

## AN IMPROVED *IN VITRO* MICRONUCLEUS ASSAY TO BIOLOGICAL DOSIMETRY

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### ABSTRACT

The biological dosimetry is widely used to estimate the absorbed dose in people occupationally or accidentally exposed to the radiation for a better medical treatment, minimizing the harmful effects. Many techniques and methods have been proposed to detect and quantify the radioinduced lesions in genetic material, among them, the micronucleus (MN) assay. In the present study, we proposed an improved *in vitro* micronucleus technique that is rapid, sensitive and with minor cell manipulations. Assays were carried out with human tumor cells (MCF-7) seeded ( $3 \times 10^4$  cells) in slides placed into Petri dishes. Adherent cells were maintained with RPMI medium, supplemented with fetal calf serum, 1 % antibiotics, cytochalasin B (2  $\mu\text{g}/\text{mL}$ ), and incubated at 37°C in the presence of 5% CO<sub>2</sub> for 72h. Cells were pre-treated for 24h with aminoguanidine, a nitric oxide synthase inhibitor. Nitric oxide is an intracellular free-radical, involved in DNA double-strand break repair mechanisms. After incubation, adherent cells on slides were briefly fixed with paraformaldehyde and stained with acridine orange (100  $\mu\text{g}/\text{mL}$ ) for analysis through fluorescence microscopy. Dye fluorescence permitted accurate discrimination between nuclei and micronuclei (bright green) and cytoplasm (red), and made possible a faster counting of binucleated cells. Aminoguanidine (2 mM) induced significant increase ( $p < 0.05$ ) in frequencies of binucleated cells with micronuclei and in the number of micronuclei per binucleated cell. Data showed that proposed modifications permit to understand an early aspect of NO inhibition and suggested an improved protocol to MN assays.

### 1. INTRODUCTION

The cytokinesis-block micronucleus assay (CBMN) is a well-established technique that was developed in the early 1980s, that was proposed as alternative to the classical chromosome aberration technique to detect and quantify genetic damage. This method is based on microscopic observations of nuclear bodies as dense chromatin bodies with no more than 1/3 of the size of main nucleus [2], called micronucleus (MN), located apart from main nuclei, originated by either unrepaired double-strand DNA breakage, or lagging whole chromosomes [1]. During interphase, the micronuclei are seen in injured cells before cytokinesis.

Genotoxic agents, such as ionizing radiation (IR) [3] or some aneugenic compounds [4] are able to induce MN mammalian cells. In *in vitro* experiments, cytokinesis blockade caused by cytochalasin B (Cyto-B), a spindle polymerization inhibitor from fungal origin, promotes accumulation of MN through cell divisions [3]. After suitable preparation and staining, MN can be scored by microscopy analysis.

Among various applications, the CBMN test can be used to dose estimation in accidental or occupational exposures, with good correlation between expected dose values and micronucleus frequency in cytochalasin-B treated cells [5]. Its properties and ease of use

puts it among biosimetrical techniques officially approved by IAEA [6]. Usual approaches requires a short period of cell culture soon after exposure to genotoxic or aneugenic agents, that is necessary for mitosis activation in primary cultures, as those from human lymphocytes [6], lasting 24-48h depending upon specific cell doubling time. After mitosis initiation, cells should be treated using Cyto-B, that impairs cytokinesis and promotes formation of binucleated cells (BNC) and accumulation of MN. For established cell lines, only Cyto-B incubation times are required.

After Cyto-B treatment, cells must be collected, washed and fixed. Fixation is usually performed after successive centrifugation steps of cell suspensions in a fixative solution of methanol and acetic acid (3:1). These steps can lead to loss of cells during washing, and can be extensively time-consuming, if considering a large number of samples.

After fixation, cells must be dropped onto glass slides in an incubation chamber, usually an humid atmosphere at 50-60°C to permit solvent evaporation and granting proper adherence of cells to slides. This procedure can overdisperses events over slides, increasing time to find relevant events and microscopic scoring time. More recent improvements require the use of a cytocentrifuge to produce less dispersed events [7].

Staining of BNC was first done using Feulgen reagent, but its affinity to some granules found in white blood cells brought some difficulties in MN scoring [3]. Another method, the May-Grünwald Giemsa staining was employed, with some advantages. After Giemsa staining, cytoplasmic regions are usually found as diaphanous and pinkish colored, and nuclei and micronuclei as dense and violet bodies [3]. Differences between cytoplasm and nucleus/micronucleus staining are given as dependent on pH and density of material. Despite its good capacity of discrimination, classic Giemsa staining can lead to misinterpretation errors, due to presence of diverse artifacts on slides [3].

