



# Evaluation of genotoxic potential of peptides used in nuclear medicine (PSMA -617 and -11, and ubiquicidine 29-41) using a flow-cytometric, semi-automated analysis of micronuclei frequency in cell cultures



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## ABSTRACT

Assays that rely on the assessment of frequency of micronuclei are important standard techniques currently used to quantify potential genotoxic damage after exposure to chemical or physical agents, such as ionizing radiation, or in pre-clinical studies, to assessment of the genotoxic potential of drugs or its components. The experiments are usually performed using conventional microscopy, but currently the protocols are being upgraded to automated approaches based on flow cytometry protocols based on the elimination of the plasma membrane by chemical agents, allowing quantification by flow cytometry. In this work, the genotoxic potential of peptides used as components of radiopharmaceuticals (PSMA-617 and 11 and Ubiquicidine) was evaluated exposing CHO-K1 cells to a wide range of concentration (0.1X and 100X the maximum allowed concentration to human adults). Incubation with PSMA-11 or UBI<sub>29-41</sub> did not induce genotoxicity. After 24 h of incubation, PSMA-617 induced genotoxicity only in non-practical concentration (100-fold). Results corroborate the safety of the pre-drugs and the wide detection range of technique.

## 1. Introduction

Prior to its commercialization, compounds such as pharmaceuticals, food additives, pesticides or their components must have their efficacy and safety assessed by standard techniques. Some assays are required following methodologies which can demonstrate accurately that the test molecule is not capable to induce a potential risk (toxicity), whether directly observable *in vitro* or *in vivo*, or in the form of inherited genetic damage, or increasing risk of oncogenesis. One major consideration is the possibility that a certain test substance, in one or more concentrations, can be prone to trigger cellular mortality. Cytotoxicity, or cellular toxicity, is preliminarily evaluated in relation to other experiments. Cytotoxicity tests are performed through pre-standardized time exposure of *in vitro* cell cultures to various dilutions of test substances. International standards consider cytotoxic any concentration of a test substance that can induce at least 10 % cell death in relation to the non-exposed controls [1].

The micronucleus frequency assays are techniques used to evaluate if chemical or physical agents (such as ionizing radiation) are able to induce genotoxic damage directly on nuclear DNA, quantifying cells bearing micronuclei, which are fragments derived from broken DNA or lag chromosomes and with similar staining to that observed in the main

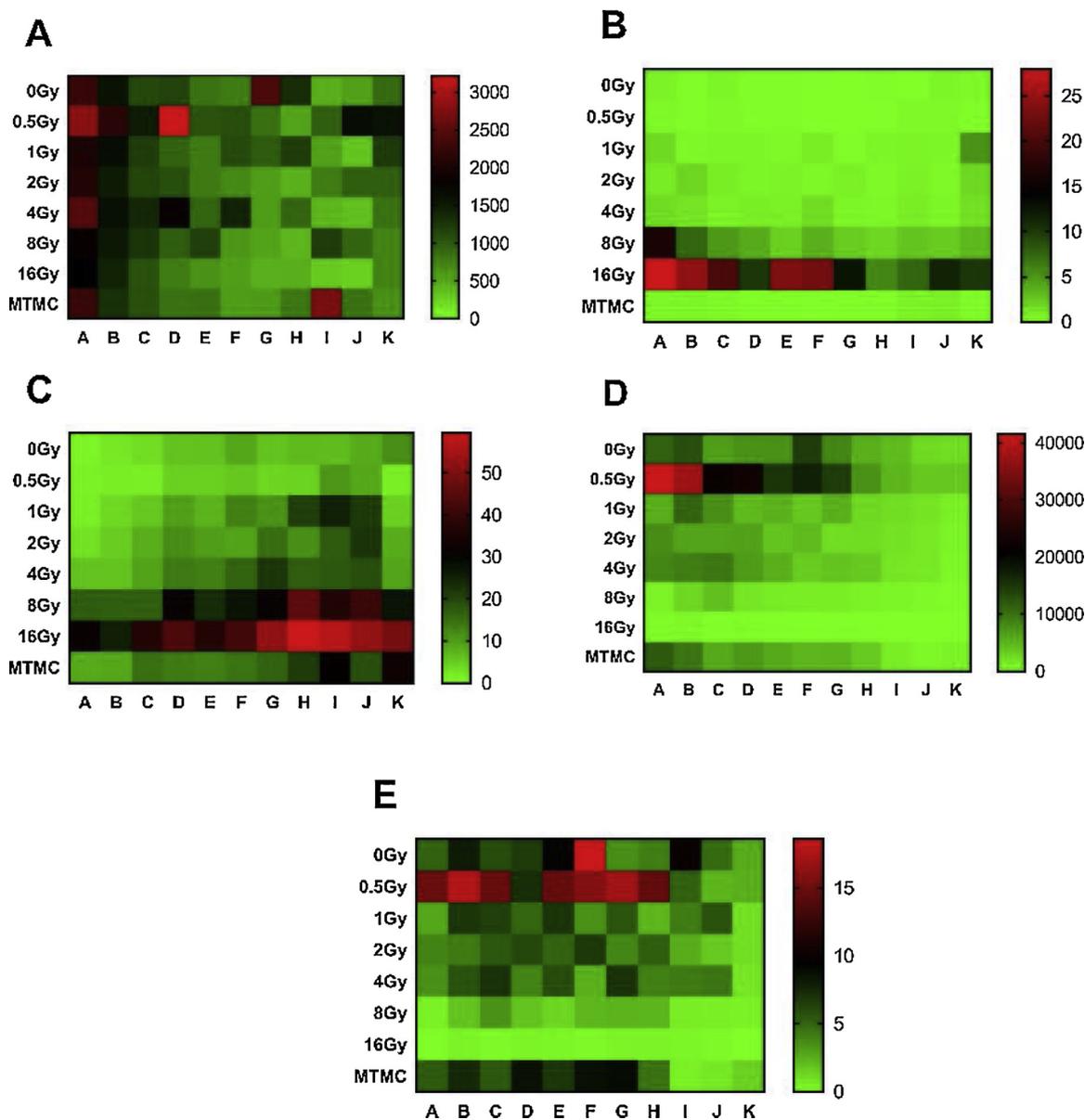
nuclei, but with 5–30% of its size [1]. These protocols are performed traditionally using conventional optical microscopy [2] or with fluorescent dyes that allow the manual counting of binucleate cells bearing micronuclei [3]. Despite its ease of execution, it is time consuming may bring some operational challenges [4]. Currently the protocols are being upgraded to automated approaches based on flow cytometry protocols based on the elimination of the plasma membrane by chemical agents, allowing quantification by flow cytometry. These modern protocols usually consider fluorescence-stained nuclei and micronuclei, and can discriminate dead or living cell nuclei by flow cytometry [5].

Prostate-specific membrane antigen (PSMA) is a cell surface protein, which is expressed in almost all prostate neoplastic formations [6]. PSMA was first identified as a homologous N-acetyl-L-aspartyl-L-glutamatopeptidase I (NAALADase I or folate hydrolase I) protein, which is active in the central nervous system, where binds to the neurotransmitter N-acetyl-laspartyl- glutamate (NAAG), producing N-acetylaspargate (NAA) and glutamate [7,8]. PSMA conjugates can be used to detect carcinogenic formations with malignancy, because its relationship with neoplastic angiogenesis, which increases its expression in the stroma adjacent to the vasculature of solid tumors.

A number of preclinical studies have evaluated the role of a small radiolabeled molecule with PSMA inhibitors for human prostate cancer

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**Fig. 1.** Variation in the amounts of events acquired between the first and last columns of the 96-well plates in the experiments. Numbers per well. (A) Beads, (B) Cell nuclei from non-viable cells, (C) Micronuclei, (D) Nuclei, (E) Nuclei/Beads Ratio.

imaging using various radionuclides for diagnostic and therapeutic purposes, such as  $^{11}\text{C}$  [9],  $^{18}\text{F}$  [10],  $^{99\text{m}}\text{Tc}$  [11] and  $^{68}\text{Ga}$  [12]. PSMA-11 can be labeled with  $^{68}\text{Ga}$  and used for diagnostic purposes, while PSMA-617 can be radiolabeled with  $^{68}\text{Ga}$ ,  $^{44}\text{Sc}$  or  $^{177}\text{Lu}$  and used for therapy [13]. Conventional imaging studies, including ultrasonography, bone scintigraphy, and computed tomography (CT), are used to detect primary and metastatic prostate cancer [6].

