in a graphic the dose-response curve showed that to obtain gamma radiation LD_{50} it is necessary to use higher radiation doses to get 50% mortality of cell population in the assay.

Comparing the results of NCI-H292 and RD cells it is evident that NCI- H292 showed to be about 8 times more resistant than RD cells. The results of RD cells showed that cell viability decreases with increasing of radiation dose.

The NCI-H292 and RD viability curves obtained in the gamma radiation LD_{50} assay is presented in the Fig.1. The LD_{50} is the radiation dose which injury or kill 50% of cell population in the assay, obtained in the intersection of the viability curve with 50% cell viability line.

RD cells LD_{50} was 435Gy and for NCI-H292 cells was higher than 1kGy.

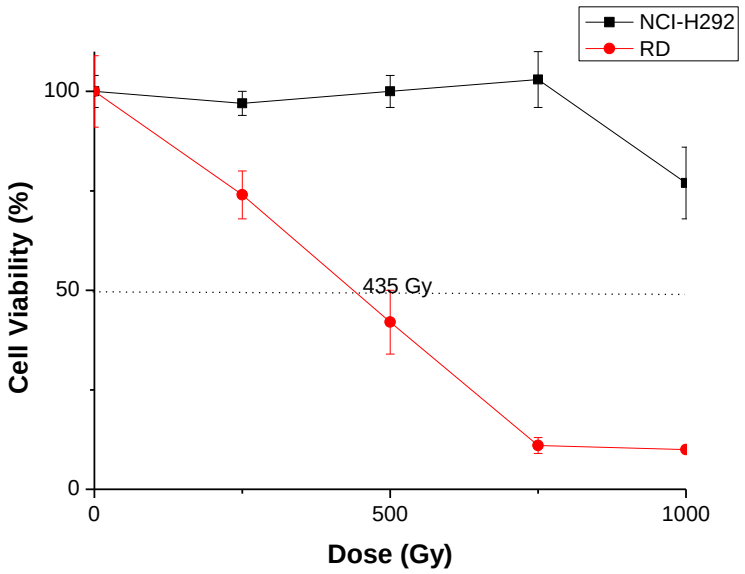

Figure 1. Viability curves of NCI-H292 cells and RD cells in the gamma radiation LD_{50} assay.

3.2 Determination of Resveratrol Cytotoxicity ($IC_{50\%}$)

In the resveratrol cytotoxicy assay, with mean OD results were calculated the cell viability percentage of each dilution, compared with cell control considered 100%. The NCI-H292 results are presented in Tab.2 and RD in Tab.3.

Table 2. The percentage of NCI-H292 cell viability in resveratrol cytotoxicity assay.

Extract Concentration (%)	Negative Control (%)	Positive Control (%)	Resveratrol (250µM/L) (%)	Resveratrol (250µM/L) (%)	Resveratrol (250µM/L) (%)	Resveratrol (250µM/L) (%)
100	118±3	16±3	36±2	39±4	39±1	40±2
50	116±6	111±5	64±8	82±2	80±8	86±5
25	110±2	116±4	67±12	81±2	85±4	87±9
12.50	97±7	112±7	77±7	85±2	88±3	87±9
6.25	94±1	120±3	83±6	94±3	98±4	98±10

Table 3. The percentage of RD cell viability in resveratrol cytotoxicity assay.

Extract Concentration (%)	Negative Control (%)	Positive Control (%)	Resveratrol (250µM/L) (%)	Resveratrol (250µM/L) (%)	Resveratrol (250µM/L) (%)	Resveratrol (250µM/L) (%)
100	124±5	14±0	14±9	13±1	14±5	13±5
50	121±8	15±1	48±24	56±3	51±2	52±12
25	115±5	72±14	61±13	75±7	61±1	77±10
12.50	115±4	73±3	73±12	82±3	82±2	77±17
6.25	106±3	70±3	108±4	114±2	113±2	118±7

Projecting the cell viability percentages in relation to concentration of solutions is obtained the cell viability curve where is possible to find the cytotoxicity index, $IC_{50\%}$ in the intersection of viability curve and 50% cell viability line. $IC_{50\%}$ is the sample extract concentration which injury or kills 50% of cell population in the assay.

