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A N   I N T E R N A T I O N A L   J O U R N A L

In vitro Response of the Human Breast Cancer Cell Line MDAMB-231 and Human Peripheral Blood Mononuclear Cells Exposed to ^{60}Co at Single Fraction

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ABSTRACT

Radiotherapy using gamma rays is a common modality of breast cancer treatment. The aim of this research is to investigate the biological response of the human breast cancer cell line MDAMB-231 and human peripheral blood mononuclear cells (PBMC) exposed in vitro to ^{60}Co irradiation at a single fraction of 10 Gy, 25 Gy and 50 Gy doses at 136,4 cGy.min^{-1} rate. Cells were irradiated at room temperature by the Theratron 80 radiotherapy system. Biological response was evaluated through cellular viability using MTT assay and nucleus damages visualized by Propidium Iodide assay and electrophoresis agarose gel after gamma irradiation. Nucleus damages induced by ^{60}Co irradiation were compared to damage caused by cell exposure to 10% methanol. The 50 Gy dose of irradiation did not stimulate nuclus damages at the same level as that affected by 10% methanol induction in the MDAMB-231. Further studies are necessary to understand these mechanisms in the MDAMB-231 human breast carcinoma cell line.

Key words: Breast Cancer, MDAMB-231, PBMC and Gamma rays

INTRODUCTION

The malignant breast tumor is the most frequent disease found mostly in 45-65 year-old women all over the world (Harris and Norton, 1997; Lester and Cotran, 1999). Breast cancer is a public health issue in South America, involving about 70.000 cases of which 30.000 are fatal according to WHO database (Jones and Burnett, 1990, Schwartsmann, 2001). Argentina is one of the countries of the world that presents a major incidence of breast cancer, while in Brazil, breast cancer also reaches a high-level of incidence, followed by skin and lung cancer (Lester and Cotran, 1999, Tessaro et al., 2001; Vasconcelos et

al., 2001). The surgical method associated with radiotherapy and chemotherapy is the primary clinical intervention in patients without distant metastasis (Way, 1991). Radiotherapy is recommended as standard treatment after breast conserving surgery using two tangential fields in 25-29 fractions of 2 Gy up to total dose of 50-58 Gy spread over a total treatment period of 33-39 days. Locoregional control is observed with a 45 Gy to 50 Gy photon dose (Moss and Cox, 1989, Fredriksson et al, 2001, Koukourakis and Yannakakis, 2001).
Radiation effects on cells have been studied *in vitro* using variable techniques (Hall, 1988). Exposure of normal cells to ionizing radiation

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leads to a variety of biological effects that include cell cycle arrest, transformation and cell death (Gomes and Milanez, 1997). Damage of DNA cells, induced by irradiation, was found through investigation of base modifications in the DNA cells exposed to low LET ^{60}Co gamma rays and high LET ^{12}C^{+6} particle radiation, generating degradation products after these irradiation types (Pouget et al, 2002). The *in vitro* effects on cancerous cells at low doses of photon irradiation were investigated, such as the MDAMB-231 human breast carcinoma and PC-3 prostate carcinoma with 5 Gy, as well as the human melanoma and squamous cell carcinoma with ^{60}Co at 7 Gy (Mantha et al, 1999, Binder et al, 2000). However, gamma rays can present radioresistance in cell lines using low doses, as found in two bladder carcinoma cells after 2 Gy ^{137}Cs exposure (Gupta et al, 2000). Human blood cells have been used for studies on ionizing radiation. Peripheral blood mononuclear cells (PBMC) irradiated with gamma rays are commonly used as feeding cells during the cloning of T lymphocytes and lymphokine activated killer cells (Chong et al, 1991). Moreover, DNA fragmentation in human lymphocytes induced by low doses of gamma rays can be measured with pulsed field gel electrophoresis, and apoptotic fragments can be detected after irradiation (Belyaev and Ringdahl, 2002)

The relationship between tumor radiosensitivity and apoptosis has been evaluated in several tumor cell lines (Zhivotovsky et al, 1999). It was proven that caspase 8 inhibitor partially prevented apoptosis in Ataxia Telangiectasia cells and in normal lymphocytes exposed to low doses of radiation, and cyclin B1 protein levels rapidly increased during gamma irradiation with a 2 Gy dose in a human Burkitt's lymphoma line, promyelocytic leukemia cell and thymocytes (Albanese and Dainak, 2000).