Ubiquidine is a synthetic cationic peptide with antimicrobial activity with affinity for the cell walls of the microorganisms [14]. Its use are being proposed as a diagnostic radiopharmaceutical ( $^{99\text{m}}\text{Tc}$ -UBI<sub>29–41</sub> or  $^{68}\text{Ga}$ -NOTA-UBI<sub>31–38</sub>) for fungal [15] or bacterial [16] infections. One of its main positive features is the ability to differentiate regions of septic inflammatory foci from aseptic inflammation, helping in the choice of treatment of patients suffering from various infections [17]. Antimicrobial peptides are important components of the immune system of all living organisms and generally contain hydrophobic and cationic amino acids, that is, molecules that have the characteristic of having a hydrophobic region (insoluble in water, but soluble in lipids and organic solvents) and a region hydrophilic (soluble in aqueous

medium), respectively. UBI is a promising peptide for the solution of cases of aseptic inflammation [18].

The work used a micronucleus scoring test based on flow-cytometry to evaluate the pharmacological safety of the three peptides.

## 2. Materials and methods

### 2.1. Cell cultures

The work used a non-tumoral established cell line derived from Chinese hamster ovary (CHO-K1, ATCC CCL-61), which is a standard lineage for genotoxicity testing [19]. The cells were cultured in 25 cm<sup>2</sup> bottles with RPMI 1640 medium with phenol red (Vitrocell-Embriolife) supplemented with 10 % fetal bovine serum (SFB-GIBCO-BRL) and 1 % antibiotics (Penicillin, Streptomycin - GIBCO-BRL), incubated at 37 °C in the presence of 5 % CO<sub>2</sub> until 70 % confluence in monolayer, with replacement of the culture medium every 48 h and subcultured every 7 days in culture. All experiments were performed using cultures subjected to at least one subculture passage after thawing, with subsequent

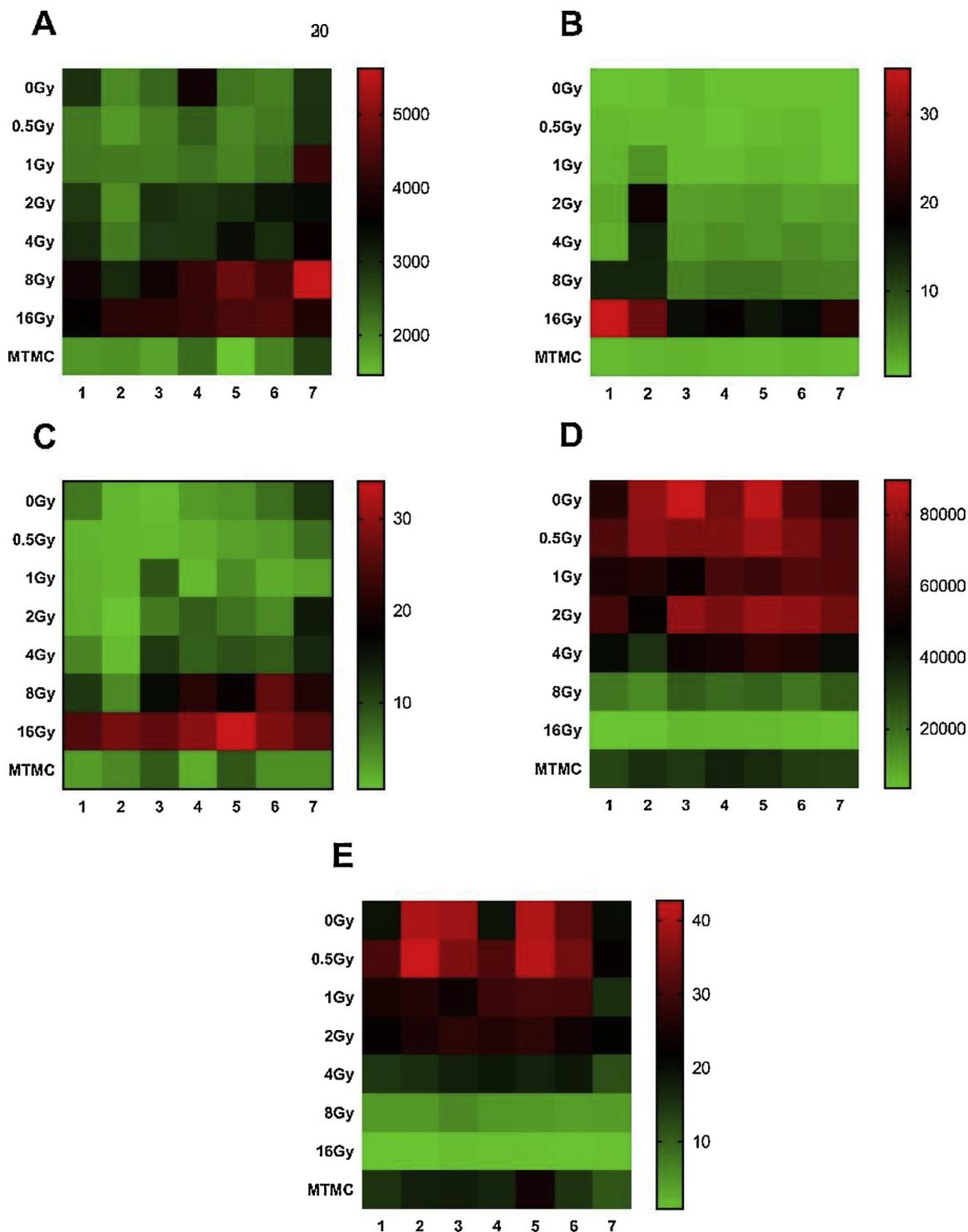


Fig. 2. Variation in the amounts of events acquired between the first and last columns of the 96-well plates in the experiments after adoption of resuspension procedure. Numbers per well. (A) Beads, (B) Cell nuclei from non-viable cells, (C) Micronuclei, (D) Nuclei, (E) Nuclei/Beads Ratio.

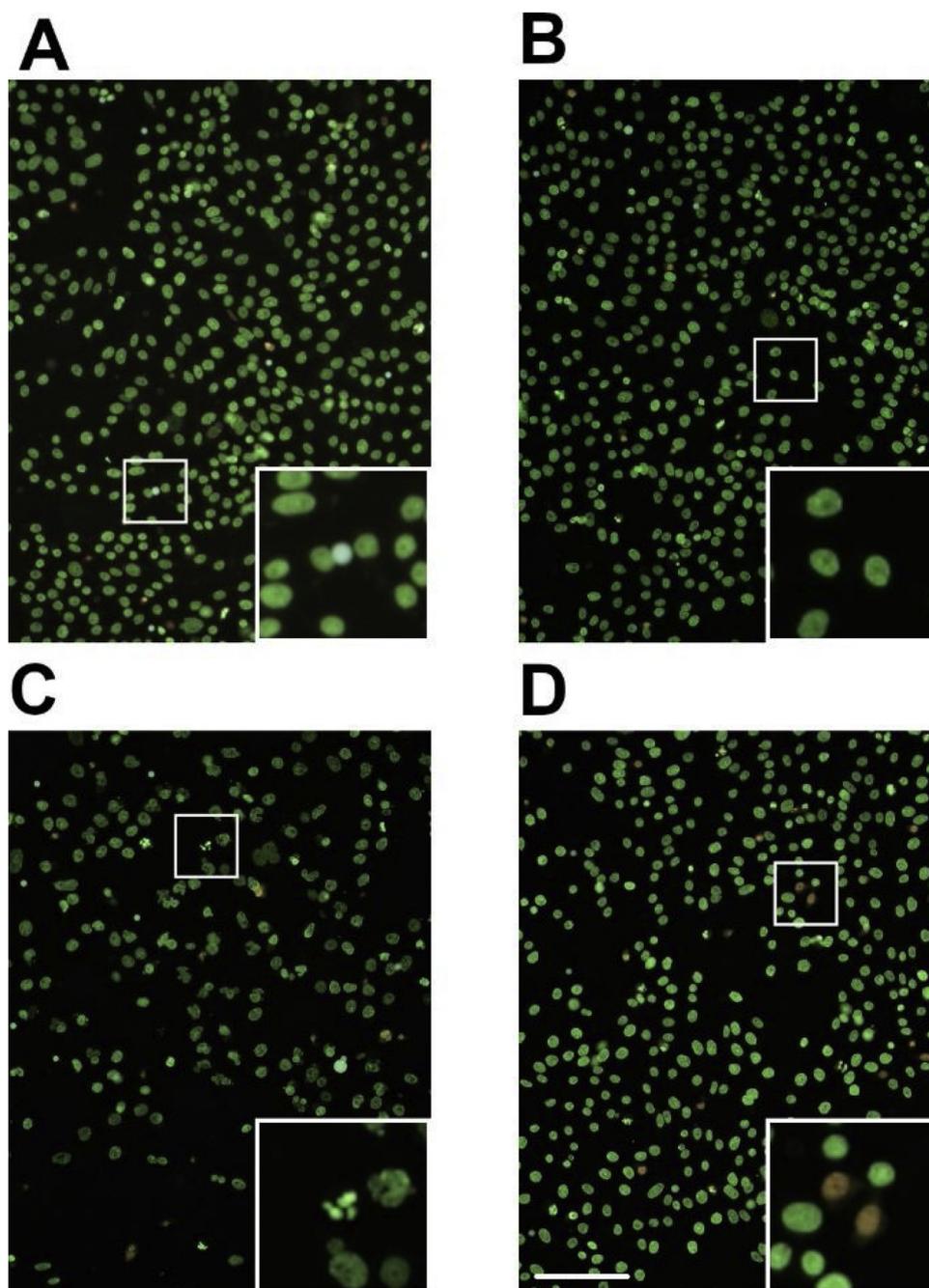
passages less than nine. For experiments, 5000 viable cells (scored by trypan blue dye exclusion) were plated in 100 µL of culture media / well.

