BJRS

BRAZILIAN JOURNAL
OF
RADIATION SCIENCES
07-2A (2019) 01-13

SBPR
Sociedade Brasileira de Proteção Radiológica

Use of 2-color flow cytometry to assess radiation-induced genotoxic damage on CHO-KI cells

Luma Ramirez de Carvalho[1], Letícia Bonfim[1], Daniel Perez Vieira[1]

[1] *Instituto de Pesquisas Energéticas e Nucleares (IPEN / CNEN - SP), 05508-000 São Paulo- SP, Brazil*

lrcarvalho@ipen.br

ABSTRACT

The micronucleus assay is an important technique used to evaluate genotoxic damage of chemical or physical agents (as ionizing radiations) on cells, based on quantification of cells bearing micronuclei, which are fragments derived from damage (breakage) of the DNA. Currently, this technique was updated to an automated approach that relies on plasma membrane dissolution to analyze fluorescent dye-labelled nuclei and micronuclei by flow cytometry. Cell suspensions were irradiated in PBS by a ^{60}Co source in doses between 0 and 16 Gy, and incubated by 72h. Cell membranes were lysed in the presence of SYTOX Green and EMA dyes, so EMA-stained nuclei could be discriminated as from dead cells, and nuclei and micronuclei could be quantified. Amounts of micronuclei (percent of events) in the samples, were found to be proportional to radiation doses, and could be fitted to a linear-quadratic model (R^2 = 0.993). Only higher doses (8 and 16 Gy) and positive control could induce relevant increases in micronucleus amounts. The incorporation EMA showed an increase in irradiated cells. Mid- to high doses (4, 8 and 16 Gy) induced reduction of cell proliferation. Experiments showed the suitability of the technique to replace traditional microscopy analysis in evaluation of the effects of ionizing radiations on cells, with possibility to use in biological dosimetry.

Keywords: **micronucleus, radiation , cytometry.**

ISSN: 2319-0612
Accepted: 2018-10-27

1. INTRODUCTION

The *in vitro* micronucleus frequency test (FMN) is one of the methodologies of choice in the development of toxicological safety tests. Its accomplishment is based on the counting of unrepaired double breaks in the DNA of cultured cells at various concentrations of test substances or incremental amounts of various aggressive agents such as ionizing radiation. In interphase cells, the product of such breaks is apparent in the form of micronuclei, which are agglomerates of DNA with similar staining to that of the main nucleus, and 5 to 30% of its size.

The increase in the proportion of cells that bearing micronuclei, as well as their quantity in the cytoplasm of the analyzed cells, are mechanistic signals of genotoxic damage. After analysis, this increase may relate the concentration of the test substance to its genotoxic potential [1].

The technique is based on the observation of accumulation of cytoplasmic micronuclei by blocking cytokinesis (CBMN), and is performed using cytochalasin B, a compound of fungal origin that can inhibit cytokinesis (cell division itself) without inhibiting karyokinesis of the cell nucleus after DNA duplication. In this way, cells treated by this compound 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. The technique is characterized as an in vitro test, and is a relatively fast and very efficient test system used to evaluate chemicals that can induce genotoxic damage, leading to the formation of micronuclei in the cytoplasm of cells.

These micronuclei may originate from acentric fragments or whole chromosomes that are unable to migrate with the rest of the chromosomes during the anaphase of cell division [2]. Micronuclei observed in such way can only be apparent in cells that have completed at least one nuclear division after exposure to the mutagen and is therefore a good indicator of chromosomal damage. Higher frequencies of MN may be related to increased DNA damage, which may result in increased risk for the development of cancer or genetic diseases. Using the same data, the cytokinesis-block

proliferation index (CBPI) and the replication index (RI) are also calculated, whose values are analyzed together with the proportions of binucleate cells with micronuclei

The test protocol is performed after exposure of cells in culture to varying concentrations of a given test substance. After this period, the cells are fixed and stained, and further subjected to analysis by light microscopy [3] or fluorescence [4]. As a microscopy technique, the analysis of the frequency of micronuclei in each sample may face certain operational challenges. 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 1/10 to 1/100 of the fluorescence of main nuclei [5]. The methodology is used not only for the quantification of genotoxic damage of chemical agents but is also being reported as an important tool for biological dosimetry of radiation [6] since its biological reflection of interaction with matter [7]. The present work used a flow cytometry testing protocol to assess genotoxic damage caused by ^{60}Co on CHO-KI cells.