The *in vitro* responses after irradiation are important for understanding the cellular effects of ionizing radiation. In the current work, the biological response of non-fractionated gamma rays doses, by the Theratron 80 radiotherapy system, on cell line MDAMB-231 and PBMC were investigated.

MATERIALS AND METHODS

Cell Culture

The MDAMB-231, a human breast carcinoma cell line obtained by ATCC, was donated by the Pharmacology Department of the Instituto de Ciências Biológicas of the Universidade Federal de Minas Gerais (ICB-UFMG). PBMC was collected from five 20-30 year-old health volunteers in heparinized blood tubes. Studies involving human specimen were conducted with the informal consent of all donors, and allowed by the "ethics committee on human subjects" at the Universidade Federal de Minas Gerais (UFMG). Cell cultures were maintained in RPMI-1640 medium (Sigma Chemical Company) supplemented with 10% Fetal Bovine Serum FBS (Laborclin®) and antibiotics gentamicin (50 µg/µL), penicillin (500 U/mL) and streptomycin (500 mg/mL), obtained from Sigma Chemical Company, in T-25 tissues culture flasks, from Costar®, in a humidified atmosphere containing 5% CO_2 at 37 ^{0}C. The medium was changed every 2-3 days.

PBMC Isolation

The 40 mL of blood were collected in heparinized blood tubes. The blood was centrifuged at 500 *g* (Jouan CR 412 centrifuge) during 40 min at 20 ^{0}C. After centrifugation, PBMC were isolated using density-gradient centrifugation in Ficoll-Hypaque (Sigma, Sanofi Winthrop). The cells were washed with 40 mL of MEM and centrifuged at 500 *g* for 10 min at 20 ^{0}C and washed twice with 20 mL of RPMI. The PBMC was plated in a T-25 flask containing RPMI supplemented with 10% FBS and maintained in a humidified atmosphere containing 5% CO_2 at 37 ^{0}C for up to five days.

^{60}Co Irradiation

The irradiation was performed by the Theratron 80 radiotherapy system (Atomic Energy Canada Limited) at the São Francisco Radiotherapy Institute (Brazil) with 20 x 20 cm^2 field at 136.4 cGy.min^{-1} dose rate and surface-source distance of 70 cm. The Theratron ^{60}Co source presented an activity of 130 TBq. The cells were exposed to doses of 10 Gy, 25 Gy and 50 Gy at room temperature for MTT assay and 10 Gy and 50 Gy for other assays. After exposure the cells were returned to the CO_2 incubator.

MTT Assay

The MDAMB-231 and PBMC cells were removed from the T-25 flasks and quantified by a hemocytometer. The $1x10^4$, $1x10^5$ and $1x10^6$ cells were loaded in a 96-well plate (Corning®) and irradiated with gamma rays. After the irradiation the cells were maintained in 5% CO_2 at 37 ^{0}C. Cellular viability was evaluated 1 hour, 24 hours, 48 hours and 72 hours after irradiation. At the indicated times, MTT (5mg/mL) from Sigma Chemical Company was added to the cells, which were incubated for 2 hours. This assay is based on the capacity of cells to reduce 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide to formazan, a purple crystal metabolized by mitochondria that can be easily visualized in the viable cells by inverse optical microscopy. This assay measures cellular viability by addition of 10% SDS-HCl to the cells and further incubation at 37 ^{0}C in 5% CO_2 for 18 hours. The solubilized formazan crystals can be determined from the optical density at 595 nm in an ELISA reader.

