

Micronucleus formation in lymphocytes after exposure to low-dose gamma and neutron radiation

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Abstract

In vitro exposure of peripheral lymphocytes to ionising radiation results in the formation of small chromosome fragments (micronuclei) that may persist for the lifetime of the cell. An increased frequency of micronuclei in a cell may be considered as a biomarker of permanent genotoxic damage. This study investigated the response of isolated lymphocytes to two types of radiation, i.e. low-LET (low linear energy transfer) and high-LET radiation.

Micronucleus formation in isolated lymphocytes was studied by employing the cytokinesis-blocked micronucleus (CBMN) assay. Cells from one donor were isolated, stimulated with a mitogen (PHA), irradiated with both gamma and neutron radiation and incubated at 37°C and 5% CO₂ for 44 hours after which cytokinesis was blocked by adding Cytochalasin B. After a total incubation period of 72 hours, cells were harvested and prepared for analysis. Micronuclei were analysed with fluorescent microscopy and dose-response curves were constructed.

Interexperimental variation between the three experiments was unusually high. This appeared to be due to different lots of PHA that were used during the study and should be investigated further. The assay was sensitive enough to distinguish between low- and high-LET radiation doses above 0.5 Gy. Lymphocytes responded to both radiation qualities in a dose responsive manner.

1. INTRODUCTION AND LITERATURE REVIEW

Ionising radiation is one of few agents that cause direct DNA breakage in a cell (Fenech, 2000). *In vitro* exposure of peripheral lymphocytes to these agents results in chromosome aberrations that may persist for the lifetime of the cell (Albertini *et al.*, 2000).

Classic cytogenetic analysis of chromosome aberrations in metaphases has been the gold standard of biological dosimetry for many years, but the complexity and tediousness of this technique has led to the development of the much faster and simpler cytokinesis-blocked micronucleus (CBMN) assay developed by Fenech and Morley (1985).

Micronuclei are small bodies in the cytoplasm resembling the nuclear material in morphology and staining pattern. They are formed when a broken chromosome or chromosome fragment does not travel to the spindle during mitosis and, therefore, are not included in either daughter nuclei (Albertini *et al.*, 2000). An increased frequency of micronuclei in a cell may be considered a biomarker of permanent genotoxic damage, reflecting either clastogenic or aneugenic modes of action (Albertini *et al.*, 2000).

Micronuclei are ideally scored in binucleated cells and can only be expressed in dividing eukaryotic cells (Fenech, 2000). They are mostly studied in cultured isolated lymphocytes stimulated to divide by phytohaemagglutinin. Cytochalasin-B is an inhibitor of actin polymerisation, preventing the formation of the microfilament ring that constricts the cytoplasm between two daughter nuclei during cytokinesis (Fenech, 2000). This action causes the accumulation of binucleated cells in almost all dividing cells. The use of Cytochalasin-B was introduced into the original micronuclei assay to enable reliable comparisons of chromosome damage between cells with different cell cycle kinetics (Fenech, 2000).

The combination of the CBMN assay with immunochemical labelling of kinetochores or pan-centromeric probes allowed identification of the most important mechanisms of micronuclei induction. Fluorescent *in situ* hybridisation (FISH) with a pan-centromeric DNA probe can be used to distinguish micronuclei originating from chromosome breakage and those from chromosome loss (Sari-Minodier *et al.*, 2002).

Despite considerable variation in methods and variables influencing the measurement, such as incubation periods, reagents and cell fixation, the induction of micronuclei is accepted as an effective biomarker of DNA damage. However, to validate the assay as a reliable biomarker of genotoxicity, the International Collaborative Project on Micronucleus frequency in Human Population (HUMN) was initiated to compare data on micronuclei frequency in different population and cell types (Fenech, 1999). As a result, a protocol has been developed and published (Kirsch-Volders *et al.*, 2003) while Fenech *et al.* (2003) compiled detailed scoring criteria for the CBMN assay, using isolated lymphocytes cultures as reference.

The CBMN assay is not only employed in the field of genetic toxicology, but also for human biomonitoring studies (Chang *et al.*, 1999; Kryscio *et al.*, 2001; Sari-Minodier *et al.*, 2002; Thierens *et al.*, 1999 and 2000; Laffon *et al.*, 2002), and in investigations of intrinsic radiosensitivity (Floyd and Cassoni, 1994; Jones *et al.*, 1994 and 1995; Vral *et al.* 2002; Widell *et al.*, 2003). The CBMN assay has been applied in various clinical studies and was able to detect cytogenetic damage in lymphocytes from patients treated with 131-Iodine (Gutierrez *et al.*, 1999).