2.2. Peptide (PSMA-11, -617 and UBI<sub>29-41</sub>) dilutions

Aliquots of all peptides were kind gifts from the Radiopharmacy Center (IPEN / CNEN-SP), which purchased the products in their commercial forms from Advanced Biochemical Compounds (ABX -

Germany) and diluted them in sterile ultrapure water at 1 µg/µL.

To obtain the final concentrations of the test compounds, calculations were carried using the parameter of the "standard man" (70 kg and 5,5 L of blood) as the final volume. Dilutions equivalent to 1/10, 1, 10 and 100 times the maximum concentrations administered to patients was used. For PSMA-617, the concentration of 100 µg / person resulted in 1.63 ng / mL (0.1x), 16.3 ng / mL (1x), 163 ng / mL (10x) and 1630 ng / mL (100x). PSMA-11 dilutions were made in 0.18 ng / mL (0.1x), 1.8 ng / mL (1x), 18 (10x) and 180 ng / mL (100x). For



**Fig. 3.** CHO-K1 cells from after lysis of membranes obtained in 4-h exposure experiments. (A) bead, (B) normal nucleus, (C) fragmented nucleus, (D) nuclei from non-viable cells. Increases: larger frame - 10X; Highlight: 30X (10X optical and 3X digital zoom). Bar in D: 50  $\mu$ m.

Ubiquitinidin (25  $\mu$ g / person) resulted in 0.45 ng / mL (0.1x), 4.5 ng / mL (1x), 45 ng / mL (10x) and 450 ng / mL (100x).

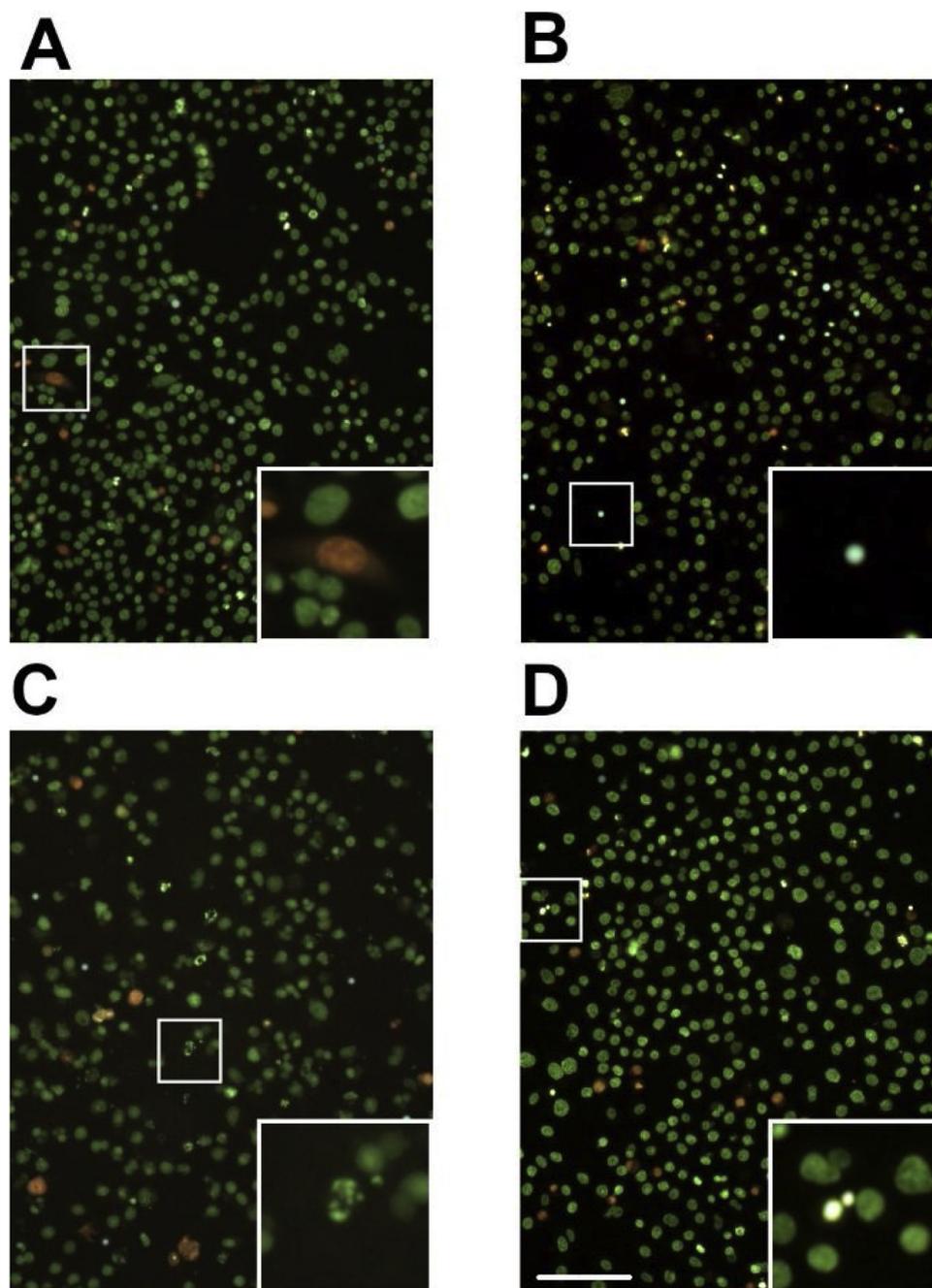
The cultures remained in contact with these concentrations of drugs for 4 or 24 h, in order to observe acute or late damage.

### 2.3. Positive controls - Chemical and physical

Colchicine (Sigma-Aldrich, CAS 50-07-7) as an aneugenic control, or Mitomycin (Sigma-Aldrich, CAS 50-07-7) as a clastogenic control, were added in final concentrations of 8.8  $\mu$ g/mL and 4  $\mu$ g/mL, respectively. As for test compounds, remained 4 or 24 h in contact with cells.

Irradiation of cells acted as a physical positive control for genotoxicity. Cells were detached from culture flasks using trypsin / EDTA solution (0.05 M / 0.05 M), washed by centrifugation in fresh culture

medium and resuspended in 1 mL PBS (Saline-phosphate buffered Saline) for gamma irradiation procedures in doses between 0.5 and 16 Gy of  $^{60}\text{Co}$  in sterile microtubes at room temperature. The irradiations were performed on a GammaCell 220 (Radiation Unit of the Canadian Atomic Energy Commission, Ltd.) at the Center for Radiation Technology (IPEN / CNEN-SP) using a metallic lead shield corresponding to 90 % radiation attenuation. With this attenuation, the dose rate did not exceed 85 Gy/h at the time of irradiation. After irradiation, the suspensions were centrifuged, resuspended in fresh media and the proportions of viable cells determined by the trypan blue exclusion method. The concentrations were adjusted to 50,000 cells / mL, with 100  $\mu$ L (5000 cells) seeded per well in 96-well plates. After seeding, the cells were incubated for 72 h under the conditions described above.



**Fig. 4.** CHO-K1 cells from after lysis of membranes obtained in 24-h exposure experiments. (A) nuclei from non-viable cells, (B) bead, (C) fragmented nucleus, (D) micronuclei. Increases: larger frame - 10X; Highlight: 30X (10X optical and 3X digital zoom). Bar in D: 50  $\mu$ m.