2. MATERIALS AND METHODS

2.1. Cell Culture

Chinese hamster ovary cells (CHO-KI, ATCC CCL-61) 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. All experiments were performed using cultures that underwent at least one subculture step after thawing, with subsequent passages less than nine.

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 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. 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. 2-color flow cytometry for MN quantification

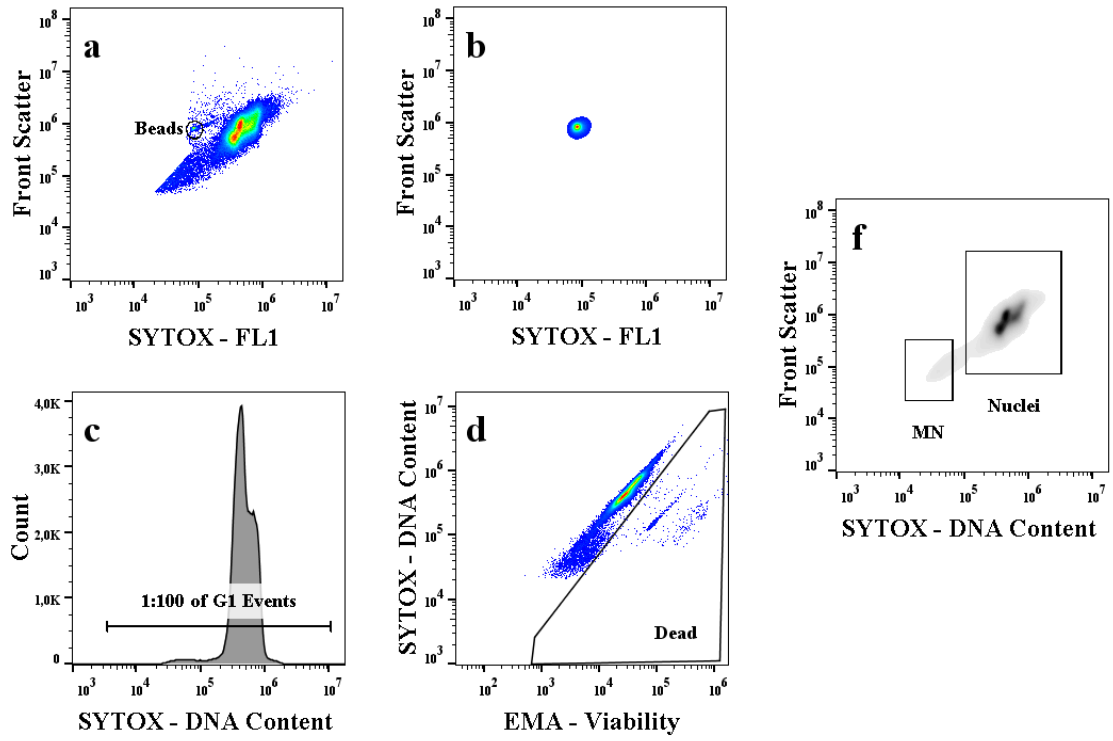
After 72 hours in culture, cells seeded in 96 well plates irradiated or treated with the described genotoxic agents or control (non-irradiated and untreated) cultures were washed in PBS and received a solution of ethidium bromide monoazide dye (Thermo-Fisher Scientific, E1374) at a concentration of 8.5 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 (37ºC), 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 (AccuCheckCouting Beads, Molecular Probes). After 30 minutes at room temperature, the material wasset ready for reading on the flow cytometer (Accuri C6, BD Biosciences).