The CBMN assay displayed high technical variability, therefore it needs to

be validated and standardised for each laboratory to ensure consistent results. The aim of this experiment was to perform the CBMN assay according to a protocol used by the radiobiology laboratory at iThemba LABS at Faure, South Africa (Slabbert, 1993, 2004) and to scrutinise the results for accuracy, repeatability and consistency. The experiment was also aimed at establishing dose response curves for ⁶⁰Co γ -rays and a 66 MeV neutron beam for doses of 0 to 4 Gy.

2. METHODOLOGY

2.1 Cell preparation

EDTA-blood from one healthy female donor was used throughout the study. Peripheral lymphocytes were sterilely isolated with Histopaque 1077 (Sigma), washed with phosphate buffered saline (PBS) and diluted in 10 ml RPMI 1640 (Roswell Park Memorial Institute), supplemented with 15% heat inactivated foetal calf serum (FCS), to produce sufficient cells for analysis. Five millilitres of cell suspension were pipetted into round-bottom culture tubes.

2.2 Irradiation

Gamma irradiation was performed using a ⁶⁰Co γ -source (Eldorado 76, Atomic Energy of Canada Ltd.), delivering a dose-rate of 0.29 Gy/min at a source surface distance (SSD) of 70 cm, a field size of 30 x 30 cm² and a gantry angle of 180°. Tubes were placed side-by-side in the centre of the radiation beam on a 0.5 mm-thick Perspex sheet with a 5 cm thick Perspex block on spacers serving as backscatter material. The dose at the position of the tubes was verified by using a 0.6 cc thimble Farmer ionisation chamber, type 2571 and a Farmer electrometer, model 2570/1 (NE Technology). The dosimetry system was calibrated against a national standard at the CSIR. The experimental set-up was also verified by irradiating seventeen calibrated thermoluminescent dosimeters (TLDs) with a dose of 2 Gy. The average TLD-dose registered after read-out in a TOLEDO dosimetry system was 2.055 Gy.

Neutron irradiation was performed with the cyclotron at iThemba LABS at Faure, using a dose rate of 0.35 Gy/min with 66 MeV protons bombarding a 19.6 mm thick beryllium target. The p(66)/Be(40) neutron beam generated had a large spectrum of energy with a maximum value of 64.15 MeV (Schreuder, 1992). A field size of 20 x 20 cm² and SSD of 150cm was used at a gantry angle of 0°. Tubes were placed side-by-side in the centre of the beam on a 10 cm thick perspex block with 2cm nylon as build-up material and 6 cm Perspex as backscatter. The dose at the position of the cells was verified by means of an 80 cc Far West (model IC-80) ionisation chamber (Far West Technology) and a BNC Portanin (model AP-2H) current digitiser (Berkeley Nucleonics, Corporation, USA).

Doses of 0.25, 0.5, 1.0, 2.0, and 4.0 Gy were applied for both types of irradiation. Control samples (0 Gy) were treated identically but sham irradiated.

2.3 Incubation and cell harvest

Immediately after irradiation, lymphocytes were stimulated to divide by adding 50 μ l of the mitogen, lyophilised phytohaemagglutinin (PHA, Gibco, 3109) at a concentration of 3 mg/ml. Three different lots of PHA were used for different experiments. Tubes were incubated at 37°C with loose lids in a

humidified atmosphere containing 5% CO₂. After 44 hours, 150 μl (3 μg/ml) of Cytochalasin B (Cyt-B, Sigma) was added to each culture tube to block cytokinesis. Cultures were then re-incubated for a further 28 hours (72 hours total incubation time). Tubes were removed from the incubator and centrifuged for 5 min at 1000 rpm. Supernatant was removed carefully and the cell pellet was gently mixed. Five millilitres of 0.075 M KCl were added drop-wise while tubes were either vortexed at low speed or shaken by hand. Tubes were centrifuged for 8 minutes after which the supernatant was discarded and the cell pellet resuspended in the remaining fluid. Five millilitres of freshly prepared Carnoy's fluid (methanol:acetic acid 3:1) were added in the same manner as the KCl. Tubes were placed in the fridge at 4°C until slides were prepared for analysis.

2.4 Slide preparation

Tubes were centrifuged, the supernatant discarded and the cells resuspended in remaining fluid. Ten drops of cell suspension were dropped onto clean, labelled, microscope slides and left on a flat surface at room temperature to dry for at least 24 hours. Slides were stained with acridine orange (TAAB Lab Equipment) at a concentration of 40 μg/ml for 1 minute, washed in distilled water and pH 6.8 buffer (Gurr) for 1 minute, covered with a cover slip, and sealed with Entalan to prevent drying. Slides were analysed immediately with a fluorescence microscope.

2.5 Microscopic analysis

Two hundred viable cells were analysed and reported as containing 1, 2, 3, 4, or more nuclei per cell. The nuclear division index (NDI) is an indicator of the efficiency of the PHA to stimulate lymphocytes in G₀ to undergo mitotic division.