#### 2.4. Negative controls

A solution of 0.9 % NaCl (NaCl, Synth Ltda. C1060, CAS, 764714-5) at the final concentration of 45  $\mu$ g/mL was used as the negative control. Wells were designated as cell control (CC), whose cells received only complete and fresh culture medium. After 4 or 24 h, the culture medium from the CC wells and negative controls was removed and the cells received fresh culture medium.

#### 2.5. Flow cytometry for micronucleus quantification

The procedure followed the protocol described elsewhere [5,20,21]. After 72 h in culture, the cells seeded in 96-well plates irradiated or in concentrations with the drugs, as well as those treated with the described genotoxic agents, were centrifuged (1500 RPM in 10 min) and

received a solution of ethidium monoazide bromide (Thermo-Fisher Scientific, E1374) at a concentration of 8.5  $\mu$ g/mL diluted in PBS supplemented with 2 % fetal bovine serum. The culture plates were opened and exposed to a blue LED light source (440–450 nm, 30 W) for 30 min for the photoactivation of the compound, which irreversibly associates the dye to the DNA of the non-viable cells having the membrane with the integrity. This procedure had the objective of efficiently labeling cells that should not be included in nucleus and micronucleus counts. After this step, the cells received PBS with 2 % fetal bovine serum and centrifuged for free dye removal.

Two lysis steps were performed to release nuclei and micronuclei and to label the DNA. The first step consisted in lysing the cells using a solution of sodium chloride (0.854 mg / mL), sodium citrate (1 mg / mL) and IGEPAL® (0.3  $\mu$ L / mL) as well as 0.4  $\mu$ M SYTOX® Green fluorescent dye (Thermo-Fisher Scientific, S7020). This solution also

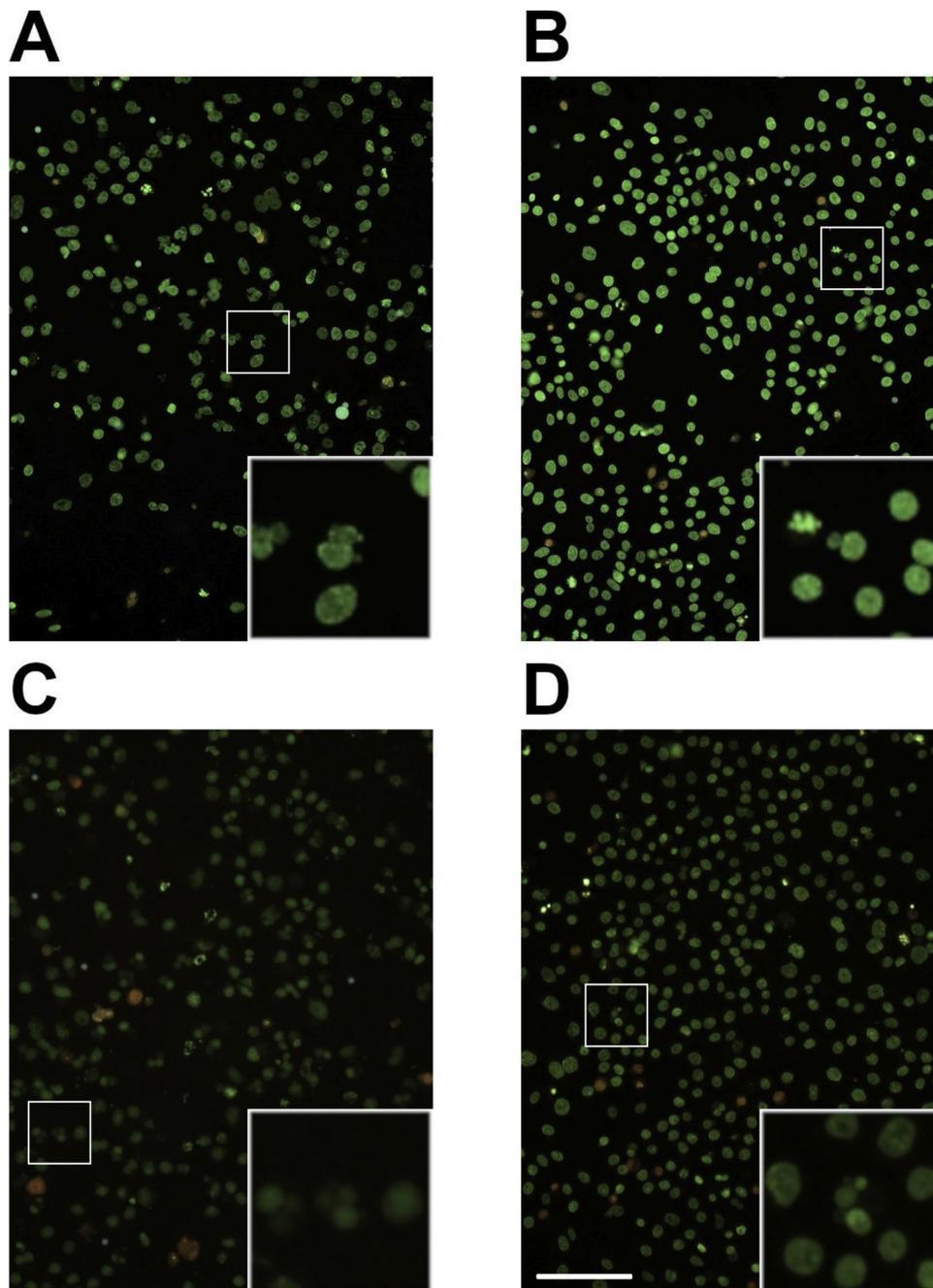


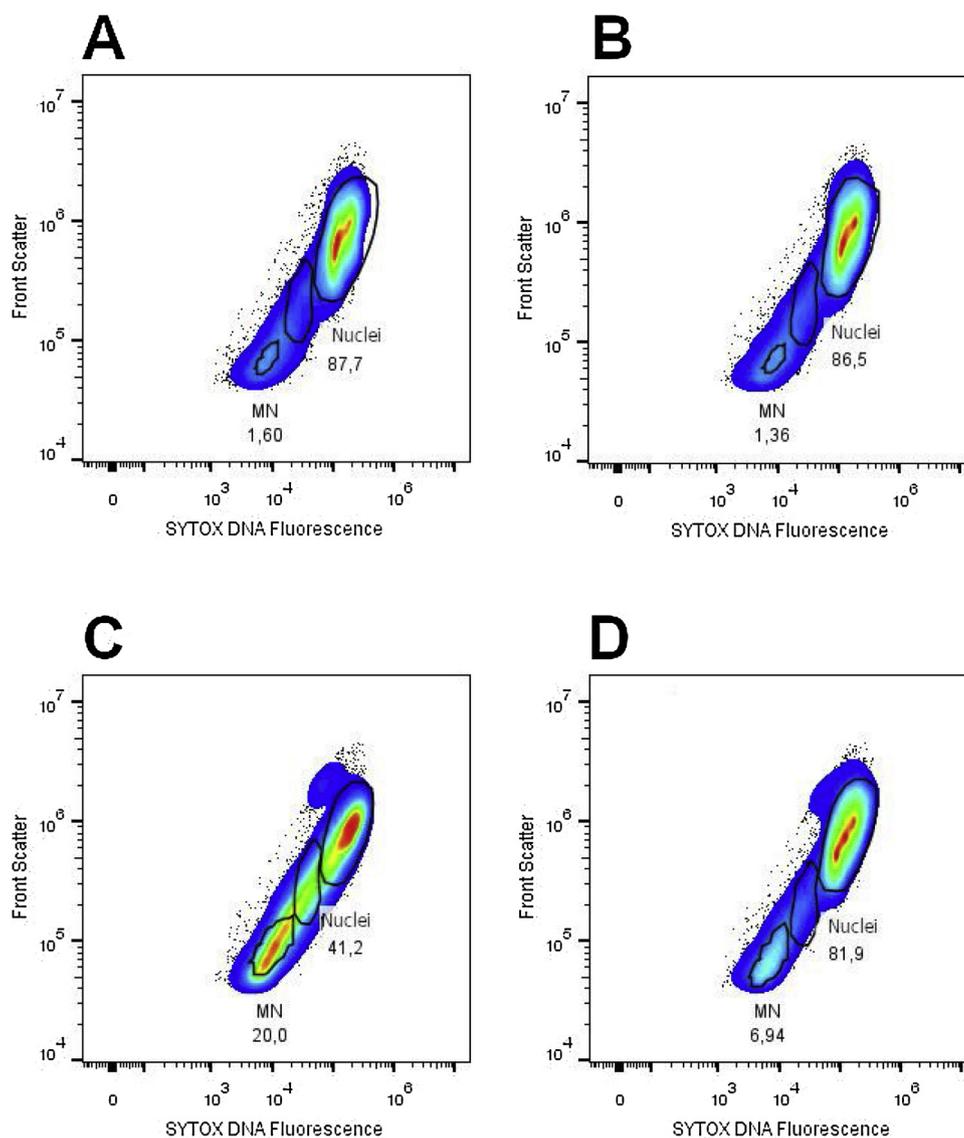
Fig. 5. Morphological findings in CHO-K1 cells after incubation with 8.8  $\mu\text{g}/\text{mL}$  of colchicine (A,C) or 4  $\mu\text{g}/\text{mL}$  of mitomycin (B,D). Top: 4 h of incubation. Bottom: 24 h. Increases: larger frame - 10X; Highlight: 30X (10X optical and 3X digital zoom). Bar in D: 50  $\mu\text{m}$ .

