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Evaluation of radiation-induced genotoxicity on human melanoma cells (SK-MEL-37) by flow cytometry

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ABSTRACT

Micronucleus assay is a test used to evaluate genotoxic damage in cells, which can be caused by various factors, like ionizing radiation. Interactions between radiation energies and DNA can cause breakage, leading to use chromosomal mutations or loss of genetic material, important events that could be induced in solid tumors to mitigate its expansion within human body. Melanoma has been described as a tumor with increased radio resistance. This work evaluated micronuclei percentages (%MN) in human melanoma cells (SK-MEL-37), irradiated by gamma radiation, with doses between 0 and 16Gy. Cell suspensions were irradiated in PBS by a 60Co source in doses between 0 and 16Gy, and incubated by 48h. Then cell membranes were lysed in the presence of SYTOX Green and EMA dyes, preserving nuclear membranes. Using this method, EMA-stained nuclei could be discriminated as those derived from dead cells, and SYTOX nuclei and micronuclei could be quantified. Micronuclei percentages were found to be proportional to dose, (R^2 = 0.997). Only the highest dose (16Gy) could induce statistically significant increase of MN ($p<0.0001$), although cultures irradiated by 4, 8 and 16Gy showed significant increase of dead cell fractions. Calculation of the nuclei-to-beads ratio showed that 8 and 16Gy could reduce melanoma cell proliferation. Results showed that although cell death and loss of proliferative capacity could be observed on cultures irradiated at lower doses, genotoxic damage could be induced only on a higher dose. Resistance to radiation-induced genotoxicity could explain a relatively high radio resistance of melanoma tumors.

Keywords: micronucleus assay, melanoma, radiation, genotoxic damage..

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1. INTRODUCTION

The National Cancer Institute (INCA) estimates about 600,000 new cases of cancer in Brazil in 2016/2017, including those characterized as non-melanoma skin [1]. The National Cancer Institute (INCA) estimates about 600,000 new cases of cancer in Brazil in 2016/2017, including those characterized as non-melanoma skin [1], that have high prevalence (more than 90% of cases of skin tumors), but low mortality that represent an object of little relevant study. Among the skin tumors of high medical relevance are melanomas. The projection of new cases in Brazil in 2016/2017 is 3000 in men and 2760 in women (1.4 and 1.3% of total cases, respectively). The mortality is relatively low, if there is treatment prior to a possible metastatization process, but after spreading through the body, the chance of cure is reduced from 92 to 68% [2].

Mortality from melanoma has doubled in the last 40 years in Brazil from 0.06 to 0.13%, in relation to the total number of cases. According to the World Health Organization, the gradual reduction of the coverage and thickness of the terrestrial ozone layer is potentially the major cause of this increase. There are an estimated 4500 new cases of melanoma in the world for loss of 10% of the filtering capacity of the layer [3].

In Brazil, mortality due to melanoma increased in general between 1979 and 2013, with special emphasis on the cases of the South region, whose observed mortality increased from 0.13 to 0.29% of the total cases, which is consonant with the fact that the region contains a predominance of caucasian populations, potentially more susceptible to the occurrence of this type of tumor. However, in the same period, the Northeast presented the highest proportional growth (0.01 to 0.06%) [4], leading to consider not only the skin tone, but also the location in relation to the Equator as a preponderant factor of occurrence of melanomas. There is a chance of underestimation of mortality by relativizing its incidence to the total number of cancer in Brazil, but the mortality calculated by the INCA in 2013 approached 25% of the cases of melanoma, an index considered high.

Primary melanomas are classified according to their origin, being characterized by cutaneous lesions that can reach the entire surface of the body and may be superficial (superficial *lentigo maligna*) or invasive (*lentigo maligna*); those of mucous origin, which can be in the head-neck, anorectal, vulvovaginal regions; and uveal, located within the eyeball [2].

