Evaluation through comet assay of DNA damage induced in human lymphocytes by alpha particles. Comparison with protons and Co-60 gamma rays


1 Autoridad Regulatoria Nuclear, Av. Del Libertador 8250, CP: C1429BNP, Ciudad de Buenos Aires, ARGENTINA. mdigiorg@cae.arn.gov.ar
3 Escuela de Ciencia y Tecnología, UNSAM, Alem 3901, CP: 1653, Villa Ballester, ARGENTINA.
4 CONICET, Rivadavia 1917, Ciudad de Buenos Aires, ARGENTINA.

Abstract

Background and purpose: Several techniques with different sensitivity to single-strand breaks and/or double strand breaks were applied to detect DNA breaks generated by high LET particles. Tests that assess DNA damage in single cells might be the appropriate tool to estimate damage induced by particles, facilitating the assessment of heterogeneity of damage in a cell population. The microgel electrophoresis (comet) assay is a sensitive method for measuring DNA damage in single cells.

The objective of this work was to evaluate the proficiency of comet assay to assess the effect of high LET radiation on peripheral blood lymphocytes, compared to protons and Co-60 gamma rays.

Materials and methods: Irradiations of blood samples were performed at TANDAR laboratory (Argentina). Thin samples of human peripheral blood were irradiated with different doses (0 – 2.5 Gy) of 20.2 MeV helium-4 particles in the track segment mode, at nearly constant LET. Data obtained were compared with the effect induced by 4 MeV protons and Co-60 gamma rays. Alkaline comet assay was applied. Comets were quantified by the Olive tail moment.

Results: Distribution of the helium-4 particles and protons were evaluated considering Poisson distribution in lymphocyte nuclei. The mean dose per nucleus per particle result 0.053 Gy for protons and 0.178 Gy for helium-4 particles. When cells are exposed to a dose of 0.1 Gy, the hit probability model predicts that 43 % of the nuclei should have experienced an alpha traversal while with protons, 85% of the nuclei should be hit. The experimental results show a biphasic response for helium-4 particles (0.1 Gy), indicating the existence of two subpopulations: unhit and hit. Distributions of tail moment as a function of fluence and experimental dose for comets induced by helium-4 particles, protons and Co-60 gamma rays were analyzed. With helium-4 irradiations, lymphocyte nuclei show an Olive tail moment distribution flattened to higher tail moments as dose increase. However, for irradiations with protons and gamma rays, at increasing doses the tail moments are shifted towards high values with the same distribution, characterized by its asymmetry.

Conclusions: The comet assay allowed to observe differences in the patterns of DNA damage induced by helium-4 particles compared to protons and gamma rays, indicating that the mean dose and the hit distribution in the lymphocyte population influence the damage induction.

INTRODUCTION

The double aim of optimizing clinical efficacy of hadrontherapy and determining radiation risk estimates for space research, converges on the assessment of the biological effects of charged particles on cells. This effect depends on the spatial distribution of ionizing events. With high LET, the ionizations are deposited in tracks and so are non-randomly distributed both within a cell and among the cells.

The relative biological effectiveness (RBE) for many end points, including mutations, cell killing and chromosome aberrations, increases as LET increases reaching a maximum around 100 keV/µm and decreasing at higher LET values. Nevertheless, RBE for double strand break induction remains around 1.0 for all radiation qualities studied. An accepted explanation is that high LET radiation tracks produce highly localized clustered damage within the DNA and also spatially separated sites of damage along the path of the radiation track. Lesions are concentrated in localized areas and are more complex due to the larger number and size of multiply damage sites when compared to low LET radiation.
Ion irradiation is heterogeneous, a single particle traversal of a cell yields a high dose and when a moderately high dose is given to a blood sample there will be cells traversed by 0, 1, 2 etc tracks. Several techniques with different sensitivity to single-strand breaks and/or double strand breaks were applied to detect DNA breaks generated by high LET particles. Tests that assess DNA damage in single cells might be the appropriate tool to estimate damage induced by particles, facilitating the assessment of heterogeneity of damage in a cell population. The microgel electrophoresis (comet) assay is a sensitive method for measuring DNA damage in single cells. The objective of this work was to evaluate the proficiency of comet assay to assess the effects of high LET radiation on peripheral blood lymphocytes, compared to protons and Co-60 gamma rays. The experience was based on the changes in the distribution of the Olive tail moment (OTM), which is considered as a sensitive indicator of DNA breakage.