contained RNase A (0.42 mg/mL, Sigma, SLB5176 V) which eliminates the chance of the dyes to associate with residual RNA molecules. After lysis for 60 min (37  $^{\circ}\text{C}$ ), the plates were centrifuged and the biological material received the second lysis solution (sucrose 85.6 mg / mL, citric acid 15 mg / mL and SYTOX<sup>®</sup> Green 0.4  $\mu\text{M}$ ).

The second lysis solution was supplemented with 5  $\mu\text{L}$  of latex beads suspension. The latex beads AccuCheck Counting Beads (Thermo-Fisher Scientific, PBC100), are about 6  $\mu\text{m}$  in diameter and which, in the flow cytometric analysis, are presented in the form of two well-characterized subpopulations, with fluorescence in the FL2 channel. The amount found across wells varied slightly ( $1054 \pm 40.48$ , Mean  $\pm$  standard error of the mean, data not shown). Thus, it was possible to calculate the relation between the number of nuclei and beads in each sample. Deviations from this ratio in relation to that found in the controls were interpreted as changes in the velocity of mitotic division.

The samples were incubated with this solution for a further 30 min in the dark. After 30 min, the material was left at room temperature for automatic event acquisition on the flow cytometer (Accuri CSampler, BD Biosciences).

The analysis followed the methodology described in the literature [20]. Briefly, events marked with EMA were excluded from the total count. The events with SYTOX<sup>®</sup> Green were evaluated according to their size (FSC) and fluorescence (FL1) for discrimination between nuclei and micronuclei. At least 20,000 events were counted in the region of the cores in each sample. The final data of this experiment consisted of percentage of micronuclei (for negative EMA and SYTOX positive), in relation to the control in comparison to control wells (non-irradiated, untreated cells). The relationship between nuclei and beads was used to analyze whether there was cell proliferation.



**Fig. 6.** Typical results derived from exposure of CHO-K1 cells for 4 h to controls: (A), Cell control, negative; (B), NaCl 45 µg/mL, Negative control; (C), Colchicine 8.8 µg/mL, positive control; (D), Mitomycin C 4 µg/mL, positive control.

## 2.6. Fluorescence microscopy

Nuclei and micronuclei from lysed cells were imaged on a Nikon Ts100 inverted microscope using fluorescence excitation (480 nm) for verification of fluorescence signals.

## 2.7. Data analysis

The data obtained were analyzed with software BD CSampler Software or FlowJo VX maintaining the gating structure described [5]. The results were expressed in relation to the amounts (fold-increases) found in the control cells (CC).

In order to evaluate the genotoxic effect of radiation exposure, the amounts of MN in relation to the controls were adjusted to the second-order polynomial function, which is usually used to evaluate the genotoxic effects clastogenic of the ionizing radiation.

Differences between groups were analyzed by one-way variance analysis (one-way ANOVA) followed by Bonferroni post-tests, using the program GraphPad Prism 7.

## 3. Results

### 3.1. Heterogeneity of events across plate

Under conditions of automatic acquisition of events, the collection of all events in 96-well plates routinely required 4–5 hours (data not shown). Due to this time, a certain variation in the number of events acquired between the first and last columns could be observed. This effect is shown in Fig. 1.

After the perception of this effect, the subsequent experiments required the suspension of the event acquisition process after the end of each column, when the contents of the wells of the next column to be analyzed were resuspended with the aid of a multichannel micropipette. The procedure added about 2 min for each column to the final acquisition time. Fig. 2 shows data acquired on plates according to protocol after addition of resuspension steps. Heatmaps representing the number of acquired events (beads, nuclei from non-viable cells, MN, nuclei and nuclei/beads ratio) after resuspension appeared to be more homogeneously dispersed across plates between replicates. Even after resuspension, higher MN frequencies observed in positive samples (controls or irradiated) were kept. Analysis showed that resuspension increased homogeneity of events without prejudice of differences

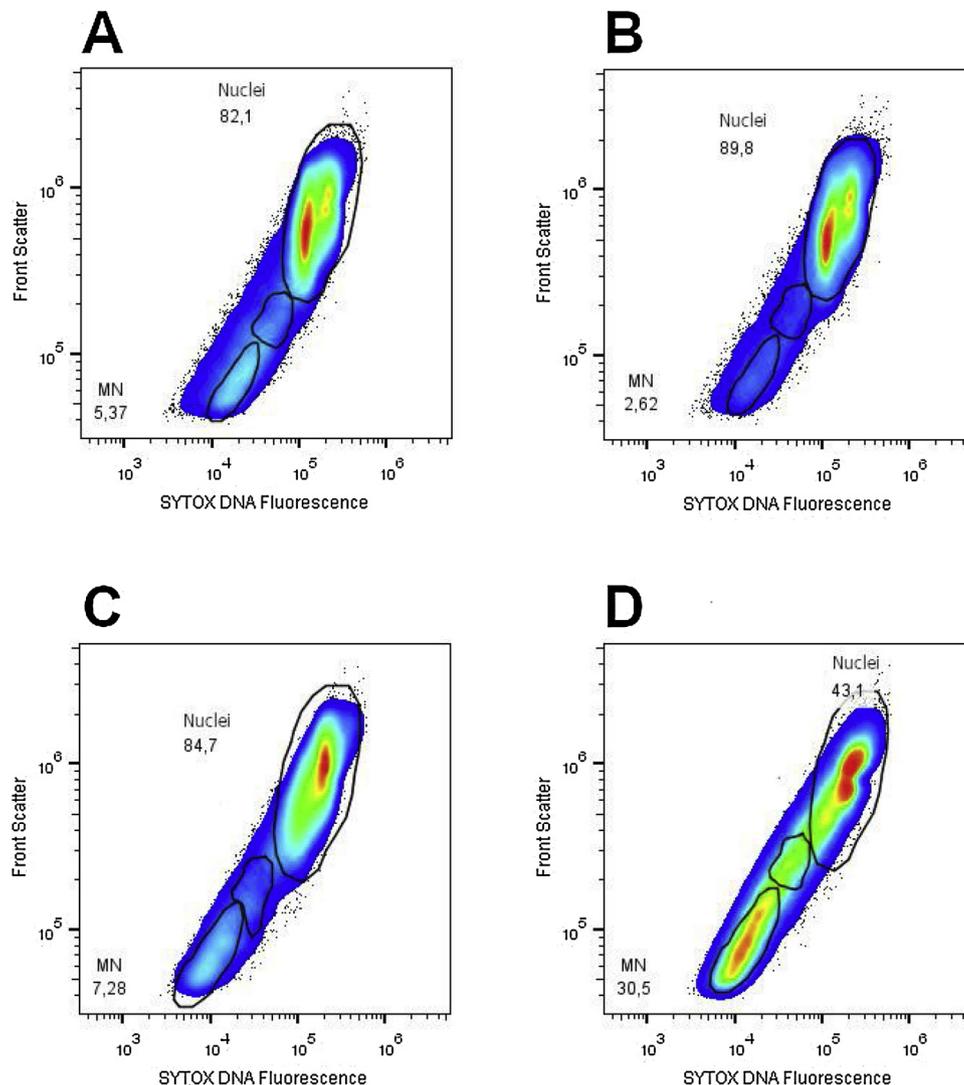


Fig. 7. Typical results derived from exposure of CHO-K1 cells for 24 h to controls: (A), Cell control, negative; (B), NaCl 45 µg/mL, Negative control; (C), Colchicine 8.8 µg/mL, positive control; (D), Mitomycin C 4 µg/mL, positive control.

between groups.