Propidium Iodide Assay

MDAMB-231 and PBMC cells were incubated for 24 hours and 72 hours after gamma irradiation with 10 Gy and 50 Gy doses. The cells were washed with 5 mL of PBS (Sigma Chemical Company) and incubated for 5 min at room temperature with 50 µg/mL Propidium Iodide (Calbiochem Inc) - a fluorescent dye that stains nuclei of damaged cells. The cells were washed again and damaged nuclei were viewed with an Olympus I x 70 Fluorescence Microscope. Photographic images were taken using Kodak film (Ektachrome, 100 ASA). Counting stained cell nuclei by hemocytometer, for each specific time and dose, yielded the number of nuclei-damaged cells. To compare nuclei damages induced by irradiation with damages induced by other methods, cells were incubated during 12 hours in RPMI supplemented with 10% methanol used for the propidium iodide assay and electrophoresis agarose gel.

DNA laddering by electrophoresis agarose gel

MDAMB-231 and PBMC DNA samples were isolated from T-25 culture flasks after 10 Gy and 50 Gy of gamma irradiation for 24 hours and 72 hours, as well as the samples treated with RPMI supplemented with 10% methanol. The cells were removed using a cell-scraper (Becton Dickinson Company). The samples were washed with 5 mL of PBS. The cells were homogenized using 250 µL of lyses buffer (50 mM Tris-HCl-20 mM EDTA-1% NP-40, pH 7,4). The samples suffered vortex stirring for 1 min and were centrifuged for 2 min at 180 g (Eppendorf Centrifuge 5415 C). Twenty µg of RNAse A was added to the supernatants (Boehringer Mannheim), which were incubated for 2 hours at 37 ^{0}C. The samples were treated with phenol-chloroform (1:1) and suffered vortex stirring for 1 min. The samples were centrifuged for 2 min at 10 000 g (Eppendorf Centrifuge 5415 C). The supernatants were collected and diluted in a 1/10 volume of the 3 M sodium acetate, pH 5.2. The DNA was precipitated with ethanol and maintained in a freezer at –20 ^{0}C overnight. The samples were centrifuged for 20 min at 10 000 g and washed with 70% ethanol. Twenty µL DEPC water was added to each sample. The samples were loaded onto 1.5% agarose gel containing 50 µg/mL ethidium bromide and run at 70 V for 60 min on a Horizon® Gibco BRL horizontal gel electrophoresis apparatus (Life Technologies™). The images were digitalized by ImageMaster® VDS (Amersham Pharmacia Biotech).

Statiscal Analysis

Data were analyzed by ANOVA or Student t test with the level of significance at $p < 0.05$.

RESULTS

Radiation Effects on the Cellular Viability

MTT assay was used to study cellular viability by mitochondrial metabolization after exposure of cells to ^{60}Co. Fig. 1 shows the formazan crystals formed by viable MDAMB-231 cells after a 50 Gy dose at the proposed times.

A discreet group of cells metabolizing MTT was observed for 24 hours and a gradual increase in MTT metabolization was observed for 48 hours and 72 hours, respectively. Cellular viability observed at 72 hours after irradiation seemed to be greater than the non-irradiated control cells. The same pattern of MTT metabolization was observed when cells were irradiated with 10 Gy and 25 Gy (data not shown).

The MDAMB-231 and PBMC cellular viability

measured by the optical density at 595 nm after gamma irradiation is presented in Fig.2. Progressive viability growth was observed in the MDAMB-231 non-irradiated control cells. Similar levels of cellular viability were observed for 1 hour, 24 hours and 48 hours after irradiation with 10 Gy and 25 Gy. An increase in cellular viability

was observed for 48 hours with a 50 Gy dose. At 72 hours after irradiation, the growth in cellular viability was equivalent for all doses tested, and with 50 Gy, viability growth was larger than that of the non-irradiated control.
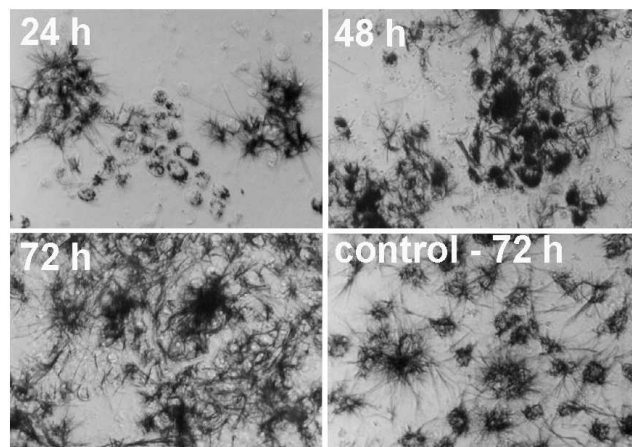