The analysis will follow the methodology described in the literature [5]. 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 latex beads (b); (c) only events with at least 1/100 of G1 fluorescence; (d) only EMA negative/SYTOX positive events were considered; (f) Nuclei and micronuclei (MN) regions.*



2.4 Event frequency homogeneity throughout plate

Data from one experiment was used to analyze homogeneity of amounts of positive events from replicates. MN, EMA-positive events and bead counts were plotted on heatmaps to depict differences between wells.

2.5 Data Analysis

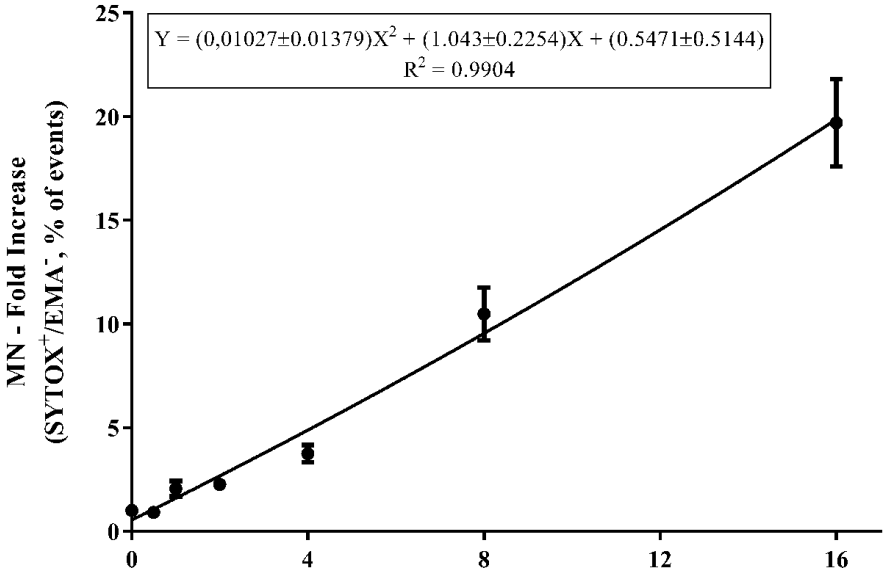
MN fold change data were fitted to a linear-quadratic (second-order polynomial) to determine whether found events could be associated to a typical clastogenic response of nuclear DNA to ionizing radiation. Differences between groups irradiated at different doses were tested using one-way ANOVA followed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

3. RESULTS AND DISCUSSION

3.1 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. 2.

Figure 2: *MN-Fold increase of CHO-KI cells irradiated from 0 to 16Gy. Inset: Regression equation obtained from data and fit coefficient. Bars: SEM.*

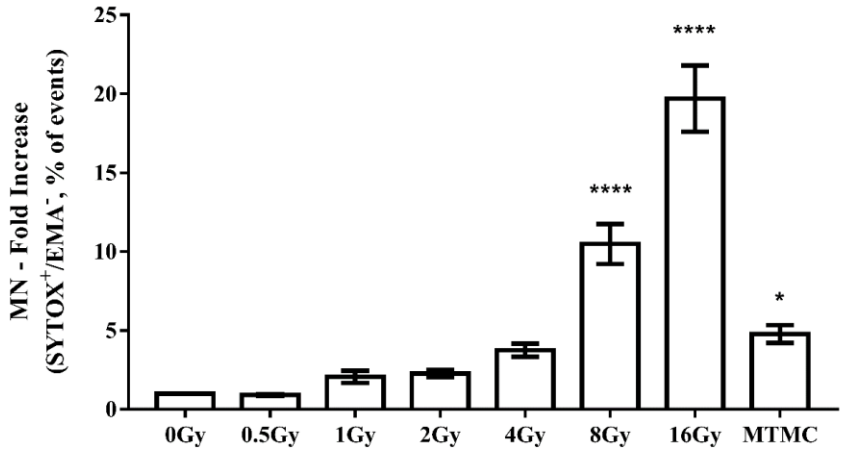


Data could be well fitted to the equation, leading to an interpretation of a good representation of a typical result from experiments of DNA aggression by ionizing radiation.