### 3.2. Negative and positive controls

Nuclear material (nuclei and micronuclei) from lysed cells were analyzed by fluorescence microscopy to confirm fluorescence staining. Samples from 4 (Fig. 3) or 24 h (Fig. 4) incubations were imaged. Qualitative analysis showed SYTOX<sup>+</sup>/EMA<sup>+</sup>, SYTOX<sup>+</sup>/EMA<sup>-</sup>, MN (micronuclei) and beads in samples.

Before resuspension steps, apparently nuclear bodies kept their localization as found before lysis. Images in Figs. 3 and 4 clearly show integer nuclei from lysed cells, viable or not, and some nuclear fragments as micronuclei. The images also are helpful showing that lysis steps were well successful removing cytoplasmic membranes despite keeping nuclear bodies apparently intact. Beads appeared as distinct quasi-spherical objects, with distinguishable fluorescence.

Some typical events with morphologically related to MN are shown in Fig. 5. Two nuclei with near micronuclei are shown in Fig. 5A. A nucleus adjacent to a considerable nuclear body is shown in Fig. 5B. Figs. 5C and 5D shows multinucleated cells.

Colchicine was found to induce higher MN percentages after 4-h incubations (Fig. 6) than after 24 h (Fig. 7). Mitomycin treatment showed an inverse effect.

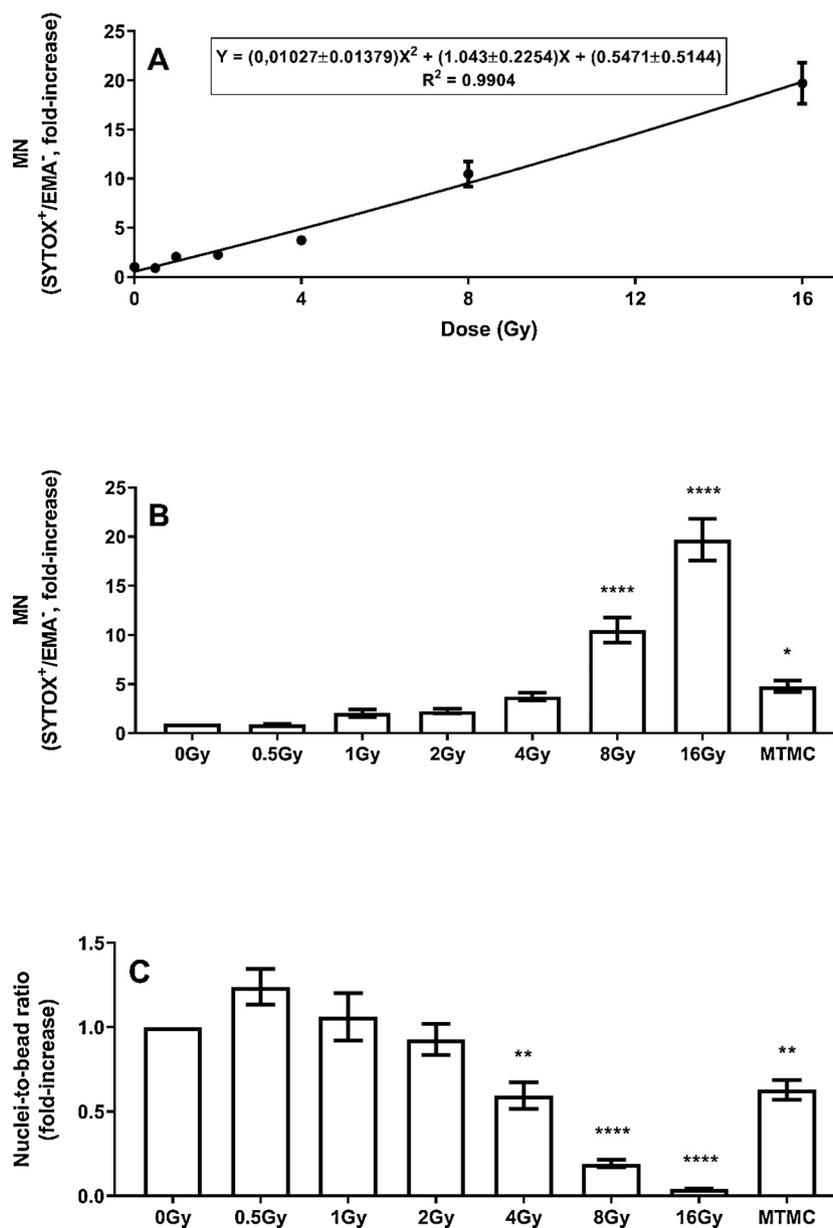
Micronuclei frequencies and nuclei-to-bead ratios (NBR) from irradiated cells are shown in Fig. 8. Increasing doses of gamma radiation from the <sup>60</sup>Co source could induce increments on micronuclei frequencies following a second-order polynomial function (8A), a typical radio induced effect. Fold increases of MN amounts were proportional to dose, following a linear-quadratic fitting ( $R^2 = 0.993$ ). Only high doses (8 and 16 Gy) induced statistically significant increases (fold-change) in MN amount (8B). Doses higher than 4 Gy were shown to significantly decreased cell proliferation rate, as measured by the nuclei-to-bead ratio (8C).

### 3.3. Testing of the genotoxic potential of exposure of cultures to PSMA-617

PSMA-617 induced increase of MN amount in higher concentrations and only after 24 h of incubation (Fig. 9).

Positive controls (mitomycin and colchicine) induced statistically different percentages of micronuclei relative to cell control (CC) after incubation of 4 and 24 h. At 24 h of exposure, the 1630 ng/mL concentration of PSMA-617 induced statistically different percentages of micronucleus relative to cell control (CC).

As found in micronucleus frequency analysis, PSMA-617 only induced changes in cell proliferation after 24 h with higher concentrations (Fig. 10).



**Fig. 8.** (A) Increases of micronuclei frequency in CHO-K1 cell cultures irradiated from 0 to 16 Gy. Inset: regression equation obtained after fit (second-order polynomial). (B) Same data plotted in columns for proper visualization of statistically significant differences. (\*):  $p < 0.05$ . (\*\*\*\*):  $p < 0.0001$ . MTMC: mitomycin (positive control). (C) Nuclei-to-bead ratios of irradiated cells cultures. (\*\*):  $p < 0.01$ . (\*\*\*\*):  $p < 0.0001$ . Dots and columns: Means. Bars: SEM (standard error of means). Gray: (absorbed dose unit); MN: micronuclei.

### 3.4. Effects of PSMA-11 treatment in cultures

PSMA-11 did not induce genotoxicity neither changes in cell proliferation rates in all tested concentrations and incubation times. Only positive controls induced statistically significant increase of MN (Fig. 11) or decrease in cell proliferation rates (Fig. 12).

### 3.5. Effects of UBI<sub>29-41</sub> treatment in cultures

The exposure of cells to peptide UBI<sub>29-41</sub> was not able to induce increases in micronucleus frequencies (Fig. 13). Some variation in cell proliferation rates was found in cultures after 24 h of treatment (4.5 and 450 ng/mL), but no statistically significance was found by group comparisons (Bonferroni) (Fig. 14).

## 4. Discussion

Previously, the micronucleus assay was done by scoring binucleated cells on microscopy slides [4] and required days for completion. Today, with the automated technique, besides counting micronuclei, it can be observed even the characteristics of the nuclei, such as size and roughness, through lasers. It is also observed the arrangement of the wells, analyzing the homogeneity of the pipetting according to the plate arrangement [22].

The micronucleus assay is one of the most commonly used tests for evaluating genotoxic damage [1]. It can be used for chemical and physical agents, such as radiation [20].