Figure 1 - MDAMB-231 cell cellular viability after gamma irradiation. Cells were plated in $1x10^6$ density for 12 hours before gamma irradiation with 10 Gy, 25 Gy and 50 Gy. At the proposed times post-irradiation viable cells were evaluated by MTT assay. Photomicrograph in optical inverse microscopy (X 400) shows formazan's crystals formed after 50Gy by mitochondrial metabolization. Several amounts of viable cells were observed in the different periods. After 72 hours formazan crystals formed in viable cells were greater than non-irradiated control cells for the period

This profile was also observed when cells were plated with other cell densities (data not shown). A reduction in cellular viability of the PBMC non-irradiated control cells was observed. For 72 hours an intense decrease in the cellular viability was observed with 50 Gy, as well as with the other cell densities, times and doses tested (data not shown).

Propidium Iodide Assay
Nuclear damages were investigated through nuclear staining with propidium iodide. Irradiated MDAMB-231 cells in phase contrast, cells stained with propidium iodide and merged images showing damaged and intact cells are presented in

Fig. 3. Nuclear damages induced by 10% methanol added to RPMI were observed in cells. An insert shows a detail of chromatin fragmentation, indicative of nuclear damage.
The percentage of damaged cells was quantified after different doses of irradiation. The percentage of nuclear damage in MDAMB-231 cells after gamma irradiation and after induction with 10% methanol viewed by fluorescence microscopy is presented in Table 1. The percentage of damaged nuclei in PBMC cells after gamma irradiation and after induction with 10% methanol can be seen in Table 2.

A MDAMB-231 Cellular Viability
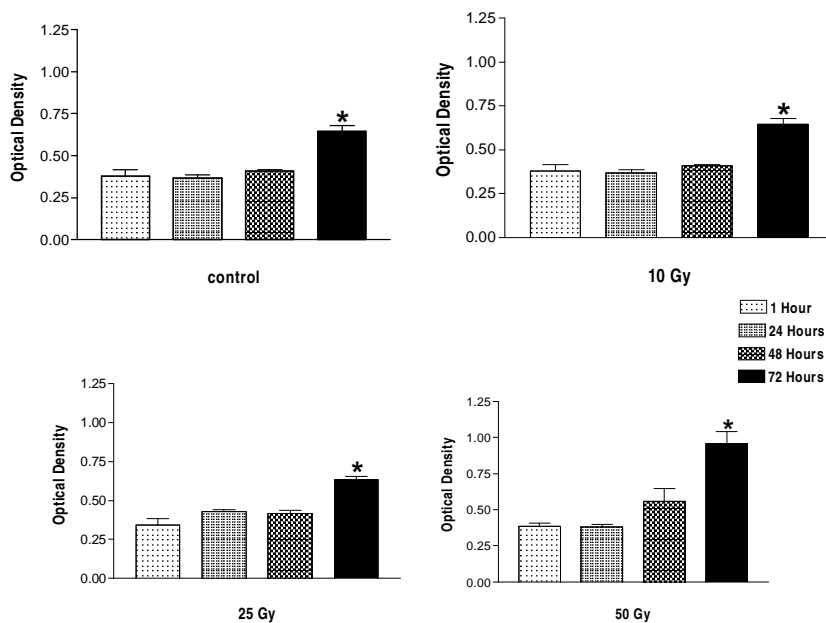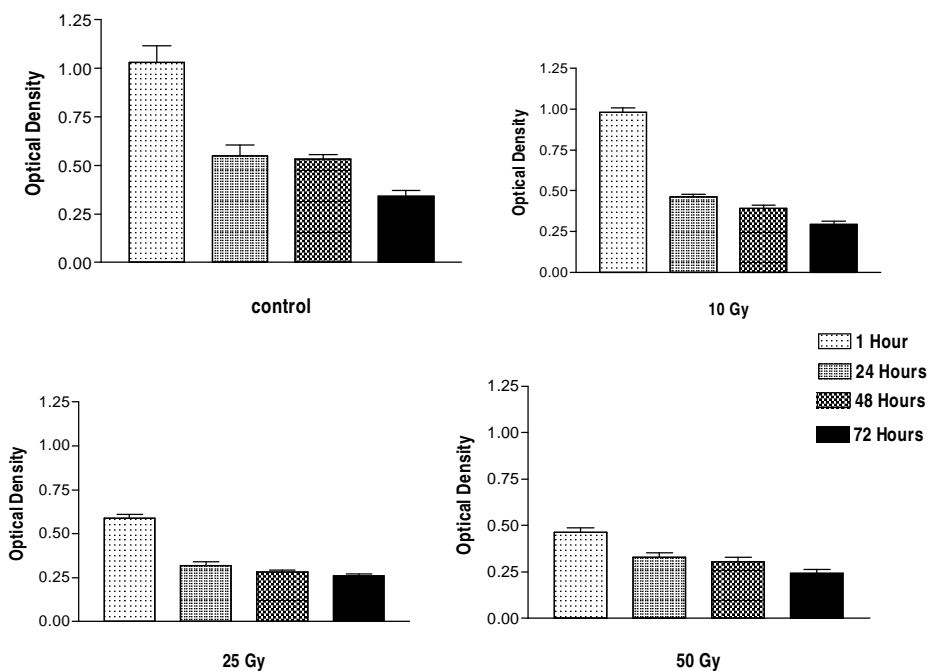