MATERIALS AND METHODS

Radiation beams

The irradiations of blood samples used the apparatus described previously [1] with some modifications [2]. The helium-4 ions and proton beams were obtained from the TANDAR 20 MV electrostatic tandem accelerator of the Atomic Energy Commission in Buenos Aires. In brief, negative helium-4 atoms were generated by a radio-frequency charge exchange source and pre-accelerated to the main accelerator. They were further accelerated to a positive high voltage where passage through a thin carbon foil stripped off the electrons to make positive ions, which were then accelerated to ground potential. A magnetic analyzer selected the required energy and the particles were focused on the detection zone and then entered the beam line. On entry the beam energy was 29.6 MeV with currents from 1 to 5 nA. The experimental beam line consists of two vacuum chambers linked by a 10 cm diameter stainless steel tube approximately 8 m long. The beam first passes through a gold scattering foil of 12.0 ± 0.5 µm in thickness, located in the first chamber. A system of anti-scatter collimators in the second chamber defines the maximum size of the beam. Scintillator screens, located in both chambers, helped to locate the beam. The system was designed to produce a beam profile homogeneous within ±1%, sufficiently wide to cover a blood specimen holder. The energies of the produced beams were 20.2 MeV for helium-4 particles and 4 MeV for protons. Both, helium-4 ions and protons passed to the same irradiation chamber. In the irradiation chamber, the beam monitor consists of a thin (25µm) aluminium-coated Mylar film from which backscattered electrons are collected on a ring held at +120 V. The backscatter current is proportional to the beam current. Finally, the beam passes out of the accelerator tube through a vacuum window of 100 µm Mylar. Uniformity achieved at the TANDAR generator was measured with a parallel plate ionization chamber Capintec PS-033 with a copper shield containing an aperture of 1.5 mm diameter.

Dosimetry

Different parallel plate ionization chambers were used to calibrate the beam monitor in terms of dose: a Capintec PS-033 for alpha particles and a Scanditronix NACP-01 for protons, previously calibrated for low energy protons in Orsay Protontherapy Center, France. The ionization chambers were calibrated according to an IAEA standard [3]. In both cases the beam monitor and ionization chamber currents were recorded remotely. The ionization chambers were located at the position of the blood samples with additional absorbers to simulate dose at the blood mid-plane. The energy spectrum of the helium-4 beam is measured using a silicon surface barrier detector. The beam energy is 20.2 MeV with an energy width (FWHM) of 0.6 MeV. An additional monitor calibration was performed using a Faraday cup, placed at the target position.
The dose was obtained through the following expression

\[ D_t = \left( \frac{N}{A} \right) \left[ \frac{S}{\rho} \right] \left( 1.602 \times 10^{-10} \right) \tag{1} \]

\( D_t \) is the calculated dose in Gy, \( N \) is the number of particles, \( A \) is the effective area of the beam (in \( \text{cm}^2 \)) and \( (S/\rho)t \) is the ICRU Report 49 [4] mass stopping power (in \( \text{MeV.cm}^2.\text{g}^{-1} \)) for alpha particles in water at the beam energy, all quantities corresponding to the irradiation position.

The ionization chambers and the Faraday cup calibrations agreed within 2%.

**Blood irradiation**

Thin samples of human peripheral blood were irradiated with 20.2 MeV helium-4 particles and 4 MeV protons, in the track segment mode, avoiding rapid changes in dose along the track associated with the Bragg peak. Thus, irradiations have been carried out at nearly constant LET [2]. Cells were also irradiated with Co-60 gamma rays at a dose rate of 0.49 Gy/min.

