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# RADIOMODIFYING EFFECT OF RESVERATROL IN HUMAN RHABDOMYOSARCOMA (RD) CELL CULTURE APPLYING THE COMET ASSAY

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## ABSTRACT

Cancer is considered a worldwide public health problem. Resveratrol is a defense polyphenol, synthesized naturally by 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. In vines this substance is found in elevated concentration. Thus, resveratrol is present in grape juice and wines, especially red wine. Red wines are the best dietary source of resveratrol.The protective effects performed by resveratrol during the process of cell damage, produced by oxidative effects of free radicals, are anti-inflammatory, anti-platelet and anti-carcinogenic activity, prevent or inhibit degenerative diseases, decrease incidence of cardiovascular diseases. Moreover, resveratrol is considered as a cell radioprotector. On the other hand, in some elevated concentrations resveratrol is considered as a radiosensitizing compound. The aim of this work was study *in vitro* the radiomodifying effect of resveratrol in human rhabdomyosarcoma (RD) cells applying the comet assay to evaluate the cellular damage and its repair capacity. In this study RD cells culture was irradiated by gamma radiation at 50 Gy and 100 Gy doses and the used resveratrol concentrations was from 15 µM to 60 µM. The protective and radioprotective effects were observed at 15 µM and 30 µM resveratrol concentrations. The resveratrol concentration of 60 µM showed cytotoxic effect to RD tumor cells and with gamma radiation presence this concentration showed no statistically significant radiosensitizing effects.

## 1. INTRODUCTION

Cancer was a relatively rare disease in ancient times. However, for the past two centuries or so, the incidence of cancer in the population has been growing drastically. This increase is due to two factors: the increasing average human lifespan and the greater exposure to carcinogenic chemical products and X-rays, such as during airplane trips at high altitude [2].

According to Brazil's National Cancer Institute [3], cancer is a growing global public health problem. Forecasts are for some 518,000 cases of cancer in Brazil in 2013.

According to Murad & Katz [4], rhabdomyosarcoma is the most common sarcoma in infancy. Although it is rare, it demonstrates a serious problem because its correct treatment is difficult. Rhabdomyosarcomas are very resistant to radiation therapy, so they require high doses. They grow from the embryonic mesenchyme, or mesodermal tissue, and can appear in any anatomical area, but most commonly occur in the genitourinary region (21%), extremities (20%), parameningeal region (nasopharynx, nasal cavity, paranasal sinuses, middle ear and infratemporal fossa) (14%), other sites on the head and neck excluding orbits (13%) and only the orbits (10%).

In the nature chemical compounds exist that are capable of inhibiting carcinogenesis at various stages, among them resveratrol (3, 4`, 5- trihydroxy-trans-stilbene), which is a polyphenol belonging to the set of compounds called phytoalexins [5]. This defensive polyphenol is synthesized by a wide range of plants in response to exposure to ultraviolet (UV) radiation or mechanical stress caused by the action of pathogens or chemical or physical agents [6].

Grape vines have a high capacity to produce resveratrol [7]. The concentration of resveratrol in wine, especially red wine, is relatively high. For non-drinkers, grape juice, especially the dark variety, is also considered an excellent source of resveratrol, although the concentration is lower than in red wine [8].

The protective effects exercised by resveratrol against damage to the cellular genetic material, produced by the oxidative effect of free radicals, promotes reactions such as induction of the antiinflammatory response [9]; antitumor activity [7]; prevention or inhibition of degenerative diseases [10]; reduced incidence of cardiovascular diseases [11]; inhibition of platelet aggregation [7], and inhibition or reduced incidence of neurodegenerative diseases such as Alzheimer's and Parkinson's diseases [12].

A chemical compound such as resveratrol that acts both as a protector and sensitizer of cells is defined as a radiomodifying compound. At low concentrations, resveratrol has a protective effect in cells, but at high concentrations it sensitizes cells [13, 14].