This work proposed some improvements to traditional CBMN assay. First, the authors cultured cells directly on slides, reducing event overdispersion and loss of cells through extensive centrifuge and wash steps. Second, acridine orange (AO) staining as performed, in order to help better discrimination between cytoplasmic and nuclear/micronuclear regions. As preliminary tests, tumor cells (MCF7) were treated with aminoguanidine (AG), a nitric oxide synthase inhibitor. Its product, nitric oxide (NO), has been related to DNA break induction in a number of publications [8]. Additionally, cells were exposed to ionizing radiation (IR) at radiotherapy doses (up to 2Gy, acute exposure) to assessment of MN induction using the improved technique through AO staining.

## **2. METHODS AND RESULTS**

### **2.1. Cell Culture**

Human breast carcinoma cells (MCF7, ATCC HTB-22) were cultured at 37°C and CO<sub>2</sub> 5% in 25cm<sup>2</sup> flasks containing RPMI1640 medium, supplemented with fetal bovine serum (10%) and antibiotic mixture (streptomycin/penicillin, 1%). Cells were subcultured after reaching 50-60% confluency, with medium replacement after 48h.

## **2.2. Experimental cell culture in glass slides**

Conventional microscope glass slides were cut into 40 x 25mm pieces, washed and sterilized to further use in cell culture conditions. To prepare the experiment, glass pieces were placed inside plastic Petri dishes. Thus 500 $\mu$ L of cell suspension ( $6 \times 10^4$  cells / mL) were seeded on glass slides and let to adhere for 24 hours. After this time, slides were washed once with phosphate buffered saline solution (PBS) and 4mL of RPMI 1640 medium was added. All experimental procedures were carried using adherent MCF7 cells on slides.

## **2.3. Aminoguanidine treatment and cytokinesis-block.**

72 hours after seeding, cells were treated or not with 1 or 2mM of aminoguanidine hydrochloride (SIGMA-Aldrich, São Paulo) diluted in culture media. Treatment was carried out by 24 hours. After this time, cells were washed once in PBS and received 4 $\mu$ g/mL cytochalasin B (SIGMA-Aldrich, São Paulo) diluted in culture medium for the next 72 hours.

## **2.4. Irradiation experiments**

Adherent cells on glass slides were prepared as above and irradiated at 0.5, 1 or 2Gy in a  $^{60}\text{Co}$  source (GAMMACELL 220 - Irradiation Unit of Canadian Atomic Energy Commission, Ltd.), using a 90% attenuator to reach a dose rate of 140Gy/h. Non-irradiated controls were processed as same as experimental samples, and were placed outside irradiation chamber during process. After irradiation, plates were washed once with PBS and Cyto-B was added as stated above.

