# ALTERNATIVE METHODS IN BIOLOGICAL DOSIMETRY: RADIOINDUCED APOPTOSIS OF BLOOD LYMPHOCYTES.

Marisa Lemes*, Márcia A. Silva*, Orlando R. Santos* & Heitor F. Andrade Jr.**

*-Supervisão de Radiobiologia, Instituto de Pesquisas Energética e Nucleares( IPEN/CNEN-SP.)
Cx.Postal 11040, CEP 05422-970, São Paulo, SP, BRAZIL

**Laboratório de Protozoologia, Inst.Medicina Tropical de São Paulo, FMUSP
Av.Dr.E.C.Aguiar, 470, CEP 05403-000, Sào Paulo, SP, BRAZIL

## ABSTRACT

Gamma rays affect cells in dose-response manner, resulting in cell death, as in cancer radiotherapy. The ionizing radiation acts by transferring energy, mainly by free radicals from water radiolysis that result in nucleic acid damage and other effects in lipids and proteins. The level of exposure is indirectly estimated by physical dosimetry, but the biological dosimetry can measure the direct radiation effect, mainly in post-dividing cells by classical cytogenetic approach. Recently, it was reported that irradiated cells develop an induced programmed death or apoptosis. With a biological dosimetric technique, we measured apoptotic cell fraction in $^{60}$Co *in vitro* irradiated blood cells from voluntary healthy donors. The DNA agarose gel electrophoresis showed a low sensitivity, because cell DNA presented the characteristic pattern only when the cells were exposed to 100 cGy or more. Using a terminal DNA labeling technique we observed that the apoptotic cell fraction proportionally increases with irradiation. Similar sensitivity was observed when compared to classical cytogenetics (3 cGy minimum detection level). These techniques are easier to perform, do not need cell culture and all cells, including interphasic ones, can be analysed, providing a good tool in biological dosimetry.

## I. INTRODUCTION

Exposure to gamma rays induce lesions of biological molecules, resulting mainly in cellular death [1, 2, 3]. This effect have been used in Medicine as in the radiotherapy of cancer, but also represents a danger to life in accidental or nuclear war exposure.

The level of exposure to ionizing radiation is usually measured by physical dosimetry, a well organized indirect approach that allow the estimation of the ionizing energy received by the body, but this technique do not allow the direct analysis of the effect in the cells. The radioinduced effect occur only by the transference of energy, without molecular isotopic exchange with the cell atoms. This results in nucleic acid damage and some other effects in lipids and proteins, mainly by indirect action of the free radicals from water radiolysis [4, 5]. Biological dosimetry, an approach that detect the effect of the radiation in the cells of a exposed proband, usually deals with the clastogenic effects of the radiation in post-dividing blood cells, in a classical cytogenetic approach. It includes the *in vitro* growth of blood lymphocytes, analyzing its chromossomal aberrations after its first cellular division, as dicentric chromossomes, double minus chromossomes, centric or acentric rings and others aberrations[4]. This technique, when used by well trained laboratories, gave maximal sensitivity of 5-10 cGy[6] but also needs the survival of the damaged cell after the exposure. Additionally, it requires the maintenance of the DNA damage during the first few divisions, because the measurement can be affected by the death of injured cell or by the repair of the damaged DNA. Some interesting techniques for estimation of radioinduced injuries like the micronucleus assay, the single cell DNA electrophoresis assay, and other sophisticated cytogenetic techniques, as hybrid premature chromatid condensation, have been devised and tested[6,7]. Moreover, all these techniques demand careful training and expertise in cytogenetics or microscopy, including the limitations above mentioned.

Recently, considerably efforts were made in the understanding of the radiation induced death, in general different for distinct cell types. It was showed that most

of the lymphoid and hematopoetic cells present a radioinduced apoptosis[8,9,10]. It could not directly related to nucleic acid damage but could be ascribed to oxidized products of lipid origin, as ceramides [2, 11, 12]. This interesting phenomenon could be reverted by phorbol ester, showing an exquisitely high activity [11]. The apoptotic cell presents intranuclear DNA cleavage in specific sites, induced by endonuclease activity. This cleavage results in relatively homogenous small DNA fragments, with multiple double strand breaks [13,14,15] that can be easily detected by agarose electrophoresis and by terminal nick end labeling [13,14,16,17,18].

Our purpose is to study quantitatively the radioinduced apoptosis as a alternative approach to biological dosimetry, using several techniques for apoptosis detection in non-cultivated *in vitro* irradiated blood lymphocytes.