Ionizing radiation interacts by direct reactions on biological macromolecules such as DNA and RNA, inducing the formation of free radicals by incidence on the various molecules inside cells. The other interaction is by indirect reactions where water is the highest substance in the cell, up to 85%, therefore with the greatest probability of interacting with ionizing radiation, resulting in the water radiolysis with formation of very reactive free radicals [15].

The water radiolysis produces the "primary products of the water radiolysis". These products are extremely reactive and capable of unleashing many toxic reactions inside cells [16].

Various methods have been developed and improved to detect and quantify damages to DNA induced by ionizing radiation. Among them is the biochemical technique known as the comet assay, ormicrogel electrophoresis assay, which was first described by Ostling & Johanson [17], utilizing neutral pH conditions for the lysis and electrophoresis of cells, which permits observing double breaks in DNA strands.

Singh *et al.* [18] described the alkaline version of the comet assay, which enables detection of breaks both in single and double strands as well as at alkali-labile sites. For this reason, the alkaline technique is more effective in detecting primary radio-induced lesions.

The aim of this study was to investigate the radiomodifying effect of resveratrol in human rhabdomyosarcoma cells (RD) by applying the comet assay to assess the damage and repair capacity of cells.

## 2. METHODOLOGY

The tumor RD cell line was obtained from the American Type Culture Collection (ATCC). The cell preparation was carried out at the Cell Culture Unit of the Adolfo Lutz Institute in São Paulo. The tumor line RD cells were cultivated in a cell culture bottle with 25 $cm^2$ using Eagle+L-15 medium, supplemented with 15% bovine fetal serum (BFS). The tumor cells were kept at 37ºC in a humid atmosphere containing 5% $CO_2$ until formation of the cell monolayer.

### 2.1. Comet Assay (Microgel Electrophoresis Assay)

The alkaline version of the comet assay was used, as described by Singh *et al* [19]. One day beforehand, it was prepared on top of cleaned histological slides, a film with normal agarose (dissolved in PBS buffer free of Ca and Mg, at 65°C) and left at room temperature to dry.

To carry out the comet assay, it was necessary to perform previous steps as: incubation of the RD cells with resveratrol and irradiation of these cells, and finally the application of the comet assay itself.

#### 2.1.1. Incubation of the RD cells with resveratrol

The resveratrol stock solution (50 mM) was used to produce three dilutions (60 µM, 30 µM and 15 µM) in Eagle+L-15 medium supplemented with 15% BFS. For each resveratrol concentration was used two RD cells culture bottles (25 $cm^2$ area). The culture medium was replaced by resveratrol solution while the control bottle received only fresh culture medium. These resveratrol solutions stayed in contact with RD cells in the respective culture bottle for 24 hours in an humid atmosphere incubator at 37ºC containing 5% $CO_2$.

#### 2.1.2. Irradiation of the RD cells

After incubation of the RD cells with resveratrol, the culture bottles were submitted to the trypsinization process. The cell suspension was centrifuged and the RD cells were resuspended in PBS without EDTA. The cell suspension was adjusted to $1x10^6$ cells/mL, and

3 mL of this was placed in 15-mL test tubes and these tubes were irradiated by different doses: 100 Gy, 50 Gy and 0 Gy (control). Immediately afterward, the irradiated tubes were placed in an ice bath. Each test with a different resveratrol concentration was accompanied by a control sample that was not irradiated.

### 2.1.3. Application of the comet assay itself

The comet assay was performed for each resveratrol concentration at 0, 24 and 48 hours after irradiation, for all radiation doses, including 0 Gy as control of cell viability.

The first microgel electrophoresis procedure was performed just after irradiation of the cells (time 0). The other samples were processed, plated in culture bottles and incubated at 37ºC in a humid atmosphere containing 5% $CO_2$ for 24 and 48 hours, to evaluate the cell repair capacity. After incubation, the cells were submitted to trypsinization and the cell suspension was adjusted and diluted in PBS without EDTA for the corresponding assay.

