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IN VITRO CELL CULTURE LETHAL DOSE SUBMITTED TO GAMMA RADIATION

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ABSTRACT

The present study was designed to evaluate the in vitro effect of gamma radiation in cell culture of mouse connective tissue exposed to different doses of gamma radiation and under several conditions. The cell viability was analyzed by neutral red uptake methodology. This assay was developed for establish a methodology to be used in the future in the study of resveratrol radioprotection. Resveratrol (3,4',5-trihydroxystilbene), a phenolic phytoalexin that occurs naturally in some spermatophytes, such as grapevines, in response to injury as fungal infections and exposure to ultraviolet light. In the wines this compound is found at high levels and is considered one of the highest antioxidant constituents. The intense antioxidant potential of resveratrol provides many pharmacological activities including cardioprotection, chemoprevention and anti-tumor effects. Our results demonstrated that ^{60}Co gamma radiation lethal dose (LD50) on NCTC clone 929 cells was about 340Gy.

1. INTRODUCTION

The physical absorption of radiation in cells that results in ionization or less likely excitation causes a chemical reaction, free-radical formation. A free-radical is an atom or molecule that has a single unpaired electron in one orbit and plays a prominent role in human health. The elements that probably will be included in free-radical formation are water and oxygen [1].

The hydroxyl radical is the most meaningful oxidizing species that results from radiation interaction with water. Water is of particular interest because it is the major component of the human body. A donation of one electron to oxygen results specially in the production of the superoxide radical ion [1].

Free radicals may undergo a variety of reactions with other molecules and other oxygen metabolites. It is important to state that such reactions may be direct or indirect.

The probability of the process of direct reactivity towards biological targets (RNA and DNA) is rather low. The free-radical is a precursor for other reactive metabolites which may lead to biological damage by several mechanisms. Its reactivity may differ significantly according to its biological environment [2].

Radioprotective agents are free-radical scavenger which prevent the free radicals from interaction with others cellular compounds and thus averting radiation injury [3].

Resveratrol (3,4',5-trihydroxystilbene) is a natural phenolic phytoalexin found in several plants such as grapes and consequently in wine as response to injury as fungal infections and exposure to ultraviolet light and chemical agents [4]. Growing evidence suggests that resveratrol exerts radioprotective effect acting as a scavenger of free radical contained in several biologic processes [5]. This property provides a prevention of human pathological processes such as human cardiovascular diseases [6] and also plays a role in both cancer prevention and therapy [7].

The objective of this research was to determine the radiation dose that induces the death of 50% of cell population in culture. This radiation dose is considered the Lethal Dose 50% (LD_{50}). This study was performed by neutral red uptake methodology [8] using mouse connective tissue fibroblasts, NCTC clone 929 cell line (ATCC CCL1), because they are a standard model for *in vitro* tests, since they are easy to cultivate and because of their favorable doubling time of about 24 hours.

Therefore, this study supports the *in vitro* future investigation of the radioprotective potential of resveratrol.

2. METHODOLOGY

The reagents were purchased from Carlo Erba and Merck and culture medium (MEM) used was produced by Cell Culture Section from Instituto Adolfo Lutz.

2.1. Cell culture

A mouse connective tissue fibroblast cell line NCTC clone 929 was cultured in minimum Eagle medium (MEM) supplemented with 10% fetal calf serum (FCS), 0.1 mM non-essential amino acids and 1 mM sodium pyruvate, and maintained at 37°C until monolayer culture. The cells were detached by 0.20% trypsin and 0,02% EDTA solution and 0.2 mL of $3.5x10^5$cell/mL cell suspension was seeded in flat-bottomed 96 microplate wells at a density of $7.0x10^4$ cells/well. The microplate was incubated for 24 hours at 37°C in a CO_2 humidified incubator.

2.2 Microplate preparation for irradiation

Comparative experiments were performed as follows:

(A) The cell irradiation was performed without substitution of culture medium (MEM);

(B) Replacement of culture medium by MEM supplemented with 5% FCS right before irradiation;

(C) Replacement of culture medium by phosphate buffer saline solution (PBS) pH 7.4 right before irradiation.

2.3 Irradiation

Irradiation of cell culture microplate was performed by a ^{60}Co gamma source (GammaCell 200, Atomic Energy of Canada, Ltd., CAN.) at a dose rate of 2.64 kGy/h, with different doses: 250, 500, 750 and 1000 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.