B PBMC Cellular Viability

Figure 2 - Measure of cellular viability of MDAMB-231 and PBMC cells through optical density at 595 nm, based on solubilized formazan crystals. Panel A, MDAMB-231 optical density in $1x10^6$ cells. Panel B, PBMC optical density in $1x10^6$ cells. Results represent the mean $\pm$ standard error (Mean $\pm$ SE) of quadruplicates from three experiments and (*) indicates a significative difference at $p < 0.05$.
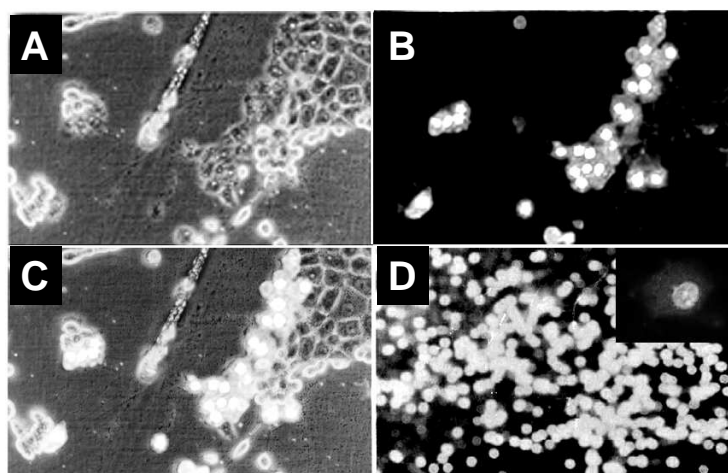
Figure 3 - Propidium iodide stained MDAMB-231 cells. Photomicrograph in inverse fluorescence microscopy (X 400). These cells were stained for 72 hours after 50 Gy gamma irradiation. Panel A, cells in phase contrast. Panel B, the fluorescence field with damaged nuclei stained by propidium iodide. Panel C, merging of these fields with damaged and intact cells positioned. Panel D, nuclear damages induced by treatment with 10% methanol in fluorescence field and an insert showing chromatin condensation after nuclear damage

Table 1 - Percentage of damaged nuclei found in MDAMB-231 cells 72 hours after irradiation by ^{60}Co.