With the processed cells (0, 24 and 48 hours after irradiation), 10 μL aliquots of the suspension were added to 90 μL of low melting agarose which was dissolved in PBS buffer, free of Ca and Mg, at 37°C. The mixture was placed on a slide containing a normal agarose layer prepared in advance. During the agarose solidification process, about 5 minutes at 4ºC, were used cover slips and after solidification the slides were placed in a cell lysis solution (2.5 M of NaCl, 100 mM of EDTA, 10 mM of Tris, 1% sodium sarcosinate, 1% Triton X-100 and 10% DMSO) for 2 hours at 4°C to remove the proteins.

After cell lysis and protein extraction, what remained on the slides were the cell nuclei, named as nucleoids. After this moment, the assay procedures were carried out in dark environment. The slides removed from the lysis solution were placed side-by-side in the tray, containing alkaline electrophoresis buffer (1 mM of EDTA and 300 mM of NaOH, pH 12), for 30 minutes, to allow expression of the breaks in the strands (single and double) and in the alkali-labile sites of the DNA.

Then slides were submitted to electrophoresis (25 V and 300 mA) for 30 minutes at 4°C (tray in an ice bath). After the electrophoreses, the slides were neutralized three times for 5 minutes each with Tris buffer (pH 7.5) and fixed in 100% ethanol for 10 minutes, after which they were stored for subsequent analysis.

The slides were stained with 50 μL of ethidium bromide (20 μg/mL) and analysed under a fluorescence microscope (Carl Zeiss) with 200 X magnification. About 100 randomly chosen comets were analyzed on each slide.

The comets were classified by the intensity of fragmentation of the nucleoids into five categories (0 - IV), according to the criterion established by Jaloszynski *et al*. [19] and Mozdarani *et al*. [20]. Comets were classified as class 0 whenhad bright head and no tails (no DNA migration), while the comets with small heads and long and/or diffuse tails were classified as category IV and those with intermediate aspects were classified in classes I, II and III.

The estimated quantity of DNA damage was determined according to the equation described by Jaloszynski *et al*. [19], varying from 0 to 400 arbitrary units, as described in Eq.1:

$$\mathbf{DD = (n1 + 2\ n2 + 3\ n3 + 4\ n4) / (\Sigma / 100)} \qquad \mathbf{(1)}$$

Where:
**DD =** damage to the DNA in arbitrary units
**n1 – n4 =** number of comets in classes I – IV
**Σ =** total number of comets analyzed, including class 0

The DD values can indicate various situations; from all undamaged cells (class 0) to all highly damaged cells (class IV). The DD values were plotted in a graphic using the GraphPad Prism5 program. The statistical analysis was by two-way ANOVA and the Bonferroni post-test, with $P > 0.05$ considered not statistically significant.

## 3. RESULTS AND DISCUSSION

The comet assay was performed to determine *in vitro* the radiomodifying effects of resveratrol. This test allows analyzing both the damage produced by the action of gamma radiation on tumor cells and the cell repair in the presence or not of resveratrol, as well as the damage induced by high concentrations of resveratrol in the absence of gamma radiation. For analysis of the DD, the assays were carried out just after irradiation of the cells, while in the cell repair analysis the assays were performed 24 and 48 hours after irradiation.

According to other authors [14, 22, 23], a resveratrol concentration of 15 µM is considered to exercise *in vitro* a protective effect, while concentrations above 15 µM induce apoptosis in tumor cells. According to the literature, 60 µM is within the resveratrol concentration range known to inhibit the metabolic activity and cell proliferation [13, 14, 21, 24]. We chose the concentration of 30 µM as intermediate between the concentrations that induce radioprotection and radiosensitizing effect.

The results of classified comets number obtained by visually counting were applied in equation **(1)** to calculate the DD values and the classified comets are shown in the Fig. 1.