After irradiation, culture medium was replaced by MEM supplemented with 5 % FCS and afterwards the microplates were returned to incubator at 37°C in a humidified 5% CO_2 atmosphere for 24 hours.

2.4 Cellular viability assessment

Once cells culture was incubated, the culture medium was replaced by culture medium containing neutral red and incubated again for 3 hours at 37°C. The dye-containing medium was discarded; the microplates were washed twice with PBS and once with 1% calcium chloride solution in 0.5% formaldehyde. Volume of 0.2 mL of extractant solution (50% ethanol in 1% acetic acid) was added in each well to promote cell rupture and neutral red release.

Cell viability was assessed by dye absorbance measured at 540 nm using an automated spectrophotometer ELISA reader type RC Sunrise - Tecan.

3. RESULTS AND DISCUSSION

To determine the LD_{50}, the percentage of cell viability was calculated from the ratio between optical densities (OD) of irradiated cells and OD of cell control. Cell control microplates were represented by the non-irradiated cells which are 100% cells survival in all experiments.

In Table 1 is presented the results of cell viability percentage of experiment A and its cell survival curve in the Fig.1. Survival curves are obtained by plotting cell viability percentage as a function of the irradiation dose. The LD_{50} is estimated in the intersection of 50% viability line with survival curve.

Table 1. Cell viability percentage in different radiation doses – Experiment A

Irradiation Dose (Gy)	0	250	500	750	1000
Cell Viability (%)	100.00 ± 16.81	63.00 ± 19.24	31.00 ± 16.02	22.50 ± 18.35	22.50 ± 19.59

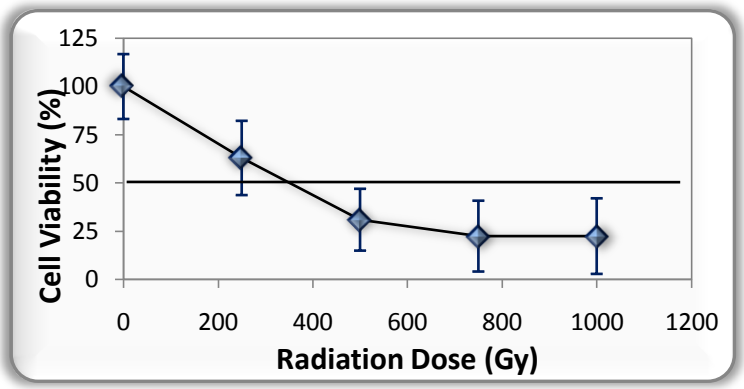

Figure 1. Radiation survival curves for NCTC clone 929. Experiment A: Cells were irradiated without replacement of culture medium.

LD_{50} of cells irradiated without substitution of culture medium was 354Gy.

Table 2 shows the results of cell viability in the experiment B where the irradiation was just after the replacement of culture medium by MEM supplemented with 5% FCS. The survival curve is presented in Fig.2, which was obtained the LD_{50} of about 391Gy, very similar to obtained in experiment A.

Table 2. Cell viability percentage in different radiation doses – Experiment B

Irradiation Dose (Gy)	0	250	500	750	1000
Cell Viability (%)	100.00 ± 16.87	59.26 ± 19.88	29.14 ± 12.24	28.59 ± 16.26	31.38 ± 19.19

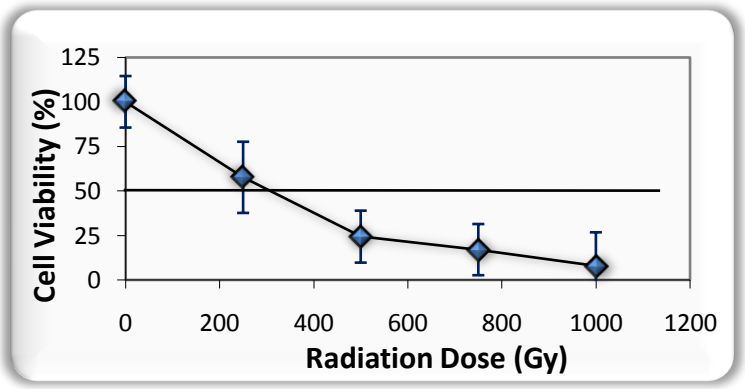

Figure 2. Radiation survival curves for NCTC clone 929. Experiment B: Replacement of medium by MEM supplemented with 5% FSC right before irradiation of cells.