## II. MATERIAL AND METHODS

Materials and reagents: All reagents were pro-analysis, with solutions made with MilliQ purified water. Reagents for molecular biology was obtained from commercial sources, cited along the specific techniques when necessary. Blood cells was purified from peripheral blood of volunteers by the classical Ficoll Hypaque technique and irradiated immediately after purification. The irradiation schedule was performed in room temperature in a GammaCell 220(Atomic Agency of Canada Ltd) homogeneous $^{60}Co$ irradiation chamber. After irradiation, the cells were maintained in 4°C until use.

DNA Agarose Gel Electrophoresis: Nucleated blood cells were purified from remaining erythrocytes by incubation in TKM1 Buffer (10 mM Tris-HCl pH 7.6; 10 mM KCl; 10 mM $MgCl_2$; 2 mM EDTA, 0.5% of Triton X-100), centrifuged, washed, and ressuspended in TKM2 buffer(10 mM Tris-HCl pH 7.6; 10 mM KCl; 10 mM $MgCl_2$; 2 mM EDTA, 0.4 M NaCl with 1% SDS). After incubation for 10 min at 55°C, one volume of NaCl 5 M was added and the suspension centrifuged to 10000 g/5 min. DNA in the supernatant was then precipitated by addition of two volumes of ethanol absolute, and centrifuged to 14000g/20min. After washing in iced 70% ethanol, the pelleted DNA was dried at room temperature. Just before electrophoresis, the dried DNA was suspended in TE buffer( Tris 1 M pH 8.0, EDTA 0,5M), incubated 30 min/65°C followed by 1h/37°C and applied to agarose 2% previously stabilized with Tris-Borate containing Ethidium bromide 0,4µg/ml. After 3hs run at 80 V, the agarose slab was trans-iluminated with UV and photographed. The molecular weights of DNA bands was compared to standards( λphage DNA cleaved with HindIII and EcoRI).

*In situ* DNA Nick End Labelling(IDNEL): The lymphocytes were separated by gradient Ficoll Hipaque, fixed with 10 volumes of Tris/HCl 0.02M pH 7.6, 0.15M NaCl(TBS) containing 4% para-formaldehyde, 80% ethanol, and centrifuged in glass slides by using a Cytospin cytocentrifuge. The specimens were rehydrated by washing in TBS and permeabilized by incubation in 20µg/ml proteinase K for 20 min. Endogenous peroxidase was inactivated with incubation in 3% $H_2O_2$ 10% methanol in TBS and washed. The slides were incubated with Terminal deoxynucleotidyl transferase(TdT) solution containing Biotin-dNTP e unlabelled dNTP at 37°C for 1.5 hours. The reaction was terminated by incubation with 0.5 EDTA pH 8 for 5 min at room temperature, blocked with 4% bovine serum albumin in TBS for 5 min, rinsed with TBS and incubated with streptavidin peroxidase conjugate. After careful washing, positive cells were revealed by incubation with diaminobenzidine-cobalt enhancing substrate. After adequate definition of this staining, the slides was counterstained with methyl green. The stained cell percentage was determined in at least 500 cells, under microscopy.

Cytogenetic Preparation : Heparinized whole blood samples were incubated in Eagle's MEM supplemented with 5% inactivated fetal calf serum, phytohemmaglutinin and bromodeoxyuridine. The incubation time was 48 hours, with added colchicine in the last two hours. Cells were hypotonized with 0,05M KCl and then fixed in methanol/acetic acid(3/1 v/v). The cells were dropped in to clean slides and placed on hot plate at 65°C and irradiated by UV rays for 30 min, in the presence of Hoechst 33258®. The cells were stained with fluorochrome plus Giemsa (FPG), and 500 mitosis analyzed under microscopy. Only chromossomal aberrations in cells undergoing their first mitosis were scored, with determination of percentage of cells with significant aberrations in at least 500 mitosis, as the percentage of mitosis with dicentric chromossomes..

Statistical analysis: The quantitative data from biological dosimetry studies were fitted in a linear quadratic model, as elsewhere described, using a Graph-Pad Prism software. The equation used was $y= A+Bx+Cx^2$, using 95% confidence intervals. Significance was considered when the probability of equality was less than 0.05, with a power of 90%.(19)

## III. RESULTS

DNA fragmentation induced by gamma radiation could be seen in the gel electrophoresis shown in Fig 1, when ladder sequences with staggered pattern could be seen only in irradiated DNA lanes. A proportional increase in intensity of these ladder pattern

could be seen with increasing radiation, but also an increasing in background DNA fragmentation occurs with higher dose.