In the Fig. 2 are shown the resveratrol cytotoxicity viability curves on NCI-H292 as well as in the Fig. 3 are shown the resveratrol cytotoxicity viability curves on RD cell line.

It was observed higher resveratrol $IC_{50\%}$ for NCI-H292 (192µM) than for RD cell line of about 128µM. These results are in accordance of LD_{50} where NCI-H292 cell line showed higher resistance than RD in the gamma irradiation assay.

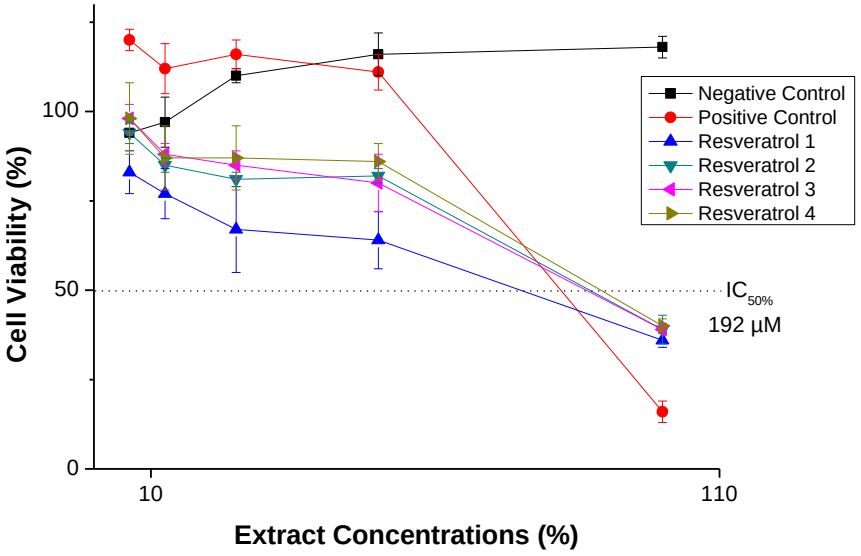

Figure 2. Viability curves of NCI-H292 cells in cytotoxicity assay by the neutral red uptake method.

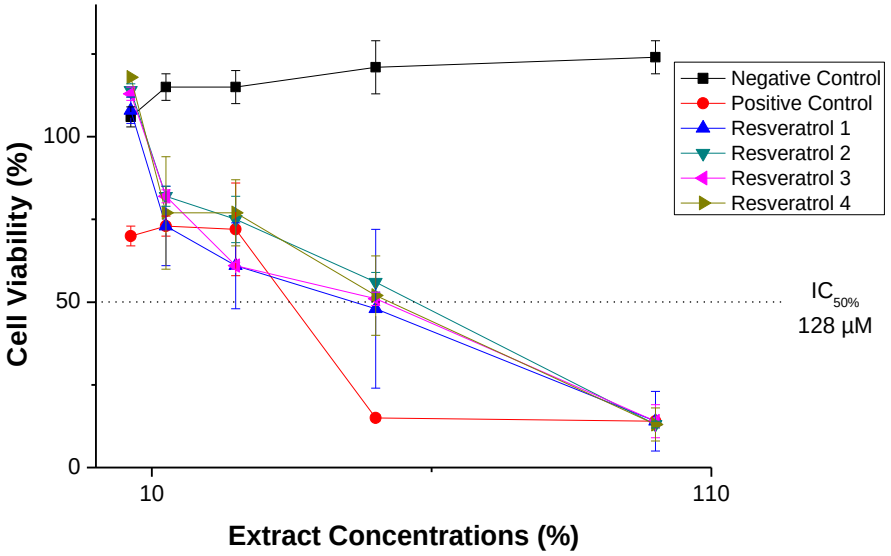

Figure 3. Viability curves of RD cells in cytotoxicity assay by the neutral red uptake method.