The diagnosis of primary melanoma (cutaneous and non-metastatic) is performed by histopathological analysis of biopsies of one or more lesions and its appearance is then classified into one of the stages: 0, 1A, 1B, 1B, 1B or 1C. Metastasis can occur from any stage, except 0, and its probability is increased according to the histological evaluation index. After the metastatic spread, the diagnosis and prognosis of the individual can be performed by evaluating sentinel lymph node biopsies, adjacent to the lesion, or by diagnostic imaging techniques [5].

In addition to surgical procedures to remove the lesion or affected adjacent tissues (especially lymph nodes), the treatment of metastatic melanoma has been evaluated using inhibitors of cell division, with very encouraging results obtained in clinical trials [6, 7] or against cell death blockers [8]. Melanoma is classically described as a tumor that exhibits relative resistance to the destructive effects of ionizing radiation both in vitro [9] and in observations from clinical studies [10]. In this way, radiotherapy has lost its importance in the treatment of this type of tumor, although a notable resurgence of the defense of the technique has appeared in recent years [11-13].

Despite the reconsideration of radiotherapy in the treatment of melanoma, several questions regarding the side effects produced due to the high doses [13,14] need to be resolved, in order not to induce comorbidities in the affected individuals or even to increase their quality of life, even in cases of the use of radiotherapy as palliative.

The appearance of increased amounts of micronuclei in cells that bearing, as well as their frequency in the cytoplasm, are consequent signals of genotoxicity. The goal of the analysis is correlate, if possible, this increase to a genotoxic potential of a test substance [15]. The technique is based on the observation of accumulation of cytoplasmic micronuclei by blocking cytokinesis (CBMN) but without inhibiting karyokinesis of the cell nucleus after DNA duplication. In this way, cells treated by a given compound would exhibit two nuclear bodies, being called binucleate cells. If such cells have been submitted to a significant genotoxic stress, and if such events could cause irreparable DNA strand breakage, binucleate cells show a dense chromatin fragment with coloration and appearance like that of the major nuclei.

After controlled exposures to test substances, cells are fixed and stained, and further subjected to analysis by light [16] or fluorescence [17] microscopy. Although it has undergone several modifications over the years, there is a need for automation of the technology for large-scale application. Some authors have used flow cytometry to quantify micronuclei, which in these cases are characterized as particles with at least 1/100 of the fluorescence of main nuclei [18]. The methodology is used not only for the quantification of genotoxic damage of chemical agents but it is being reported as an important tool for biological dosimetry of radiation [19] since its biological reflection of interaction with matter [20]. The present work used this technique to assess radiation

induced genotoxic stress on human melanoma cells (SK-MEL-37). The test protocol was useful to detect (radiation-induced) cytotoxicity and changes in cell division, as detected by preferential staining with EMA and by calculating the nuclei-to-bead ratios and proven to be suitable to study clastogenic effects of radiation on *in vitro* human melanoma model.

2. MATERIALS AND METHODS

2.1 CELL CULTURE

Sloane-Kettering human melanoma cells (SK-MEL-37, ExPASy RRID: CVCL_3878) were cultured in 25cm² bottles in RPMI 1640 medium with phenol red (Vitrocell-Embriolife) supplemented with 10% Fetal Bovine Serum (SFB-GIBCO-BRL) and 1% of Antibiotics (Penicillin, Streptomycin - GIBCO-BRL), incubated at 37° C in the presence of 5% CO_2 until approximately 70% confluence in monolayer, with replacement of the culture medium every 48 hours and subcultured every 7 days in culture.

2.2. IRRADIATION PROCEDURES

Cells were removed from culture bottles using trypsin / EDTA solution (0.05 / 0.05M), washed by centrifugation in fresh culture medium and resuspended in 1mL PBS (Phosphate Buffered Saline Buffered) for gamma irradiation procedures at doses between 0.5 and 16Gy (or otherwise when cited) of ^{60}Co within sterile microtubes and at room temperature. The irradiations were performed on a GammaCell 220 (Irradiation Unit of Canadian Atomic Energy Commission, Ltd.) equipment at the IPEN / CNEN-SP Radiation Technology Center using a metallic lead shield corresponding to 90% radiation attenuation (dose rate: 90Gy/h). Following irradiation, the suspensions were centrifuged and the proportions of viable cells determined by trypan blue exclusion method. Concentrations were adjusted to give 50,000 cell / mL suspensions, with 100µL (5000 cells) seeded per well in 96-well plates. After plating the cells were incubated for 72 hours under the conditions described above. As controls, control wells received mitomycin (Sigma-Aldrich, CAS 50-07-7) at a concentration of 2.5 µg / mL. After 4 hours of incubation as described, the wells were washed with PBS and received fresh culture medium and continued in culture for 24 hours.