3.2 Statistical differences of MN-fold increase between groups.

Statistically significant differences could be observed between groups, as shown in Fig. 3.

Figure 3: *MN-Fold increase of CHO-KI cells irradiated from 0 to 16Gy. (*): p<0.05. (****): p<0.0001. MTMC: mitomycin control. Bars: SEM.*

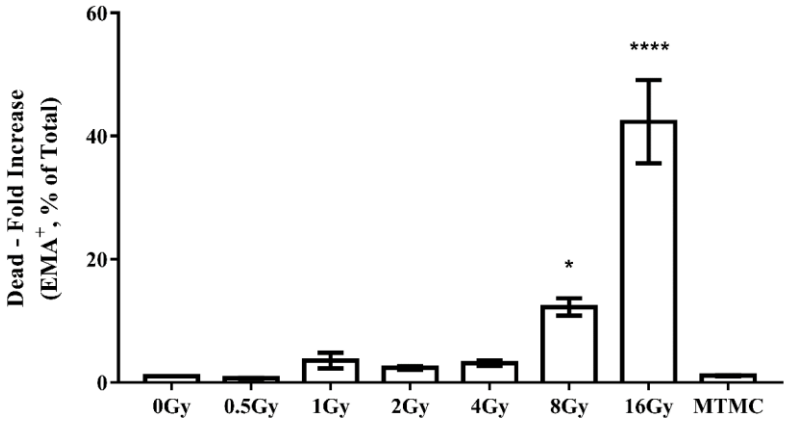


The technique is currently being used to genotoxic evaluation of chemicals on adherent cells [8]. Radiation clastogenic effects on DNA of cells could be found on CHO-KI cultures 72h after irradiation. Only higher radiation doses (8 and 16Gy) or positive control (mitomycin) could induce significant increase 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. Although these considerations, data can be further used to the construction of a standard curve for biological dosimetry, using peripheral lymphocytes instead of CHO-KI cells.

3.3 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. 4.

Figure 4: *Nuclei and micronuclei from dead cells (fold increase) of CHO-KI cells irradiated from 0 to 16Gy. (*): p<0.05. (****): p<0.0001. MTMC: mitomycin control. Bars: SEM.*

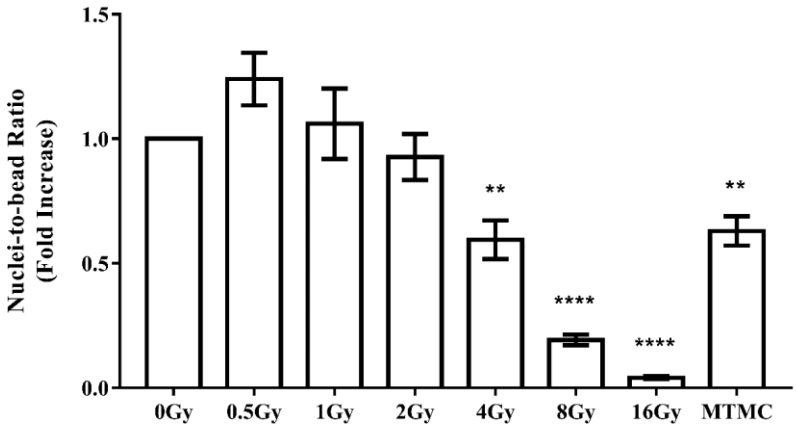


After 72h, only cultures irradiated by 8 or 16Gy showed significant shifts on cell viability. CHO-KI cells are somewhat resistant to radioinduced apoptosis or necrosis when irradiated by doses up to 5Gy [9]. Using this parameter, the present results can be considered consistent and good representative of the physiologic behavior of CHO-KI when gamma-irradiated. As expected, mitomycin did not induce cytotoxicity.

3.4 Changes in cell proliferation

Radiation-induced changes in cell proliferation are shown on Fig. 5.