| Cell treatment | Number of nuclei viewed (*) | | Damage (%) |
	Phase contrast	Fluorescence field	
Control	25,5 ± 3,1	1,0 ± 0,1	3,9
10 Gy	25,2 ± 3,1	14,2 ± 1,7	56,4
50 Gy	27,5 ± 3,4	17,7 ± 2,2	64,5
10% Methanol	19,0 ± 1,1	18,0 ± 1,1	95,0

(*) The data show MDAMB-231 cells quantified by hemocytometer, as well as the percentage of damaged nuclei stained by propidium iodide at room temperature after gamma irradiation. Results represent a standard error (Mean ± SE) from four experiments. Significative difference at $p < 0.05$.

Table 2 - Percentage of damaged nuclei found in PBMC cells 72 hours after irradiation by ^{60}Co.

| Cell treatment | Number of nuclei viewed (*) | | Damage (%) |
	Phase contrast	Fluorescence field	
Control	17,5 ± 2,1	7,5 ± 0,9	42,8
10 Gy	21,0 ± 2,6	18,7 ± 2,3	89,2
50 Gy	23,7 ± 2,8	22,5 ± 2,8	94,7
10% Methanol	20,0 ± 0,0	20,0 ± 0,0	100,0

(*) The data show PBMC cells quantified by hemocytometer as well as percentage of nuclei damaged stained by propidium iodide at room temperature after gamma irradiation. Results represent a standard error (Mean ± SE) from four experiments. Significative difference at $p < 0.05$.
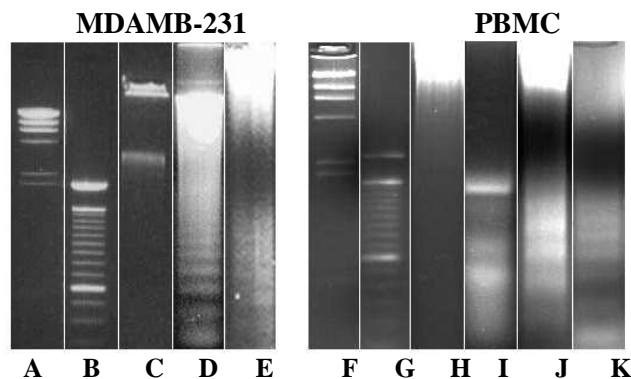
MDAMB-231   **PBMC**

Figure 4 - MDAMB-231 and PBMC cell DNA laddering. (A) Lambda Hind; (B) 100 bp (Gibco®); (C) DNA of non-irradiated MDAMB-231 cell; (D) DNA fragmentation of MDAMB-231 treated with 10% methanol; (E) DNA fragmentation of MDAMB-231 after 72 hours and 50 Gy gamma irradiation; (F) and (G) Lambda Hind and 100 bp ,respectively; (H) DNA of PBMC non-irradiated cells; (I) DNA fragmentation of PBMC cells treated with 10% methanol; (J) DNA fragmentation of PBMC cells after 72 hours and 10 Gy gamma irradiation; (K) DNA fragmentation of PBMC cells after 50 Gy for the period.

DNA laddering by electrophoresis agarose gel

DNA laddering of MDAMB-231 and PBMC cells after gamma irradiation, as well as after treatment with 10% methanol are shown in Fig. 4. DNA laddering was observed for 72 hours and 50 Gy only in the MDAMB-231. DNA fragmentation of MDAMB-231 induced by gamma irradiation with a 50 Gy dose did not reproduce the DNA fragmentation induced by 10% methanol. However, DNA fragmentation in PBMC cells was observed with 10 Gy and 50 Gy, as well as DNA fragmentation induced by 10% methanol.