2.3. CLONOGENIC POTENTIAL ANALYSIS

SK-MEL-37 suspensions were irradiated at 0, 1, 2, 4, 8 or 10Gy and seeded in triplicates (600 cells/well, 3mL) on 60 mm cell culture dishes and let to grow (37ºC, 5% CO_2) for 10-12 days. At this time, clonal colonies of non-irradiated cultures showed 50-60 cells/colony. Plates were washed twice with warm (310K) PBS and colonies were fixed with PBS + 4% paraformaldehyde for 1800 seconds. Fixed material was washed twice with PBS and stained using a standard May-Grünewald Giemsa protocol (1800 seconds). Colonies were manually counted. Numbers of colonies from cells irradiated at each dose were used for determination of plating efficiencies (PE) (1) or survival fractions (SF) (2), using following formula:

$$PE = (\text{Number of counted colonies} / 600) \times 100 \quad (1)$$

$$SF = \text{Number of counted colonies} / 600 \times (\text{Mean PE of controls}/100) \quad (2)$$

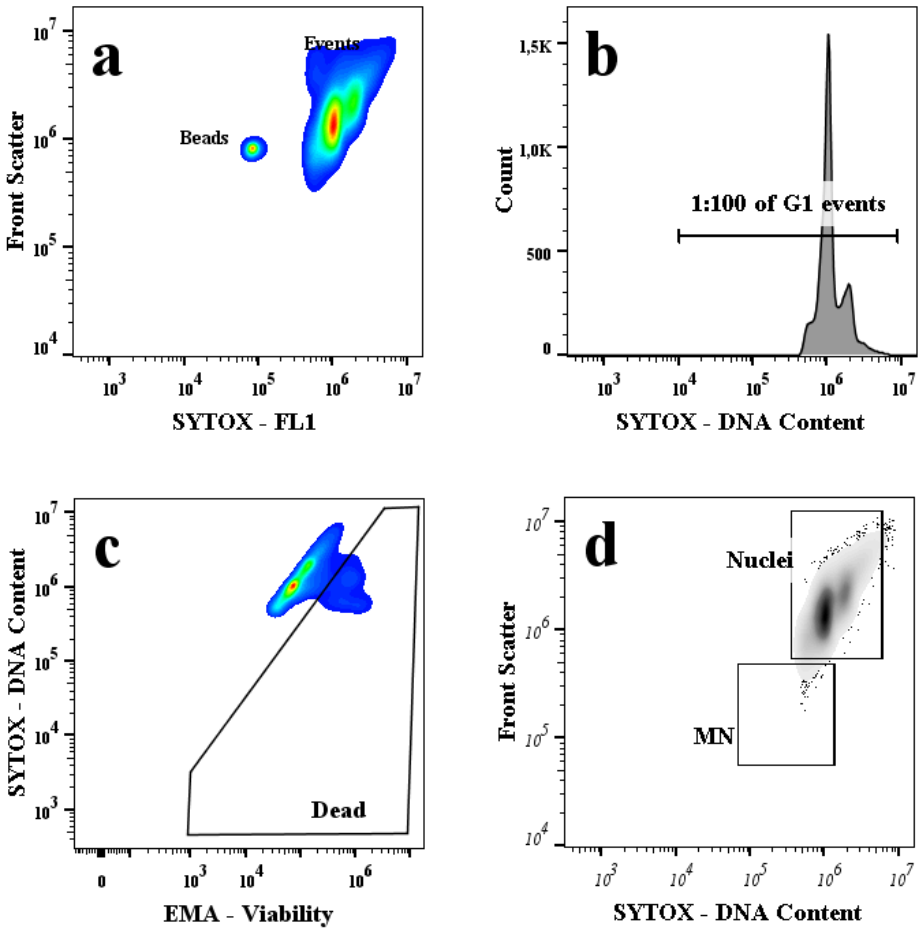
2.4.-Color Flow Cytometry for MN Quantification