Freshly-drawn, heparinized blood obtained from healthy adults, was dispensed onto plastic discs with a circular inner trough of 26.46 mm in diameter and 60 – 70 \( \mu \text{m} \) in depth. The blood sample was held in position by a Mylar foil 4 \( \mu \text{m} \) thick. The sample-holding discs were placed in a sample wheel, which rotated at 10 revolutions per minute. Each position on the wheel passed through the beam for the same number of times. They passed about 2.5 cm from the beam exit window. Between the samples and the window there was a pneumatically operated shutter (4 bar of pressure) and a beam shaper with a 4\(^\circ\) angle, used at 0.05 Gy and 0.1 Gy helium ions and protons from 0.1 to 1.5 Gy. The beam shaper was replaced by a 10\(^\circ\) angle for helium doses from 0.5 Gy to 3 Gy and protons of 2 Gy. The doses, shown in the table of results, were given in less than 5 minutes.

**Comet assay**

Alkaline Comet assay was performed according to Singh [5] and Tice [6] technique with modifications. A layer of 1.5% normal melting agarose was prepared on microscope slides. After cell irradiation, \( \approx 25,000 \) cells in 50 \( \mu \text{l} \) were mixed with 120 \( \mu \text{l} \) of 0.5% low melting agarose. The suspension was pipetted onto the precoated slides. Slides were immersed in cold lysis solution at pH 10 (2.5 M NaCl, 100 mM Na\(_2\)EDTA, 10 mM Tris pH 10, 1% Triton X–100, 10% DMSO) and kept at 4 \( ^\circ \text{C} \) for 60 min. To allow denaturation of DNA, the slides were placed in alkaline electrophoresis buffer at pH 13 (1mM Na\(_2\)EDTA / 300 mM NaOH) and left for 25 min. Subsequently, were transferred to an electrophoresis tank with fresh alkaline electrophoresis buffer and electrophoresis was performed at a field strength of 1.33 V/cm for 25 min at 4 \( ^\circ \text{C} \) (20 V – 125 mA). Slides were neutralized in 0.4 M Tris pH 7.5 for 5 min and stained with 20 \( \mu \text{g/ml} \) ethidium bromide. For visualization of DNA damage, observations were made using a 20x objective on a epifluorescent microscope equipped with an excitation filter of 510-560 nm and a barrier filter of 590 nm. One to two hundred comets on duplicated slides were analyzed. Images were captured with a digital camera with networking capability and analyzed by an image analysis software, CASP [7]. DNA damage was quantified by the Olive tail moment (OTM) [8], whose distribution was adjusted by a two-parameter Weibull model [9]. OTM is the product of the distance (in x direction) between the center of gravity of the head and the center of gravity of the tail and the percent tail DNA.

**RESULTS AND DISCUSSION**

Dosimetric data of the lymphocyte irradiations are shown in Table I., being

\[ \text{Fluence [particles/cm}^2\text{]} = \frac{D\text{[Gy]}}{\text{Stopping power[MeV. cm}^2/\text{g}]} \times 1.602 \times 10^{10} \]

The mean number of impacts per nucleus, \( \lambda = \frac{(D\text{[Gy]}) \times \text{Lymphocyte nucleus area [cm}^2\text{]}}{(1.602 \times 10^{-10} \times \text{Stopping power [MeV. cm}^2/\text{g}])} \).

The mean dose per nucleus per particle was calculated assuming a spherical cell nucleus of 6 \( \mu \text{m} \) diameter, a mean lymphocyte radius crossed by one ion (\( = 4/3 \pi \)) and a density of water \( \rho = 1 \); the mean dose per particle in Gy is 0.00566L, where L (LET) is in keV\( \mu \text{m}^{-1} \).