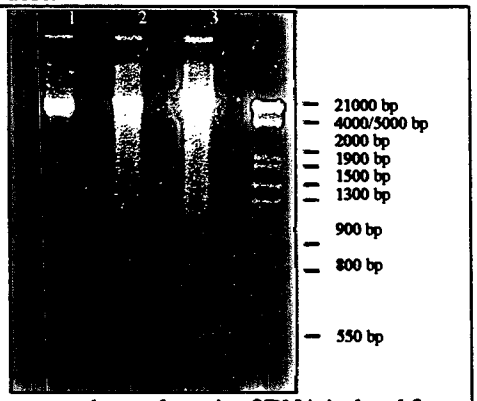


Figure 1. Agarose electrophoresis of DNA isolated from irradiated blood cells. Lane 1: control cells, lane 2: 200 cGy irradiated cells, lane 3: 600 cGy irradiated cells. Ethidium bromide fluorescent staining. Numbers represents standards M.W. markers.

The apoptotic cell determination was made as a quantitatite analysis of apoptotic cell fraction. As seen in Figure 2, the apoptotic cells are easily identified by their brown staining, allowing the estimation of percentage of stained nuclei.

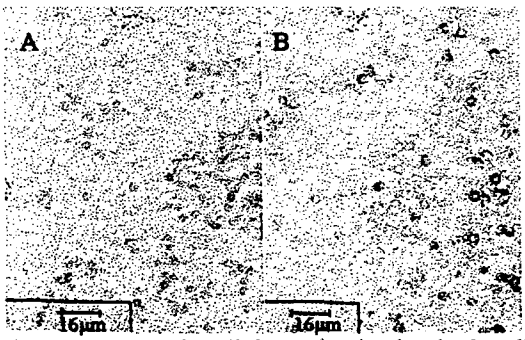


Figure 2. Apoptotic cell determination by single cell DNA nick end labelling , with Tdt and biotinylated d-nucleotides. A- Control cell, non irradiated, B- Irradiated cells, 600 cGy.

The quantitative analysis of frequency of apoptotic cells in irradiated blood lymphocytes assayed by IDNEL was showed in Figure 3, with comparison with the chromossomal aberrations found in similarly irradiated blood lymphocytes, expressed as dicentric fraction. All data were expressed as percentage of positive cells, detected in at least 500 cells or mitosis

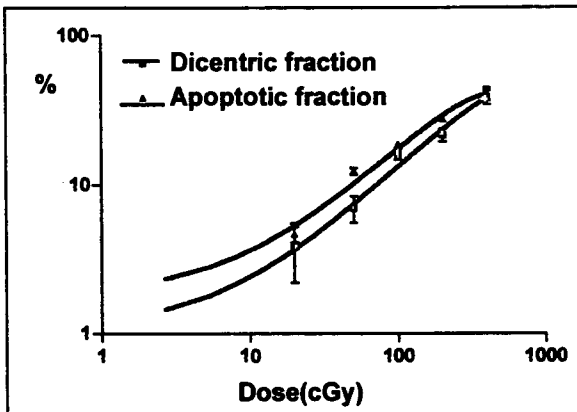


Figure 3. Apoptotic and dicentric fractions in irradiated lymphocytes. Bars represents Standard Errors,. Line represent fitting with the defined equation. Red: apoptotic fraction Green: dicentric fraction.

The correlation and fitting of the two phenomena and their increase with radiation dose was elevated, with $r^2$ higher than 95% in both cases($p<0.0001$), using the mathematical approach as described in Methods. The detection level was 3 cGy for apoptotic cells technique and 10 cGy for chromossomal aberration technique, as determined by 95% confidence interval analysis of controls cells and the curve fitting. There are no statistical significance between these levels in the two techniques.

## IV. DISCUSSION

Our data shows positive and linear correlation between apoptotic cell fraction and the quantitative exposure to gamma radiation. The apoptosis induced by radiation could be detected both in isolated DNA from irradiated cells and, by a more sensitive method, the IDNEL [16], allowed a fair quantification similar to conventional cytogenetic approach.

Biological dosimetry was attempted by several approaches, and cytogenetics was used extensively in most studies. The death of irradiated cell was early attributed to a non-specific DNA damage, that could be only chemically measured, without a roughly characterization or quantification. Despite its high sensitivity, the cytogenetic approach are restricted to surviving cell fraction, losing the diyng cells, that could be represent a interesting population for biological dosimetry [4, 6].

Only recently, apoptosis, a early described pathological phenomena, was associated to radiation, and several studies was conducted dealing with its biological and biochemical basis. At our knowledge, its use as a biological dosimeter for radiation exposure was here

originally described, despite some suggestions by others authors [2, 9, 10].
The study of apoptosis could be made in interphase lymphocytes, without the need of cell culture and, despite its relatively high complexity, with faster results. Usually, conventional time-consuming chromossomal techniques need also a very carefull trained observer, with hours of microscopic work [6]. The IDNEL allow the use of less trained observer, with faster results and, if adequately standardized, probably will result in a more sensitive technique for biological detection of radiation exposure.

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