After 72 hours in culture, cells seeded in 96 well plates irradiated or treated with the described genotoxic agents or control (non-irradiated and untreated) cultures were washed in PBS and received a solution of ethidium bromide monoazide dye (Thermo-Fisher Scientific, E1374) at a concentration of 8.5 µg / ml diluted in PBS supplemented with 2% fetal bovine serum. The culture plates were opened and exposed to a blue led light source (440-450nm, 30W) source for 30 minutes for photoactivation of the compound, which was irreversibly associated only with the DNA of non-viable cells. This procedure aimed to efficiently label cells that should not be included in the counting of nuclei and micronuclei, in addition to providing some measure of cytotoxicity. After this step, the cells received PBS with 2% fetal bovine serum and centrifuged for removal of free dye. Two lysis steps were performed to release nuclei and micronuclei and to stain its DNA. The first step consisted in lysing the cells using a solution with sodium chloride (0.854mg / mL), sodium citrate (1mg / mL) and IGEPAL (0.3 µL/mL), as well as 0.4µM SYTOX Green fluorescent dye (Thermo-Fisher Scientific, S7020). After lysis for 60 minutes 310K), the plates were centrifuged and received the second lysis solution (sucrose 85.6mg / mL, citric acid 15mg / mL and SYTOX Green 0.4µM). Second lysis solutions were supplemented with 5µL/well of fluorescent latex beads (AccuCheck Couting Beads, Molecular Probes). After 30 minutes at room temperature, the materi-

al was set ready for reading on the flow cytometer (Accuri C6, BD Biosciences).

The analysis followed the methodology described in the literature [19]. Briefly, events marked with EMA were excluded from the total count. Events with SYTOX were evaluated according to their size (FSC) and fluorescence (FL1) for discrimination between nuclei and micronuclei. At least 20000 events gated on nuclei region were counted in each sample. Data was collected from two independent experiments in octuplicates as percentages of EMA-positive events and SYTOX-positive MN events and results were given as fold-changes comparing to control wells (non-irradiated, non-treated cells). Nuclei-to-bead ratios (NBP) score was used to find cell division ratios differences between groups. Gating strategy to analysis is depicted on Fig. 1.

Figure 1: *Gating strategy. (a) FSC x FL1 to gate events and latex beads; (b) only events with at least 1/100 of G1 fluorescence; (c) only EMA negative/SYTOX positive events were considered; (d) Nuclei and micronuclei (MN) regions.*



2.5. Data Analysis

Survival fraction data was fitted to an exponential function (3) to plot the cell survival curve. Dose values were transformed into its neperian logs (base "e") and fitted to a sigmoid dose-response curve (4) to radiation DL_{50} calculation.

$$SF = e(\alpha X - \beta X^2), \text{ where "X" are radiation dose values} \quad (3)$$