Figure 5: *Nuclei-to-bead ratios (fold increase) of CHO-KI cells irradiated from 0 to 16Gy. (**): p<0.01. (****): p<0.0001. MTMC: mitomycin control. Bars: SEM.*

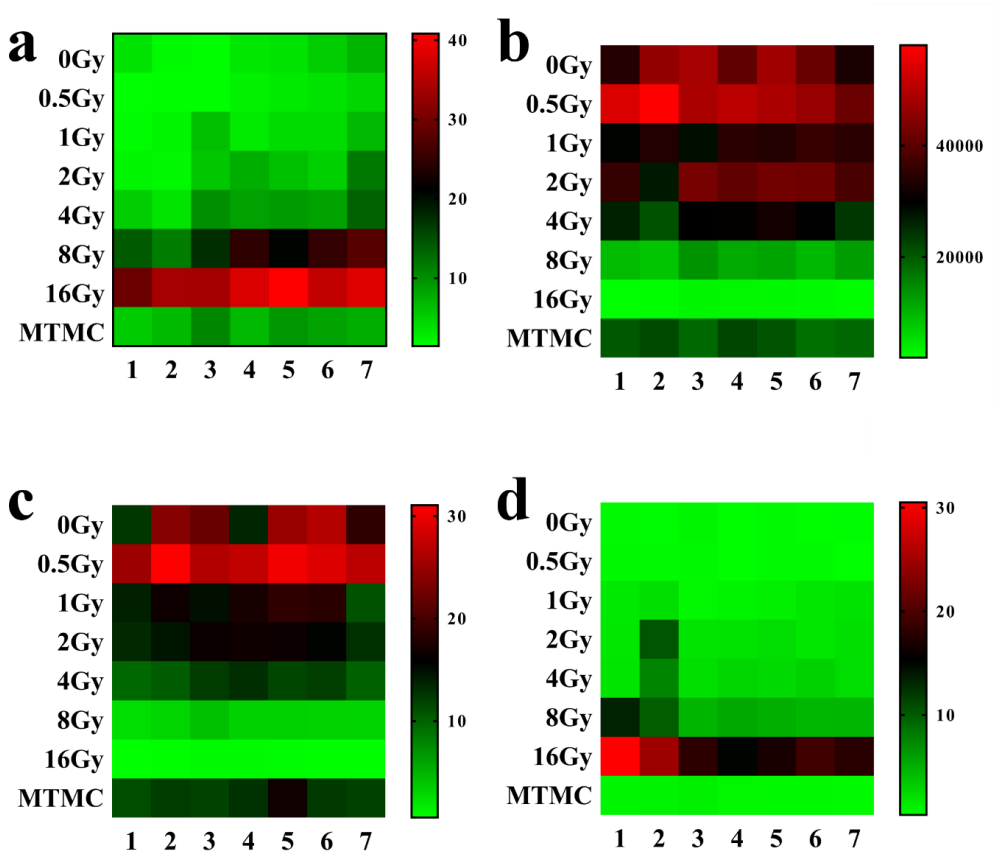


Not only higher (8-16Gy), but also 4Gy dose induced reduction of cell proliferation on irradiated cultures. Data from this analysis could be a useful surrogate for CBPI (cytokinesis-blocking proliferation index) analysis, as lysis of cell membranes prevent from analysis of binucleated cells. Using a nuclei-to-bead ratio, where bead concentrations are homogeneously dispersed across wells (approx. 2000 beads/well, data not shown), the analysis can determine if any radiation dose or chemical exposure reduced or increased the number of nuclei on a given sample. As expected, mitomycin induced a significant reduction of cell proliferation. Despite not statistically significant, an increase of proliferation could be annotated from cells irradiated by 0.5Gy. A possible hormetical effect could be studied in further works.

3.5 Event homogeneity across plate

To test if automatic sampling could produce some variability in replicates from same sample in wells across one plate, data was plotted on heatmaps, as shown in Fig. 6.