## **2.5. Fixation and staining**

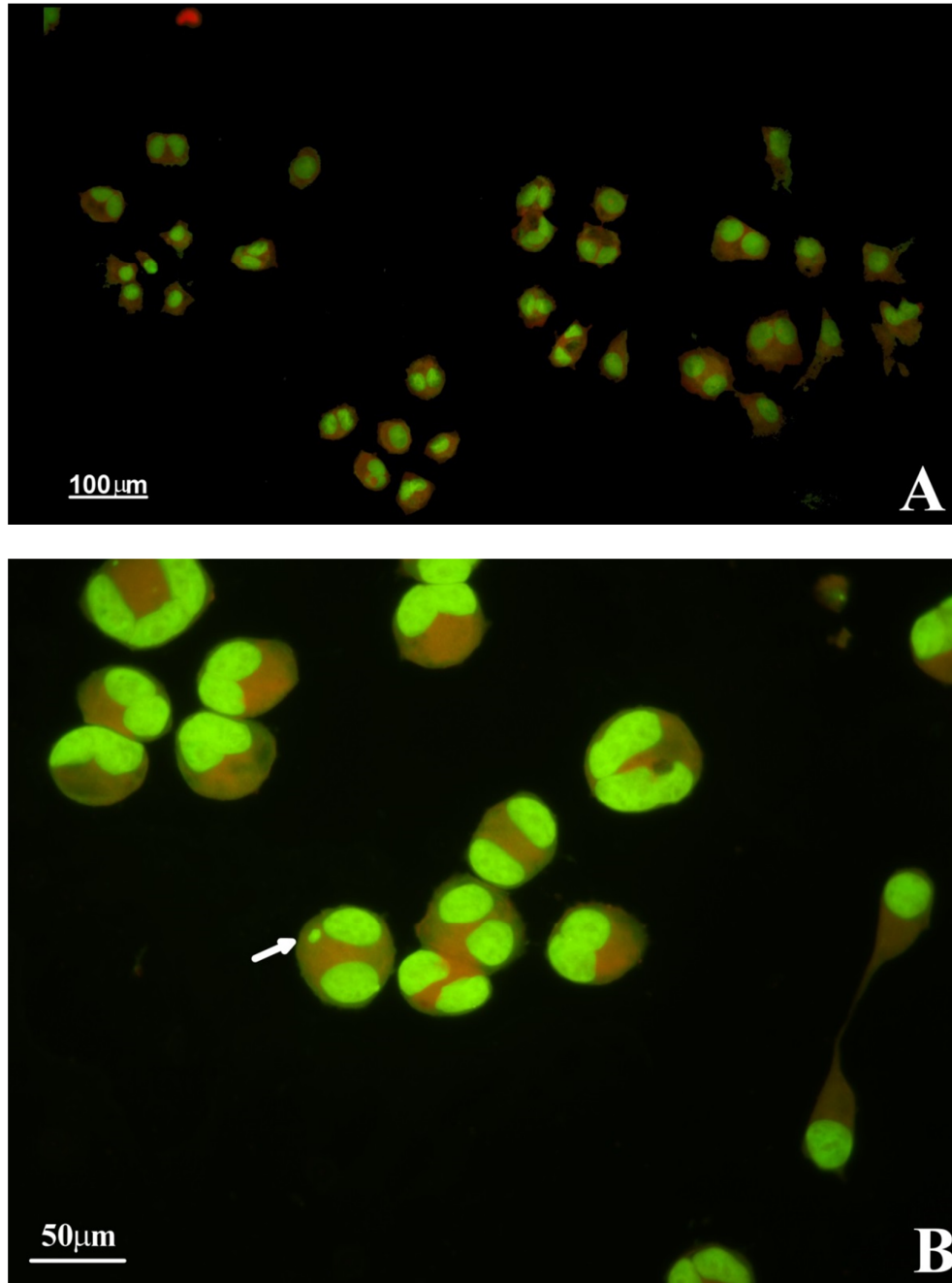
After treatment, cultured cells on slides were washed once in PBS and fixed for 15 minute with 4% formaldehyde in PBS. Fixed cells were washed three times in PBS to remove excess formaldehyde and let to dry in room temperature. Just before microscopic visualization, fixed cells on slides were stained with 100 $\mu$ L of acridine orange solution (0,003% in PBS) and covered with proper coverslips.

## **2.6. Microscopic visualization and event scoring.**

Glass pieces with stained cells were mounted on top of conventional glass slides and visualized through fluorescence microscopy (Nikon 80i) using convenient filter block (Ex: 450-490nm; Em: 515nm). In this procedure, acridine orange in cytoplasm fluoresces in bright red color, and nucleus and micronucleus emits a strong green fluorescence. Only binucleated cells were considered in this study. A minimum of 1000 cells (binucleated cells, exhibiting or not micronuclei) were counted per slide. Scores of binucleated cells (BN) with one or more than one micronuclei were analyzed. Counts were converted as percentages of total binucleated cells and plotted in bar graphs. One-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison tests were used to assess statistically significant differences between percentages of cells with one or more than one micronuclei in treated or control groups. Graph plotting and statistical analysis were performed using GraphPad Prism 5.0 software.

## 2.7. Results

### 2.7.1. Acridine orange staining



**Figure 1: MCF7 cells after acridine orange staining. (A) 10X; (B) 20X. Cytoplasm was shown in red, and chromatin bodies (nuclei/micronuclei) in green. Arrow in (B) shows micronucleus in a binucleated cell.**

Acridine orange staining improved observation of nuclear and micronuclear bodies. The staining could help in proper discrimination between cytoplasm, nuclei and micronuclei, without any artifact interferences (Fig.1B). Moreover, binucleated cells were shown in a less disperse manner (Fig.1A), reducing scoring times.