DISCUSSION

The results suggest a radioresistent behavior of non-fractionated ^{60}Co protocol for the MDAMB-231 cell line at the level of a clinical radiotherapy dose for tumor control. Indeed, the clinical control dose for human breast cancer did not decrease cellular viability in the MDAMB-231 cell line presenting high mitochondrial metabolization. These results suggest that MDAMB-231 cells are able to efficiently correct the damages induced by irradiation and to stay viable, while PBMC cells present an intense decrease in the cellular viability after irradiation. Radioresistance of MDAMB-231 cells were demonstrated by post-irradiation MTT assay in comparison with radiosensitivity of other cell lines (Hunáková et al., 2000; Andrade et al., 2003). Other studies obtained radiation resistant cell clones of MDAMB-231 selected after gamma fractioned irradiation up to doses of 60 Gy with fractions of 4 Gy/fraction (Pearce et al, 2001). These cells line can be resistant to a fractioned dose as a single fraction gamma irradiation.

A dose as high as 50 Gy gamma irradiation at single fraction should be enough to induce nuclear damages in the MDAMB-231 similar to damages induced by 10% methanol, but this effect did not occur. The results show a higher level of damaged nuclei induced by gamma irradiation on PBMC, similar to the damaged nuclei induced by 10% methanol, demonstrating a radiation sensitivity of the PBMC. Comparing the rate of nuclear damages between PBMC and MDAMB-231, gamma irradiation and 10% methanol induced more cellular damages in PBMC, while this fact was not observed to MDAMB-231 cells. Sensitivity of human lymphocytes o photons exposure has already been observed t (Nakamura et al, 1991). The percentages of nuclei damaged after irradiation were significantly higher than that of the non-irradiated control (p < 0.05). However, these percentages of damaged nuclei induced by gamma irradiation were low compared to damages induced by 10% methanol, suggesting an efficacious repair mechanism of the MDAMB-231 cells after gamma irradiation of the surviving cells. The DNA

fragmentations induced by 10% methanol were better viewed when compared to DNA fragmentations induced by gamma irradiation in MDAMB-231. These data show few ladders, suggesting that it is difficult for the irradiation to break the DNA of these cells. This fact reinforce the the observation that a low level of nuclear damages was induced after MDAMB-231 gamma irradiation when compared to the treatment with 10% methanol. The assays employed show that the MDAMB-231 cell line was able to survive in spite of having received high doses of gamma rays. In addition, the treatment with 10% methanol was more effective than ^{60}Co in causing damages to MDAMB-231 cell line.

The bystander effect, presented by irradiated cells *in vitro*, describes the biological responses of surrounding cells not directly targeted by a radiation insult, as well as the tumor *in vivo* (Zhivotovsky et al, 1999). On the other hand, radioresistance of tumor cells is a common phenomenon and intrinsic radioresistance of tumor cells has been correlated to a poorer prognosis after radiotherapy (Girinsky et al, 1992, West et al, 1993). Indeed, several breast cancer cases present failure after radiation therapy in spite of the use of standard protocols (Jobsen et al, 2001, Levitt et al, 2003, Andrade et al, 2004). Further studies are necessary to understand the mechanisms of radioresistance in the human breast carcinoma cell line MDAMB-231 to contribute to breast cancer radiation therapy treatment.

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RESUMO

Radioterapia utilizando radiação gama é uma modalidade comum no tratamento do câncer de mama. A proposta deste estudo é investigar a resposta biológica *in vitro* da linhagem celular MDAMB-231 de câncer de mama humano e células do sangue periférico humano (PBMC) expostas à irradiação pelo Co[60] em frações simples de 10Gy, 25Gy e 50Gy e 136,4cGy min^{−1} rate. As células foram irradiadas a temperatura ambiente usando o equipamento de radioterapia Theratron 80 radiotherapy system. A resposta biológica, após irradiação gama, foi avaliada através do ensaio do MTT para viabilidade celular e o do ensaio com Iodeto de Propídio para visualização do dano nuclear, além da eletroforese em gel de agarose. Os danos nucleares induzidos pelo Co[60] foram comparados aos danos causados pela exposição das células à solução de metanol a 10%. Nós observamos que a dose de 50Gy não estimulou a mesma quantidade de danos nucleares que a solução de metanol a 10% nas células MDAMB-231. Maiores estudos são necessários para a compreensão destes mecanismos nas células de câncer de mama humano MDAMB-231.

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