Figure 6: *(a) Micronuclei (% of total valid events), (b) Nuclei (absolute counts), (c) Nuclei-to-bead ratios and (d) EMA+ events (from dead cells, % of total valid events) found on test plate. Squares represent wells and numbers on horizontal axis represent column numbersin test plate.*



Some preliminary experiments showed inconsistency of nuclei and bead counting across test plates. The authors found to be useful perform readings one column at time, stopping automatic event acquisition after one reading and resuspending samples using a multichannel pipette. After the adoption of this procedures, events showed to be more heterogeneously disposed on test plate, as shown in figure.

4. CONCLUSION

Data will help further developments of a flow cytometry-based automated approach to micronucleus frequency analysis for biological dosimetry assessment. Future studies may rely on analysis using peripheral lymphocytes as radiation damage sensors.

5. ACKNOWLEDGMENT

This work was funded by IPEN following the 3rd Intern Funding Program (2016). Authors wish to thank Eng. Elizabeth Sebastiana Ribeiro Somessari and Eng. Carlos Gaia da Silveira from Center of Radiation Technology (CTR-IPEN/CNEN-SP) for very helpful assistance on irradiation procedures. Luma Ramirez de Carvalho was a CAPES fellow (PROEX0041041)

REFERENCES

1. SPEIT, G.; ZELLER, J.; NEUSS, S. The in vivo or ex vivo origin of micronuclei measured in human biomonitoring studies. **Mutagenesis**, v. 26, n. 1, p. 107–110, 2011.

2. OECD – Organisation for Economic Co-operation and Development. **Oecd guideline for the testing of chemicals draft proposal for a new guideline 487 : in vitro micronucleus test**. 2012. Available at: < http://www.oecd.org/env/ehs/testing/TG487%20Oct%202012%20updated%2029oct.pdf>. Last accessed: 22 Nov 2017.

3. HEDDLE, J. A. et al. Reflections on the development of micronucleus assays. **Mutagenesis**, v. 26, n. 1, p. 3–10, 2011.

4. OCAMPO, I.Z.; PASSOS, P.Q.S.P.; de CARVALHO, L.R.; LIRA DA CRUZ, C.A.; ESTEVES-PEDRO, N.M.; da SILVA, F.M.; HIGA, O.Z.; DIAS, L.A.; OKAZAKI, K.; VIEIRA, D.P. In vitro cytotoxic and genotoxic evaluation of peptides used in nuclear

medicine (DOTATATE and Ubiquicidin(29-41)) in CHO-K1 cells. **Cytotechnology,** v. 6, p. 2301-2310, 2016.

5. BRYCE, S. M; BEMIS, J.C.; AVLASEVICH, S.L.; DERTINGER, S.D. In vitro micronucleus assay scored by flow cytometry provides a comprehensive evaluation of cytogenetic damage and cytotoxicity. **Mutation Research - Genetic Toxicology and Environmental Mutagenesis**, v. 630, p. 78–91, 2007.

6. GARTY, G.; BIGELOW, A.W.; REPNI, M.; TURNER, H.C.; BIAN, D.; BALAJEE, A.S.; LYULKO, O.V.; TAVERAS, M.; YAO, Y.L.; BRENNER, D.J. An automated imaging system for radiation biodosimetry. **Microscopy Research and Technique**, v. 598, p. 587–598, 2015.

7. HALL, E. J. et al. Basic radiobiology. **American journal of clinical oncology**., v. 11, p. 220–252.

8. BRYCE, S.M.; SHI, J.; NICOLETTE, J.; DIEHL, M.; SONDERS, P.; AVLASEVICH, S.; RAJA, S.; BEMIS, J.C.; DERTINGER, S.D. High content flow cytometric micronucleus scoring method is applicable to attachment cell lines**. Environ Mol Mutagen**, v. 51, p.260-266, 2010.

9. SANTOS, G.S.; TSUTSUMI, S.; VIEIRA, D.P.; BARTOLINI, P.; OKAZAKI, K. Effect of Brazilian propolis (AF-08) on genotoxicity, cytotoxicity and clonogenic death of Chinese hamster ovary (CHO-K1) cells irradiated with (60)Co gamma-radiation. **Mutat Res Genet Toxicol Environ Mutagen**, v. 762, p.17-23, 2014.