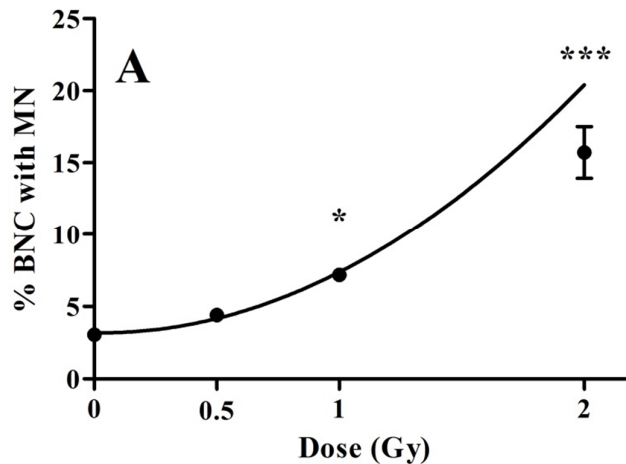
### 2.7.2. Micronucleus scoring on irradiated MCF7 cells

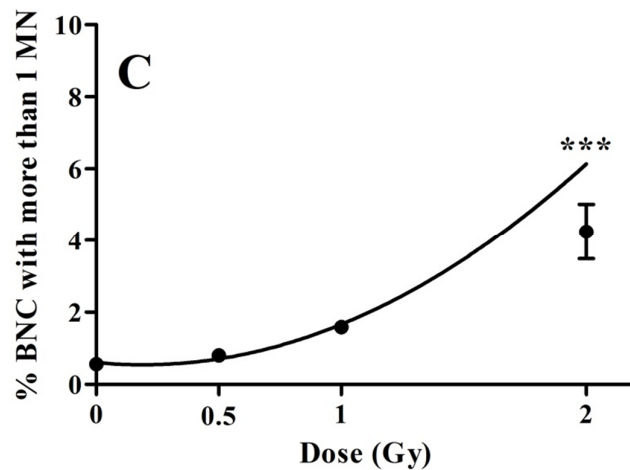
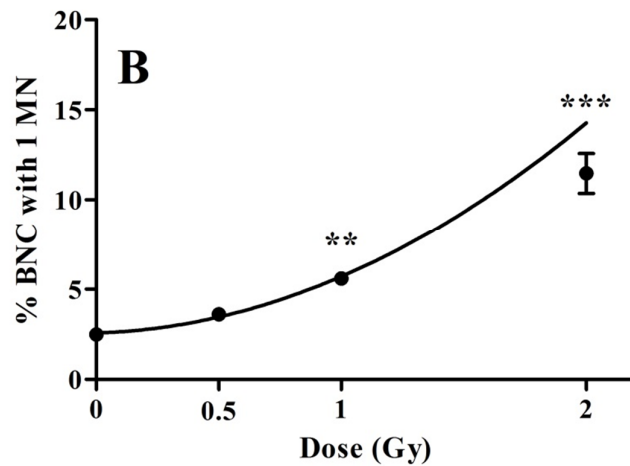
To assess whether the proposed modifications could interfere in proper scoring of micronuclei, irradiated cultures were used to build dose-response curves. Data obtained were fitted to a quadratic model  $y = \alpha x^2 + \beta x + c$ , where  $y$  is the percentage of BNC showing one or more micronuclei,  $\alpha$  and  $\beta$  the quadratic and linear coefficients of the model, respectively, and  $x$  is the absorbed dose in Gy. In this case, well fitted data means that observed biological responses were in conformity to expected biological response. The  $R^2$  values obtained from experiments showed good fitting of data to the model. Table1 shows the coefficients of the fitted curves: total percentage of BNC with micronuclei, with only one MN and more than one MN.

**Table 1: Coefficients of quadratic regression model for dose-response curves of micronucleus (MN) induction in MCF7 binucleated cells (BNC) after exposure to gamma radiation ( $^{60}\text{Co}$ ).**

	Mean±SD			$R^2$
	$\alpha$	$\beta$	c	
% Total BNC with MN	2.261±0.1316	1.831±0.2838	3.025±0.1118	0.9920
% BNC with 1 MN	2.717±0.2462	0.4137±0.5153	2.574±0.2117	0.9827
% BNC with more than 1 MN	1.709±0.1747	-0.6527±0.3656	0.608±0.1502	0.9641

The proposed modified CBMN method can detect MN induction on irradiated cells fitted to a quadratic model as expected. Data of MN scoring of MCF7 cells are shown in Figs. 2A, 2B and 2C.