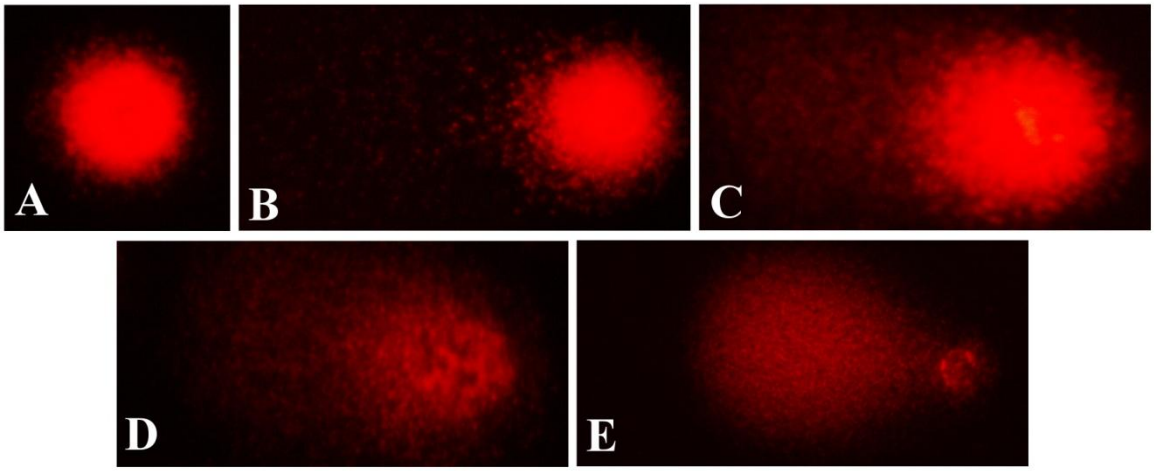


**Figure 1 – Micrographs of RD cells, with visual classifications of the comets: (A) comet class 0; (B) comet class I; (C) comet class II; (D) comet class III and (E) comet class IV. Events were visualized under 10 X magnification.**

Table 1 present the DD values of non-irradiated RD cells at different resveratrol concentrations (0 µM, 15 µM, 30 µM and 60 µM) and analyzed at different times (0 h, 24 h and 48 h).

**Table 1 – DD values for the RD cells at different resveratrol concentrations and not irradiated (0 Gy),**

| Resveratrol concentration (µM) | DD (au) | | |
|---|---|---|---|
| | Evaluation Time | | |
| | **0 h** | **24 h** | **48 h** |
| 0 | 35±11 | 30±12 | 26±7 |
| 15 | 20±2 | 13±6 | 11±5 |
| 30 | 19±5 | 19±7 | 13±4 |
| 60 | 45±33 | 56±29 | 48±30 |

In the RD culture with resveratrol at 60 µM concentration not submitted to radiation and analyzed immediately (0 h), induced a statistically significant increase in the DD, evidencing the intrinsic cytotoxicity of resveratrol. The other concentrations (15 µM and 30 µM) significantly diminished the DD number, showing the protective effect exercised by resveratrol on the RD cells. , The cell repair was noted in all concentrations of resveratrol when analysed at 24 h and 48 h after incubation as well as in the control cells. Comparing Thecell repair capacity of 60 µM resveratrol compared with control and with the other concentrations showed statistically no significant as shown in Fig. 2.