$$SF = Bottom + (Top-Bottom)/(1+e^{((LogDL50-X)*HillSlope))} \quad (4)$$

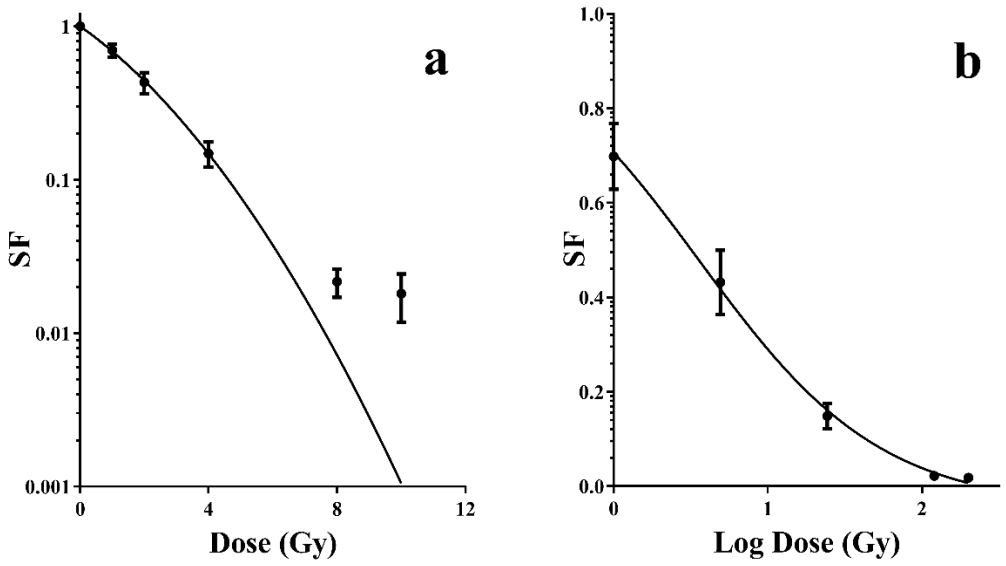
MN fold change data were fitted to a linear-quadratic function (second-order polynomial) to determine whether found events could be associated to a typical clastogenic response of nuclear DNA to ionizing radiation. Differences between groups irradiated at different doses were tested using one-way ANOVA followed by Bonferroni post-tests.

3. RESULTS AND DISCUSSION

3.1 Survival Fraction and DL_{50} of irradiated human melanoma cells

Cell survival curve obtained from irradiated cultures is shown in Fig. 2a. Same data was fitted to a sigmoidal dose-response model, as shown in Fig. 2b.

Figure 2*: Survival fraction (SF) values of SK-MEL-37 irradiated at doses from 0 to 16Gy fitted to a colony-growth (a) and sigmoidal dose response (b). Bars: SEM***.**



Coefficients of data for (3) and (4) equations are shown in Table 1.

Table 1: Mean and standard error values of coefficients obtained from data of survival fraction values of SK-MEL-37 cells irradiated from 0 to 10Gy. (α) and (β) are linear and quadratic coefficients (same as in (3)).

Equation	Coefficient	Mean ± SEM
(3)	α	-0.3398 ± 0.07017
	β	0.03461 ± 0.029
	β/α (radiosensitivity coefficient)	-0.1019 ± 0.1045
	R^2	0.9347
(4)	DL_{50}	3.596
	R^2	0.9982

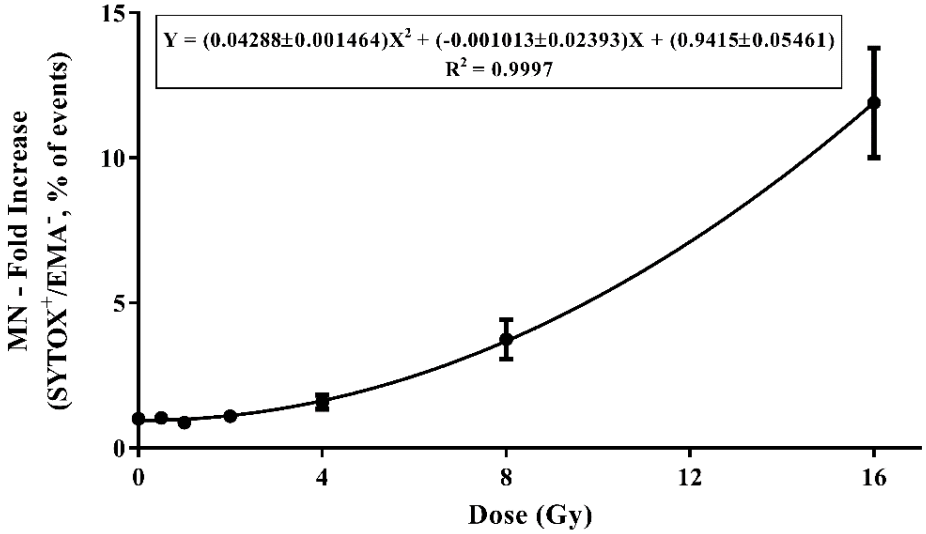
The ratio of linear and quadratic coefficients (β/α) is taken as a classical parameter of cell radiosensitivity [21]. (DL_{50}) was the necessary radiation dose (calculated) to induce 50% of cell mortality in experiments and given in Gray (Gy). R^2 values are fit coefficients for each equation, with no SEM values.