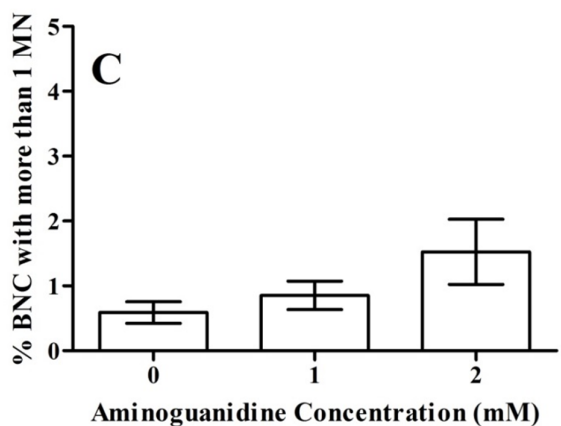
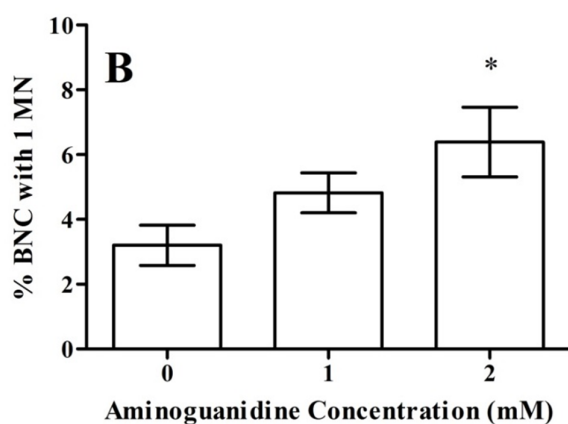
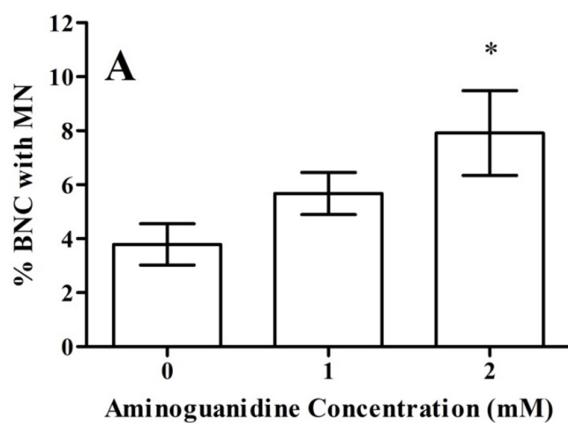


**Figure 2: Dose-response curves of micronucleus (MN) induction of irradiated MCF7 cells. Data are shown as percentages of total binucleated cells (BNC) (A): Total percent of BNC with MN; (B): Percent of BNC with only one MN inside cytoplasm; (C): Percentage of BNC with more than one MN. Error bars represent standard error means (SEM). Statistically significant differences comparing to non-irradiated controls (Dunnett post-hoc test) are represented as (\*) $p < 0.05$ ; (\*\*)  $p < 0.01$ ; (\*\*\*)  $p < 0.001$ .**

The proposed MN scoring could only detect MN increase in doses higher than 0.5Gy, as shown in Fig.2A and 2B. More expressive DNA damage, represented as BNC with more than one MN, only could be detected in 2Gy irradiated cells (Fig.2C).

### 2.7.3. Micronucleus scoring on MCF7 cells treated with aminoguanidine

Micronucleus scoring of cells treated with aminoguanidine (1 or 2mM) is presented on Fig.3.



**Figure 3: Micronucleus (MN) frequencies on binucleated cells (BNC) treated with aminoguanidine (C). (A): Total percent of BNC with MN; (B): Percent of BNC with only one MN inside cytoplasm; (C): Percentage of BNC with more than one MN. Error bars represent standard error means (SEM). Statistically significant differences comparing to untreated controls (Dunnett post-hoc test) are represented as (\*) $p < 0.05$ .**

Aminoguanidine treatment increased MN proportions at 2mM concentration, as depicted on Fig.3A and 3B. Percentage of total BNC with MN, and BNC with only one MN were increased when this concentration was used. Multiple DNA damage (BNC with more than one MN) was not induced by any of AG treatments in this study (1 or 2mM).

### 3. CONCLUSIONS

The CBMN method can be reproduced with good quality after the introduction of the fluorescent staining method with more specific staining characteristics. Experiments had been carried out entirely on slides, using adherent cells, that were simpler and more convenient for scoring, and acridine orange staining had been helpful in microscopy scoring, and results were in conformity with expectations. The presented staining technique have been used in a considerable number of papers, and is becoming a standard procedure [3, 9]. Moreover, data obtained showed that aminoguanidina administration (2mM) apparently induced MN formation in cells what can suggest an important role of nitric oxide inhibition on radioinduced DNA strand breaks. Further with AG-treated cells exposed to ionizing radiation are planned to evaluate the MN induction.

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