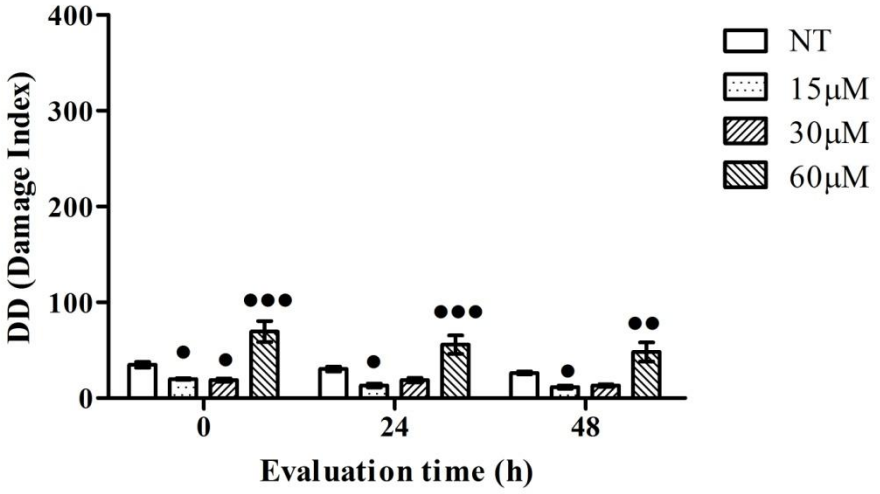


**Figure 2 – Control comet assay of the RD cells not exposed to radiation: DNA damage (DD) after incubation with resveratrol at different concentrations and analyzed at different times: • P <0.05; •• P <0.01; ••• P < 0.001. NT= not treated = control**

The DD values of the RD cells irradiated at 50 Gy and 100 Gy doses and obtained 0, 24 and 48 hours after irradiation, at different resveratrol concentrations are presented in Table 2.

**Table 2 – DD values for RD cells incubated in different resveratrol concentrations irradiated at 50 Gy and 100 Gy doses, obtained 0, 24 and 48 hours after irradiation.**

| Resveratrol concentration (µM) | DD (au) | | | | | |
|---|---|---|---|---|---|---|
| | Evaluation time | | | | | |
| | **50 Gy** | | | **100 Gy** | | |
| | **0 h** | **24 h** | **48 h** | **0 h** | **24 h** | **48 h** |
| 0 | 302±19 | 272±17 | 248±40 | 336±17 | 319±14 | 292±18 |
| 15 | 286±32 | 253±28 | 204±63 | 328±17 | 281±13 | 249±29 |
| 30 | 283±16 | 247±20 | 236±15 | 315±13 | 281±19 | 269±18 |
| 60 | 301±21 | 299±17 | 274±23 | 329±33 | 334±8 | 308±8 |

The data of the RD cells irradiated at 50 Gy dose, in the presence of different resveratrol concentrations, showed that only at 15 µM resveratrol concentration showed significantly reduction of the DD of RD cells, 48 h after irradiation, indicating resveratrol radioprotective property. The other resveratrol concentrations did not have statistically significant effects by the used method (Fig. 3).

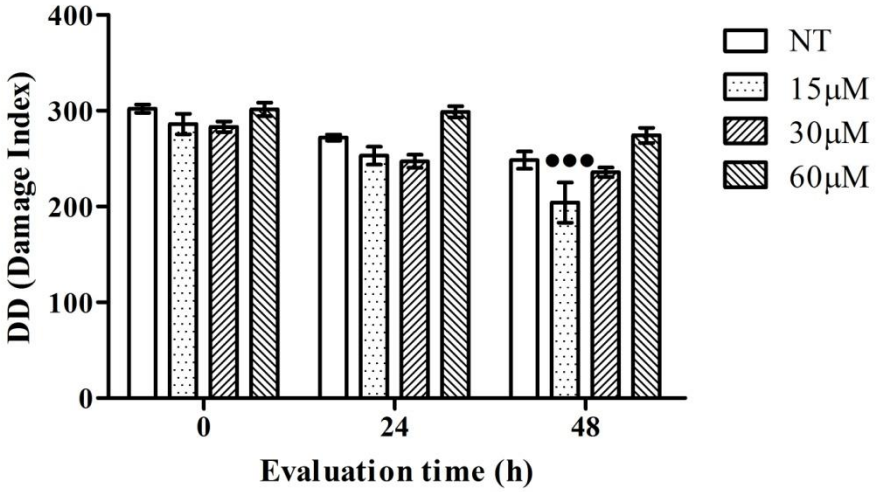


**Figure 3 – Comet assay: damage to the DNA of the RD cells incubated in different resveratrol concentrations, irradiated at 50 Gy dose, analysed 0, 24 and 48 hours after irradiation••• P < 0.001,**
**NT= not treated.**

The RD cells in the presence of 30 µM resveratrol and irradiated at 100 Gy, showed a small decline in the DD soon after irradiation (time=0 h) and 24 h after irradiation a significant reduction of DD was observed at 15 µM and 30 µM resveratrol concentration, probably due to repair process attenuating the radioprotective effect andthis process continued for 48 h, especially in the 15 µM concentration. Resveratrol at 60 µM presented a no statistically significant results, however a visually increase in the DD after 24 h as shown in Fig.4. It was observed a cell repair reduction at 24 h and 48 h. This probably occurred because 60 µM resveratrol prevents or impairs cell repair.