At first, it was chosen the NCI-H292 (human mucoepidermoid pulmonary carcinoma) cells to be the tumor cell line for this work. After the determination of LD_{50} and $IC_{50\%}$ this cell line showed to be resistant not only to gamma radiation but also to the resveratrol

cytotoxicity, it was changed the tumor cell line NCI-H292 to RD (human rhabdomyosarcoma), with good results.

4. CONCLUSION

In the determination of gamma radiation lethal dose (LD_{50}) the NCI-H292 tumor cell line showed high resistance to gamma radiation, compared with RD cell line, showed LD_{50} of about 435 Gy.

The cytotoxicity index of resveratrol was 192µM for NCI-H292 and 128µM for RD tumor cell lines. In this assay it was noticed the same behavior about NCI-H292 resistance.

These tests are part of the preliminary study to determine the *in vitro* radiosensitizing effect of resveratrol in tumor cell culture.

More experiments will be performed in order to show the resveratrol radiosensitizing effect.

5. REFERENCES

[1] INSTITUTO NACIONAL DO CÂNCER (INCA/PRO-ONCO) – MINISTÉRIO DA SAÚDE. Estimativa 2010: Incidência de Câncer no Brasil. Rio de Janeiro, Brasil (2010).

[2] J. A. Baur; D. A. Sinclair. *Therapeutic potential of resveratrol: the in vivo evidence*. Nature Review Drug Discovery, **Volume 5**, pp. 493-506 (2006).

[3] P. Jeandet; A.C. Douillet-Breuil; R. Bessis; S. Debord; M. Sbaghi; M. Adrian. *Phytoalexin from the Vitaceae: biosynthesis, phytoalexin gene expression in transgenic plants, antifungal activity, and metabolism*. Journal of Agricultural and Food Chemistry, **Volume 50**, pp. 2731-2741 (2002).

[4] H. D. Van Etten; J. W. Mansfield; J. A. Bailey; E. E. Farmer. *Two classes of plant antibiotics: phytoalexins versus "phytoanticipins"*. Plant Cell, **Volume 6**, pp. 1191-1192 (1994).

[5] L. Frémont. Biological effects of resveratrol. Life Sciences, **Volume 66**, pp. 663-673 (2000).

[6] C. K. Sautter; S. Denardin; A. O. Alves; C. A. Mallmann; N. G. Penna; L. H. Hecktheuer. *Determinação de resveratrol em sucos de uva no Brasil.* Ciência e Tecnologia dos Almientos, **Volume 25**, pp. 437-442 (2005).

[7] S. S. Leaonard; C. Xia; B. H. Jiang; T. Stinefelt; H. Klandorf; G. K. Harris; X. Shi. *Resveratrol scanvenges reactive oxygen species and effects radical-induced cellular responses.* Biochemical and Biophysical Research Communications, **Volume 309**, pp. 1017-1026 (2003).

[8] V. Vingtdeux; U. Dreses-Werringloer; H. Zhao; P. Davies; P. Marambaud. *Review: Therapeutic potential of resveratrol in Alzheimer´s disease.* BMC Neuroscience, **Volume 9**, Suppl. 2 – S6 (2008).

[9] D. M. Goldberg; S. E. Hahn; J. G. Parkes. *Beyond alcohol: beverage consumption and cardiovascular mortality.* Clinica Chimica Acta, **Volume 237**, pp. 155-187 (1995).

[10] S. Kondo. *Mutation and cancer in relation to the atomic-bomb radiation effects.* Japonese Journal Cancer Research, **Volume 79**, pp. 785-99 (1988).

[11] S. O. Rogero; A. B. Lugão; T. I. Ikeda; A. S. Cruz. *Teste in vitro de Citotoxicidade: estudo comparativo entre duas metodologias*. Materials Research, **Volume 6**, pp. 317-320 (2003).

[12] ISO document 10993-5. *Biological evaluation of medical devices, Part 5, Tests for cytotoxicity: in vitro method* (2009).