Analysis allowed the calculation of the DL50 of gamma radiation on human melanoma cells, 3.596Gy.

3.1. MN Induction by ^{60}Co radiation

MN fold-increases could be fitted to the classical model of DNA aggression by radiation, as shown in Fig. 3.

Figure 3: *MN-Fold increase of SK-MEL-37 cells irradiated from 0 to 16Gy. Inset: Regression equation obtained from data and fit coefficient. Bars: SEM.*

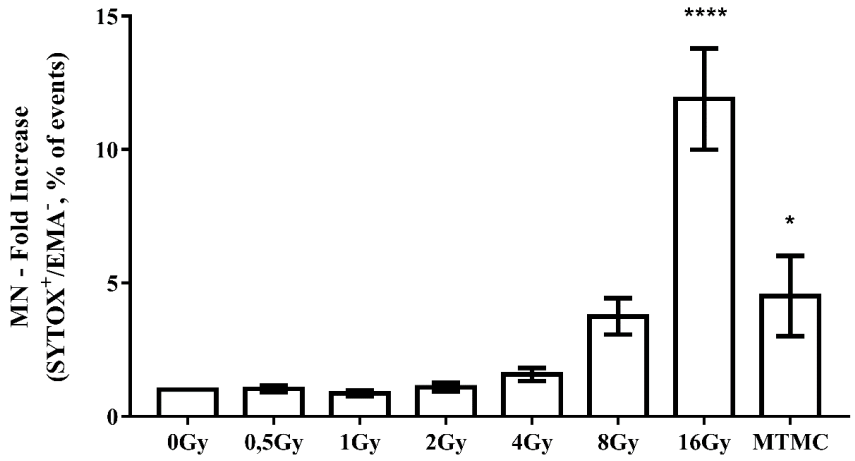


Data could be well fitted to the equation, leading to an interpretation of a good representation of a typical result from experiments of DNA aggression by ionizing radiation.

3.3. Statistical Differences of MN-Fold Increase Between Groups

Statistically significant differences could be observed between groups, as shown in Fig. 4.

Figure 4: *MN-Fold increase of SK-MEL-37 cells irradiated from 0 to 16Gy. (*): p<0.05. (****): p<0.0001. MTMC: mitomycin control. Bars: SEM.*



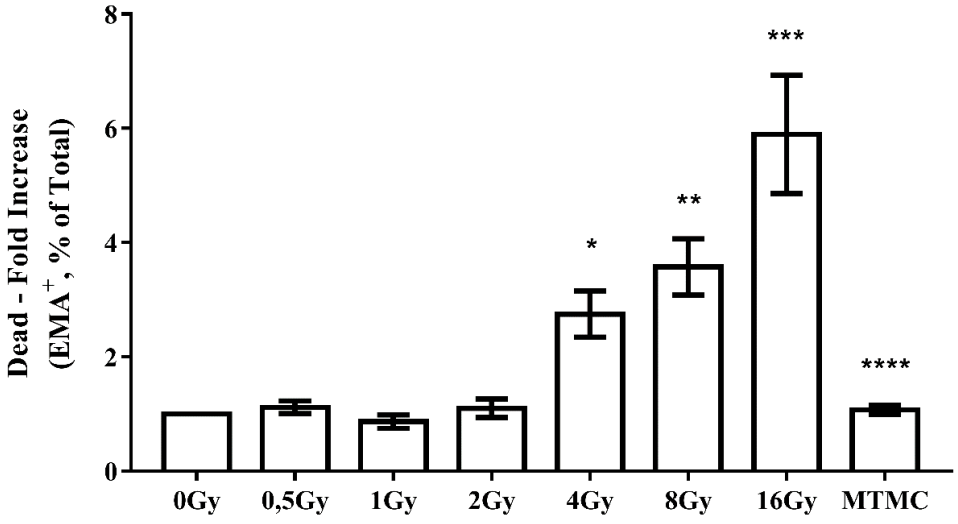
Radiation clastogenic effects on DNA of cells could be found on SK-MEL-37 cultures 72h after irradiation. Only the highest radiation dose (16Gy) or positive control (mitomycin) could induce