The aim of the work was to show that a faster and more feasible technique such flow cytometry could replace the traditional microscopy slide scoring to assess micronuclei frequencies. Although no experiments were made to test the sensitivity of technique, some considerations can be raised. CHO-K1 cells are reported to have a basal level of

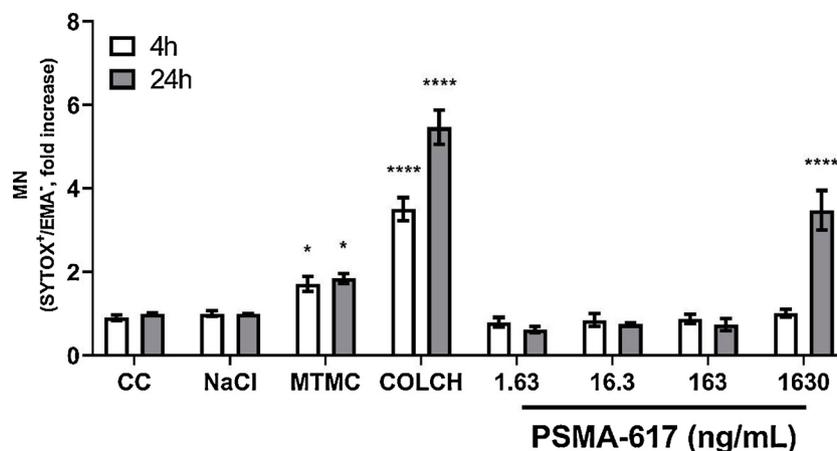


Fig. 9. Micronucleus frequency in CHO-K1 cell cultures treated with concentrations of PMSA-617 and controls for 4 (white) or 24 (gray) hours. (\*):  $p < 0.05$ . (\*\*\*\*):  $p < 0.0001$ . CC: control cells; NaCl: NaCl 45  $\mu\text{g/mL}$  (negative control); MTMC: Mitomycin (positive control); COLCH: colchicine (positive control). Bars: SEM.

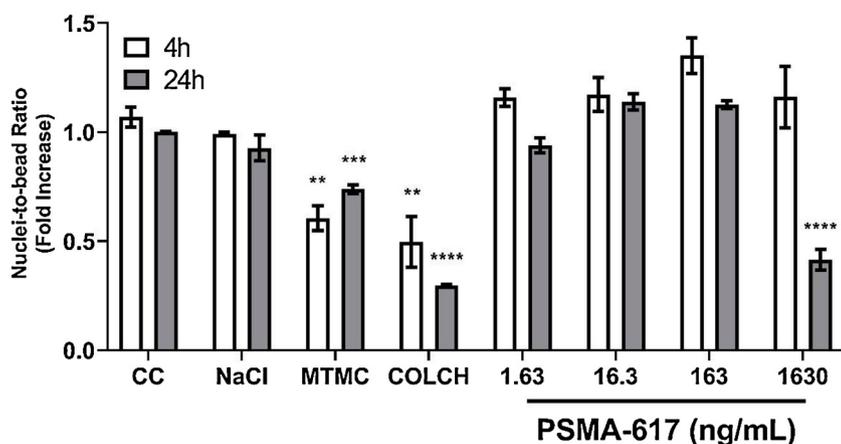


Fig. 10. Nuclei-to-bead ratios, representing cell proliferation rates, in CHO-K1 cell cultures treated with concentrations of PMSA-617 and controls for 4 (white) or 24 (grey) hours. (\*\*):  $p < 0.01$ . (\*\*\*):  $p < 0.001$ . (\*\*\*\*):  $p < 0.0001$ . CC: control cells; NaCl: NaCl 45  $\mu\text{g/mL}$  (negative control); MTMC: Mitomycin (positive control); COLCH: colchicine (positive control). Bars: SEM.

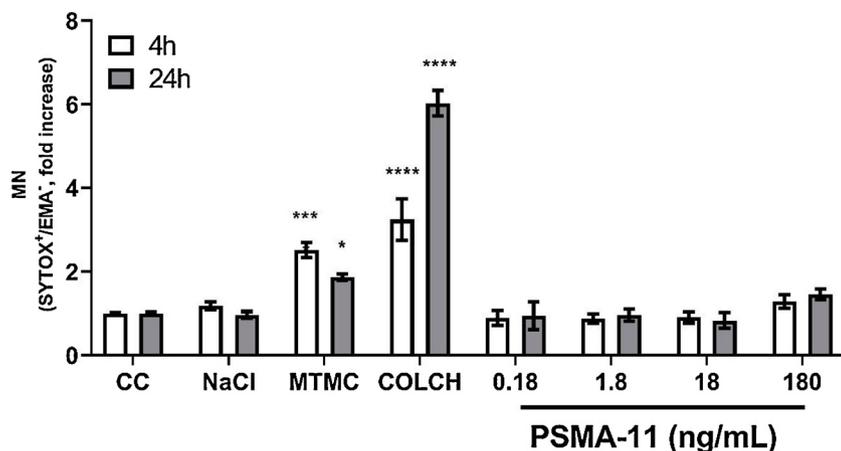


Fig. 11. Micronucleus frequency in CHO-K1 cell cultures treated with concentrations of PMSA-11 and controls for 4 (white) or 24 (grey) hours. (\*):  $p < 0.05$ . (\*\*\*):  $p < 0.001$ . (\*\*\*\*):  $p < 0.0001$ . CC: control cells; NaCl: NaCl 45  $\mu\text{g/mL}$  (negative control); MTMC: Mitomycin (positive control); COLCH: colchicine (positive control). Bars: SEM.

MN frequency between below 0.5 [23] and 1.7–2.0 % of binucleated cells [4] in experiments using microscopy. Using flow cytometry, control cultures of CHO-K1 exhibited  $1.65 \pm 0.49$  % [24] and a broader study showed  $0.72 \pm 0.26$  % (mean  $\pm$  SD of percentages of MN obtained in all control cultures) [25]. In Fig. 5A, a typical plot of MN found in control cells shows 1,6 % of total events acquired when at least  $2 \times 10^5$  events were captured inside the nuclei gates, a number that cannot be directly compared to findings of MN frequencies using other techniques because it was collected using another sampling technique (MN%/1000 binucleated cells vs. MN%/20000 nuclei events). Nevertheless, the frequency observed in the present experiments can be

considered low, which would delimit the minimum theoretical limits of detection of the technique used. The used protocol was compared the traditional scoring on microscopy slides and found to be as sensitive as traditional scoring [20,26], or even more sensitive [27].

Higher radiation doses (8 and 16 Gy) may induce a significant increase in the percentage of micronuclei, and its occurrence could be fitted to a linear-quadratic response. The procedure can also be suitable to construct a standard curve for biological dosimetry, using peripheral lymphocytes instead of CHO-KI cells [28,29].

For pharmacological safety reasons, experiments were carried out at higher concentrations than those practicable, thus proving that the test

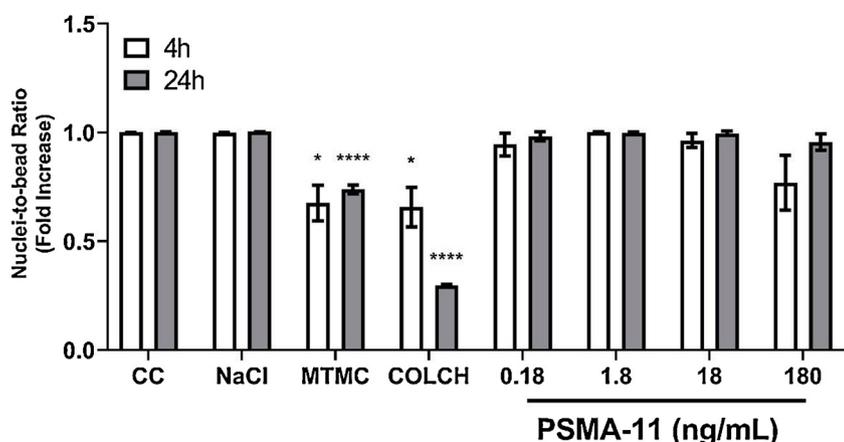


Fig. 12. Nuclei-to-bead ratios, representing cell proliferation rates, in CHO-K1 cell cultures treated with concentrations of PSMA-11 and controls for 4 (white) or 24 (gray) hours. (\*):  $p < 0.05$ . (\*\*\*\*):  $p < 0.0001$ . CC: control cells; NaCl: NaCl 45  $\mu\text{g/mL}$  (negative control); MTMC: Mitomycin (positive control); COLCH: colchicine (positive control). Bars: SEM.