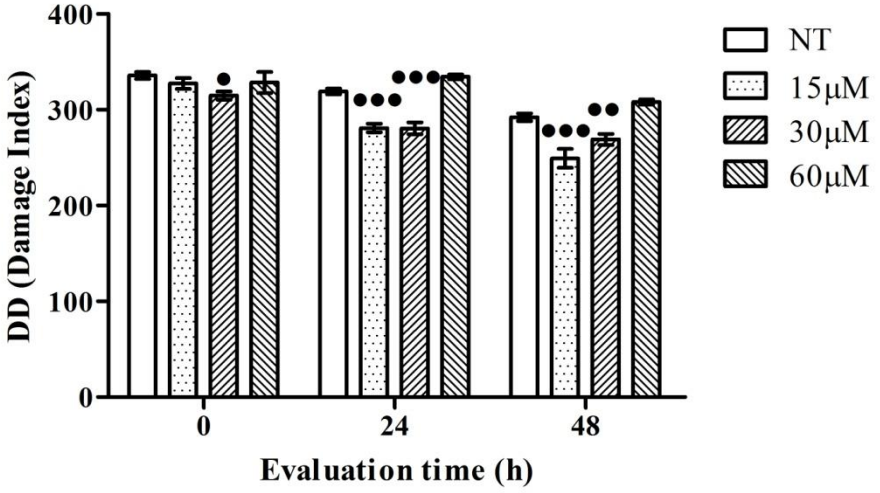


**Figure 4 - Comet assay: damage to the DNA of the RD cells after incubation with resveratrol at different concentration and irradiated at a dose of 100 Gy: • P < 0.05; •• P < 0.01; ••• P < 0.001.**

The data obtained showed a radioprotective effect of resveratrol at the concentrations of 15 µM and 30 µM, but this was only statistically significant at 15 µM, in conformity with other literature results [14, 22, 27, 28]. Resveratrol at 60 µM concentration showed a cytotoxic effect on the tumor cells and appeared to exercise a radiosensitizing effect, but it was not statistically significant. Ferry-Dumazet *et al*. [25] showed that a high concentration of resveratrol (60 µM) can inhibit cell growth and induce apoptosis, both in normal and tumor cell lines. According to Chow *et al*. [21], 50 µM resveratrol reduces the proliferation of RD cells by around 50%. They also assessed concentrations of 70 and 100 µM and observed that the RD cells proliferation reduction increased with increasing resveratrol concentration.

The results of this study reaffirm that resveratrol acts as a radiomodifying compound on tumor-line RD cells, with a radioprotective effect at low concentrations and radiosensitizing effect at high concentrations, as also found by Chow *et al*. e Mukherjee *et al*. [21,26]. We found that resveratrol acts as cell protector at 15 µM and shows sensitizing effect at 60 µM.

## 4. CONCLUSION

We observed that when the evaluation time increases the DD decreases, proving the repair capacity of RD tumor cells and that comet assay can evaluate the cell damage as the cell repair.

The resveratrol concentration of 15 µM clearly showed its cell protective and radioprotective effects as30 µM concentration, but not significant as in the concentration of 15 µM.

The resveratrol concentration of 60 µM showed a cytotoxic effect in cell culture, increasing significantly the DD of RD cells. The radiosensitizing effect of resveratrol was not statistically significant; however, visually isnoted the increase of RD cells DD in the presence of ionizing radiation. In the 60 µM resveratrol concentration it was also observed some cells difficulty to perform the rapair, especially at 100 Gy radiation dose.

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