The results of the last experiment, C with replacement of the culture medium by PBS pH 7.4, are presented in Table 3 and Fig.3. The obtained LD_{50} was about 278Gy. This experiment was done to verify the presence of some radioprotection components in the culture medium. It was observed that in the presence of MEM the DL_{50} was around 20% higher.

Table 3. Cell viability percentage in different radiation doses – Experiment C

Irradiation Dose (Gy)	0	250	500	750	1000
Cell Viability (%)	100.00 ± 14.45	57.65 ± 19.96	24.40 ± 14.57	17.11 ± 14.35	8.01 ± 18.82

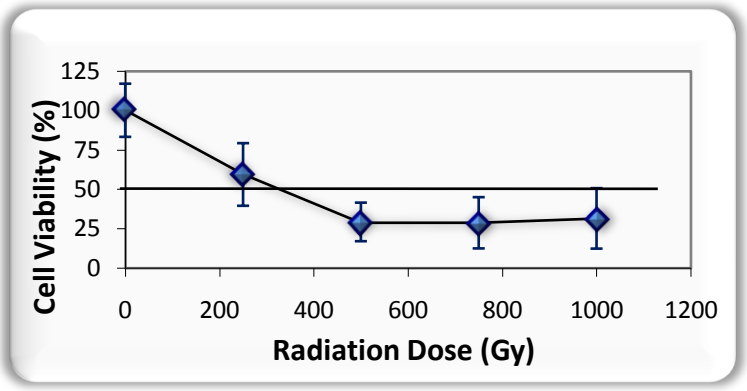

Figure 3. Radiation survival curves for NCTC clone 929. Experiment C: Replacement of medium by saline solution right before irradiation.

4. CONCLUSIONS

The *in vitro* test of NCTC clone 929 cell line LD_{50} for gamma radiation by neutral red uptake methodology showed an average result of about 340Gy. The result obtained by method performed with the irradiation of cell in MEM will be used in the resveratrol radioprotective *in vitro* test. The resveratrol radioprotective and cancer-preventive potential properties need to be investigated to reduce or prevent the radiation harmful effect to the population at risk.

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REFERENCES

1. F. A. Mettler Jr.; Upton, A. C. *Medical Effects of Ionizing Radiation*, W. B. Saunders Company, Philadelphia & United States of America (1995).
2. S. B. Dowd; E. R. Tilson, *Practical Radiation Protection and Applied Radiobiology*, W. B. Saunders Company, Philadelphia & United States of America (1999).
3. L. F. Fajardo; M. Berthrong; R. E. Anderson. *Radiation Pathology*, Oxford University Press, Hong Kong & Japan (2001).
4. S. Pervaiz, "Resveratrol: from grapevines to mammalian biology", The FASEB Journal, **Vol. 17**, pp. 1975 – 1985 (2003).
5. S. S. Leonard; C. Xia; B. H. Jiang; B. Stinefelt; H. Klandorf; G. K. Harris; X. Shi, "Resveratrol scavenges reactive oxygen species and effects radical-induced cellular response", *Biochemical and Biophysical Research Communications*, **Vol. 309**, pp. 1017-1026 (2003).

6. A. A. Chaves; M. S. Joshi; C. M. Coyle; J. E. Brady; S. J. Dech; B. L. Schanbacher; R. Baliga; A. Basuray; J. A. Bauer, "Vasoprotective endothelial effects of a standardized grape product in human", *Vascular Pharmacology*, **Vol. 50**, pp. 20-26 (2009).
7. S. Pervaiz, "Chemotherapeutic potential of the chemopreventive phytoalexin resveratrol", *Drug Resistance Updates*, **Vol. 7**, pp. 333-344 (2004).
8. G. Ciapetti; D. Granchi; E. Verri; L. Savarino; D. Cavedagna; A. Pizzoferrato, "Application of a combination of neutral and amido black staining for rapid, reliable cytotoxicity testing of biomaterials", *Biomaterials*, **Vol. 17**, pp.1259-1264 (1996).