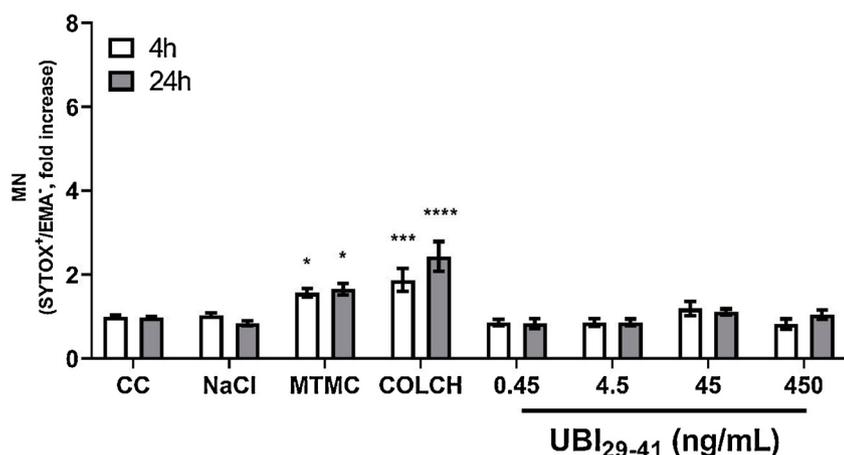


Fig. 13. Micronucleus frequency in CHO-K1 cell cultures treated with concentrations of UBI<sub>29-41</sub> and controls for 4 (white) or 24 (gray) hours. (\*):  $p < 0.05$ . (\*\*):  $p < 0.001$ . (\*\*\*\*):  $p < 0.0001$ . CC: control cells; NaCl: NaCl 45  $\mu\text{g/mL}$  (negative control); MTMC: Mitomycin (positive control); COLCH: colchicine (positive control). Bars: SEM.

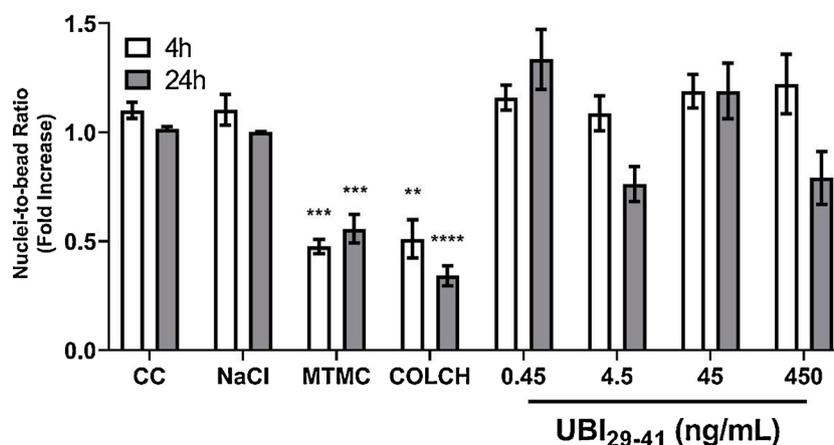


Fig. 14. Micronucleus frequency in CHO-K1 cell cultures treated with concentrations of UBI<sub>29-41</sub> and controls for 4 (A) or 24 (B) hours. (\*\*):  $p < 0.01$ . (\*\*\*):  $p < 0.001$ . (\*\*\*\*):  $p < 0.0001$ . CC: control cells; NaCl: NaCl 45  $\mu\text{g/mL}$  (negative control); MTMC: Mitomycin (positive control); COLCH: colchicine (positive control). Bars: SEM.

compounds do not present appreciable genotoxicity.

Some genotoxicity of PSMA-617 was identified only in the 100x concentration, after 24 h of exposure. For PSMA-11 and UBI, only positive controls induced damage, meaning that even above the dose limit per patient, these concentrations were not capable of damaging the DNA.

The amount of non-viable cells should not be considered in genotoxicity assays, as the presence of genotoxic damage in cells that cannot continue in their life cycles is considered irrelevant from the point of view of carcinogenesis. When a given cell carries DNA damage,

however extensive, will not continue to reproduce, and such damage has no chance of propagation to its progeny. Accounting for these events would lead to deviations that may induce overestimation of unrepaired DNA damage [30]. No drug concentration induced a significant increase in the number of non-viable cells (data not shown).

Using a nuclei-to-bead ratio, where the beads are homogeneously dispersed between the wells of plates, the analysis can determine if any radiation dose or chemical exposure reduced or increased the number of nuclei in a sample, and thus, a reasonable measure of cell proliferation when comparing ratios from cell controls. The data of this

analysis can replace the analysis of CBPI (proliferation index by blocking cytokinesis), which is impracticable in this context, since the lysis of cell membranes makes impossible the analysis of binucleate cells [5]. Positive controls reduced proliferation, with mitomycin being an alkylating agent (clastogenic control), that is, inducing direct breaks in the DNA that are not repaired, the proliferation was appeared to be reduced. The effect of the positive control colchicine, although inducing significant genotoxic damage, was not necessarily reflected in the reduction of proliferation. According to other study [31], the micronuclei in colchicine can be reversed over time, being a non-disjunction (aneugenic) and reflecting on proliferation.

Colchicine also showed higher induction of genotoxic damage than mitomycin in the percentage of micronuclei. Its effects are related to the formation of microtubules, which causes tubulin polymerization and allows the mitotic division [32], and interacting with proteins such as cyclin  $\beta$  [33] regulating the cycle (increasing or decreasing cell activity), inducing oscillations directly linked to cell division. Thus, increase of micronuclei may be linked in this interaction of tubulin with cyclin. Incubations for 24 h apparently reduced the genotoxic effect of mitomycin, which cannot be observed in the present study under these conditions. The recent OECD incubation suggestion [1] shows that cells must be exposed to the test chemical with metabolic activation for 3–6 hours and exposed at a time equivalent to about 1.5–2.0 cycles the treatment. The present study chose for a 24-h incubation as an attempt to simulate the maximum time of presence of drugs in the body, as well as using concentrations much larger than those practicable.

Clastogenicity and aneugenicity describe two types of DNA damage characterized by structural or numerical factors in changes in chromosomes. DNA damage can be chromosomal, affecting both the sister chromatids of a chromosome, or involving only one of the two chromatids. Aneugenic DNA damage is considered indirect or secondary damage, as opposed to clastogenicity that is considered a direct DNA damage [34]. Different types of DNA damage are accumulated due to clastogenicity, including insertions, deletions, translocations, inversions and chromatid aberrations [35].

No in-depth analysis of the direct DNA damage caused by these drugs was done. It is important to take as example the  $^{18}\text{F}$ -FLT ( $^{18}\text{F}$ -Fluorothymidine or Fludesoxythymidine) as an analogous nucleoside of thymidine and one of the nucleic acid building blocks (RNA or DNA). FLT is only poorly incorporated into DNA and thymidine kinase 1 can be regulated even during an inhibition of DNA synthesis [36]. In addition, increased uptake of  $^{18}\text{F}$ -FLT may be stimulated by the cell repair mechanism or by the recovery pathway of pyrimidine metabolism. That is, for this molecule, there is evidence of the mechanism of DNA association that could be the effector of the observed breaks, because it is a nucleoside that would be incorporated into the DNA by the own progression of the step of DNA synthesis (phase S) of the cell cycle. For peptide molecules such an association is not direct.

However, there are studies with PSMA [37], which describe it as possessing folate hydrolase enzymatic activity on the surface of the cells that may be responsible for the cellular reception of the poly-Y-glutamate folate released in the medium interstitial by dead cells within the tumor. This action allows the folate free form to participate in the synthesis and DNA methylation and polyamine synthesis, important for cell proliferation. Methylation is an inducing factor of DNA fragility [38]. However, it is a study made with non-tumor cells, so that it is not certain that this folate can react with all types of cells, even to non-tumor cells. About the UBI class of peptides, no studies were found until the present moment that discuss about.

Although there was a variety of analyzes for genotoxicity, the micronucleus assay was chosen to be relatively faster than the other assays, and more effective for flow cytometric analysis, available in the laboratory. The Ames test, for example, would analyze a gene mutation consisting of a reversal of bacterial mutation, which relies on amino acids to form colonies, exclusive to bacterial cells. In the absence of amino acids, the colonies do not have an ability to grow in culture [39].

In the present study, it would not be possible to work with CHO-K1 cells in this context, although.

Through the present experiments, it was not possible to detect phenomena that could be interpreted as derivatives of genotoxicity, nor mechanisms capable of inducing it, in treated CHO-K1 cells with concentrations between 10–1000% of the concentrations of use of UBI<sub>29–41</sub> or of the forms -11 and -617 of PSMA in adults, nor even in incubations for 24 h.

## 5. Conclusion

The present experiments did not detect *in vitro* genotoxicity of PSMA-617 and PSMA-11 even in concentrations up to 10 times the maximum concentration used in patients, regardless of the exposure time. At concentrations 100 times greater than those used, it induces small significant damage provided that the incubation lasts for up to 24 h in PSMA-617. Ubiquicidin did not show genotoxicity at any concentration. In addition, the technique was able to detect micronuclei in a sensitive way in irradiated cultures, showing its usefulness also in biological dosimetry of the ionizing radiations.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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