The mean dose per nucleus per particle resulted 0.053 Gy for protons and 0.178 Gy for helium-4 particles.
Experimental dose = “accumulated dose” [counts] (beam monitor)/ calibration factor [counts/Gy], where: calibration factor = accumulated dose/ (ionizing chamber dose x geometric factor) and the geometric factor = 4/360 or 10/360, according to the beam shaper angle.

Table I. Dosimetric data for protons and helium-4 particles used for lymphocyte irradiations

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>H^+</td>
<td>4</td>
<td>94.04</td>
<td>9.4</td>
<td>7.45 x 10^6</td>
<td>2.1</td>
<td>0.112</td>
<td>0.112</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2.05 x 10^7</td>
<td>5.8</td>
<td>0.309</td>
<td>0.309</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>4.69 x 10^7</td>
<td>13.3</td>
<td>0.707</td>
<td>0.707</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>6.72 x 10^7</td>
<td>19</td>
<td>1.013</td>
<td>1.013</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>10^8</td>
<td>28.4</td>
<td>1.515</td>
<td>1.515</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.33 x10^8</td>
<td>37.7</td>
<td>2.011</td>
<td>2.011</td>
</tr>
<tr>
<td>4He^2+</td>
<td>20.2</td>
<td>314</td>
<td>31.4</td>
<td>1.01 x 10^6</td>
<td>0.3</td>
<td>0.051</td>
<td>0.051</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2.07 x 10^6</td>
<td>0.6</td>
<td>0.104</td>
<td>0.104</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.01 x 10^7</td>
<td>2.9</td>
<td>0.511</td>
<td>0.511</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2.01 x 10^7</td>
<td>5.7</td>
<td>1.010</td>
<td>1.010</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>4.07 x 10^7</td>
<td>11.5</td>
<td>2.050</td>
<td>2.050</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>5.02 x 10^7</td>
<td>14.2</td>
<td>2.526</td>
<td>2.526</td>
</tr>
</tbody>
</table>

Hit probability calculations were evaluated assuming Poisson distribution in lymphocyte nuclei: 
\[P(n) = \frac{(e^{-\lambda} \lambda^n)}{n!}\]
where, \(P(n)\) is the probability for one nucleus to be hit by \(n\) ions and \(\lambda\) is the mean number of impacts per nucleus.

Table II. Calculated hit probability for lymphocyte nuclei receiving a 0.1 Gy (4 MeV) proton dose

<table>
<thead>
<tr>
<th>Number of nuclei hits</th>
<th>Hit fraction (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>15</td>
</tr>
<tr>
<td>1</td>
<td>29</td>
</tr>
<tr>
<td>2</td>
<td>27</td>
</tr>
<tr>
<td>3 or more</td>
<td>29</td>
</tr>
<tr>
<td>Nuclei hit (%)</td>
<td>85</td>
</tr>
</tbody>
</table>

Table III. Calculated hit probability for lymphocyte nuclei receiving a 0.1 Gy α particle dose

<table>
<thead>
<tr>
<th>Number of nuclei hits</th>
<th>Hit fraction (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>57</td>
</tr>
<tr>
<td>1</td>
<td>32</td>
</tr>
<tr>
<td>2</td>
<td>9</td>
</tr>
<tr>
<td>3 or more</td>
<td>2</td>
</tr>
<tr>
<td>Nuclei hit (%)</td>
<td>43</td>
</tr>
</tbody>
</table>
1a. - Control
1b. - Co-60
1c. - Protons
1d. - Alpha particles

Figure 1. Probability distribution of OTM for unirradiated lymphocytes and exposed to 0.1 Gy
As the histograms obtained from the comet assay data (OTM) show a distinctive asymmetry, Weibull distributions were applied to fit comet experimental data obtained from lymphocytes exposed to 0.1 Gy of helium-4 particles, protons and Co-60 gamma rays (Figure 1).