significant increase on MN fold-changes, despite its occurrence could be associated to a linear-quadratic response. Further development would be arranged to turn the technique more sensitive, being able to detect more discrete differences between doses.

3.4. Assessment of Radiation-Induced Cytotoxicity

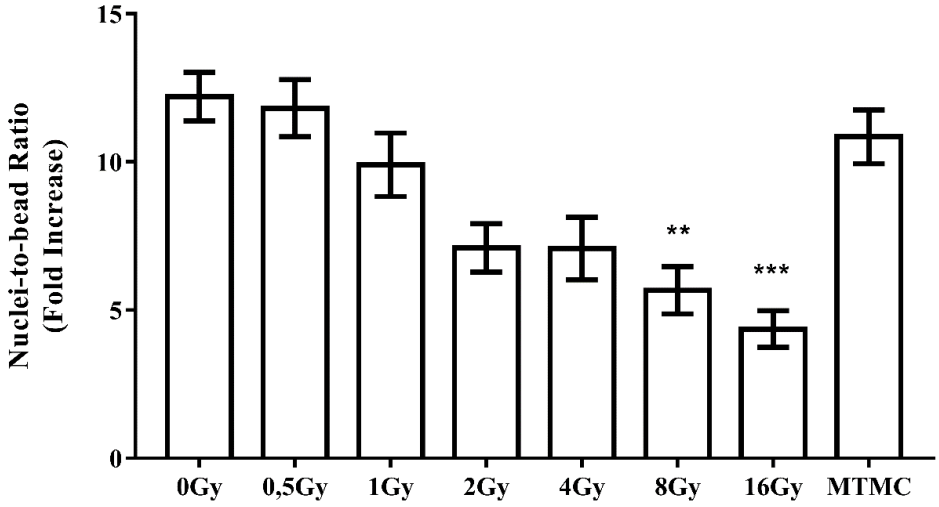
$EMA^+/SYTOX^+$ events were representative to nuclei and micronuclei from cells considered unviable at the time of experiment. This differentiation is important specially when comparing flow cytometry results to those obtained using microscopy scoring, the last relying on binucleated (and thus, viable and proliferating) cells. Quantification of these events can also be used to cytotoxicity testing. Events from unviable cells were shown on Fig. 5.

Figure 5: *Nuclei and micronuclei from dead cells (fold increase) of SK-MEL-37 cells irradiated from 0 to 16Gy. (*): p<0.05. (**): p<0.01. (****): p<0.0001. MTMC: mitomycin control. Bars: SEM.*



3.5. Changes in cell proliferation

Figure 5: *Radiation-induced changes in cell proliferation.*



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4. CONCLUSION

Human melanoma SK-MEL-37 cells showed increased radiosensitivity when radiation-induced genotoxicity data were analyzed. In the present experiments, only the higher dose (16Gy) could induce genotoxic damage. Further work should include experiments to test DNA repair mechanisms, as quantification of phosphor ATM/ATX complexes, γ-H2AX formation or even p53 phosphorylation.

The technique detected cell death to a very acceptable extent, in cultures irradiated at doses of at least 4Gy. The results are coherent to those obtained with clonogenic potential experiments, which resolved a very close value of DL50 of 3.596Gy. The flow cytometry technique has proven to be suitable to geno- and cytotoxicity of irradiated human melanoma cell cultures.

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