The two-parameter Weibull model implies that the probability density function has the following dependence:

\[
f(x) = \left( \frac{\beta}{\alpha} \right) \left( \frac{x}{\alpha} \right)^{\beta-1} \exp \left( -\left( \frac{x}{\alpha} \right)^{\beta} \right)
\]

where, \( \alpha \) is the scale parameter or characteristic value of the variable \( x \), here the OTM, and \( \beta \) is the shape parameter.

The integral of the probability density function \( f(x) \) is the probability distribution \( P(x) \). The probability distribution is normalized so that the total area under the curve equals one.

Figure 1a shows the probability distribution for unirradiated lymphocytes (control), Figure 1b to 1d represent the probability distribution for lymphocytes irradiated with 0.1 Gy of Co-60 gamma rays, protons and alpha particles.

When cells are exposed to a dose of 0.1 Gy, the hit probability model predicts that 43% of the nuclei should have experienced an alpha traversal (Table III) while with protons, 85% of the nuclei should be hit (Table II). The comet experimental results show a biphasic response for 0.1 Gy helium-4 particles, indicating the existence of two subpopulations: unhit and hit, that is consistent with the predictions. In Fig. 1d, the first profile (area under the curve) indicates that 51% of the lymphocytes corresponds to the unhit subpopulation (control, see Fig. 1a), the second profile shows that 49% are hit cells.

Histograms in Fig. 2 represent the distributions of OTM as a function of the experimental dose for comets induced by helium-4 particles, protons and Co-60 gamma rays irradiations.

For irradiations with gamma rays, at increasing doses the tail moments are shifted towards high values with the same distribution, which is characterized by its asymmetry. When cells are irradiated with 4 MeV protons (9.4 KeV/\( \mu m \)), the comet distribution profiles follow a similar pattern but with higher dispersion of comet distributions. Fluences of 7.45x10\(^6\) and 2.05x10\(^7\), involving doses of 0.112 and 0.309 Gy respectively, do not induce a significant change in DNA damage distribution compared with control. With fluences of 6.72 x10\(^7\), 10\(^8\) and 1.33x10\(^8\) (doses: 1.013, 1.515 and 2.011 Gy) a significant shift to higher OTM is observed.

After irradiations with 20.2 MeV helium-4 particles (31.4 KeV/\( \mu m \)), the shift to higher OTM at increasing mean dose is more pronounced and a broadening in comet distribution is observed. As an example, 19 hits per nucleus are required for 4 MeV protons to produce a 1 Gy dose. For the same mean dose, 5.7 hits 20.2 MeV alpha particles cause a significant increase in DNA damage, evaluated through OTM of lymphocytes comets (see Fig.2). Thus, this indicates that for exposures to an ion of moderate LET, the mean dose and the hit distribution in the lymphocyte population influence the damage induction.
Figure 2. Distribution of OTM as a function of the mean dose for comets of human lymphocytes irradiated with Co-60 γ rays, 4 MeV protons and 20.2 MeV α particles.
CONCLUSIONS

At present, few studies have applied comet assay to evaluate the effects of high LET ions on cells [10]. The comet assay provided information of the heterogeneity of DNA damage induced by alpha particles in single human lymphocytes, allowing to identify and quantify hit and unhit subpopulations at low doses (0.1 Gy).

With helium-4 irradiations, it was observed a clear shift and dispersion (broadening) of comet distributions towards high OTMs with increasing fluence and mean dose. These marked variations in DNA damage suggest that each lymphocyte nucleus might be crossed by different number of particles, and thus, receive different doses. However, such a dispersion was not observed after irradiations with low LET radiation (Co-60 gamma rays) because increasing doses induce more breaks in every cell, resulting in a shift of comet distribution with the same profile.

The comet assay allowed to observe differences in the patterns of DNA damage induced by helium-4 particles compared to protons and gamma rays, indicating that for exposures to an ion of moderate LET, the mean dose and the hit distribution in the lymphocyte population influence the damage induction.

REFERENCES