The formula for the calculation of the NDI is:

$$NDI = \frac{N_1 \times 1 + N_2 \times 2 + N_3 \times 3 + N_4 \times 4}{\text{total cells counted}}$$

where N₁₋₄ indicates scorable cells containing 1 to 4 nuclei.

Five hundred binucleated (BN) cells per slide were examined for the presence of micronuclei and were reported as containing 0, 1, 2, 3, 4 or more micronuclei per BN cell.

The formula for the calculation of the total number of micronuclei present in the cell sample was as follows:

$$MN/500 \text{ cells} = \frac{BN_1 \times 1 + BN_2 \times 2 + BN_3 \times 3 + BN_4 \times 4}{\text{total BN cells counted}} \times 500$$

where BN₁₋₄ indicates binucleated cells containing 1 to 4 micronuclei.

The total number of micronuclei was calculated for each dose point.

2.6 Scoring criteria

The scoring criteria adopted by the HUMN project were used for analysis of micronuclei in this study (Fenech *et al.*, 2003). Only binucleated cells with both nuclei situated in the same cytoplasm were scored. The nuclei were more or less equal in size with intact cytoplasm. The diameter of the micronuclei was not greater than a third of the main nucleus and had the same staining intensity as the main nucleus. Micronuclei could touch but not overlap the nucleus. Apoptotic and necrotic cells were identified by chromatin condensation and cytoplasmic vacuoles respectively and were excluded from the study.

2.7 Statistical analysis

Dose-response curves were generated with Microsoft® Excel 2000 (9.0.2720). Standard deviations and averages were calculated using the STDEV and AVERAGE statistical functions respectively. Coefficients of variation were calculated using the following formula:

$$CV = SD/Mean \times 100$$

p-Values were calculated using the two-sample student's t-test (TTEST function), assuming unequal variances.

Analysis of dose-response curves was performed with GraphPad Prism version 3.0 for Windows (GraphPad Software, San Diego, California, USA).

3. RESULTS

Three experiments were carried out in which lymphocytes were exposed to gamma and neutron doses ranging from 0 to 4 Gy. Two slides were prepared per dose point and 500 cells were scored per slide. Dose-response curves were compiled by plotting the number of micronuclei per 500 binucleated cells as function of radiation dose (Figure 4). The nuclear division index (NDI) and the number of micronuclei per 500 binucleated cells were calculated according to the formulas explained in section 2.5 (microscopic analysis).

To compare the micronucleus yield distributions relative to Poisson distribution, the relative variance (σ²/y), and the dispersion index (μ) were determined. The average relative variance for the three experiments for gammas and neutrons were both 1.08 and the dispersion index was 1.16 and 1.87 for gammas and neutrons respectively. Negative μ values indicate under dispersion and positive μ values indicate over dispersion of an observed Poisson distribution. A μ value of >1.96 is regarded as significantly different from Poisson.

The MN data has been analysed by means of a second-degree polynomial fit. The coefficients reflect the alpha and beta parameters of a linear quadratic model (Table 1). Alpha values represent lethal damage and beta values repairable damage.

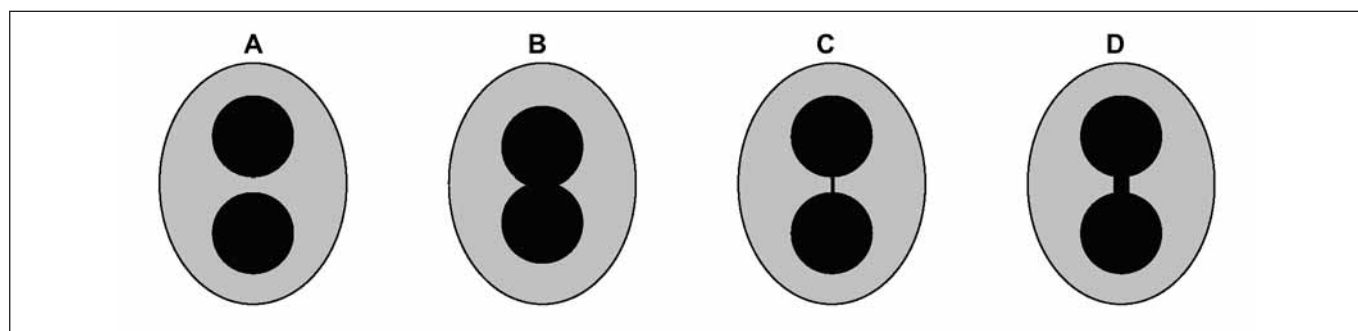


Figure 1: Criteria for choosing binucleated (BN) cells in the CBMN assay. A: Ideal BN cell; B: BN cell with touching nuclei; C & D: BN cell with nucleoplasmic bridges. All of the above cells can be scored for micronuclei (Redrawn from Fenech, 2003).

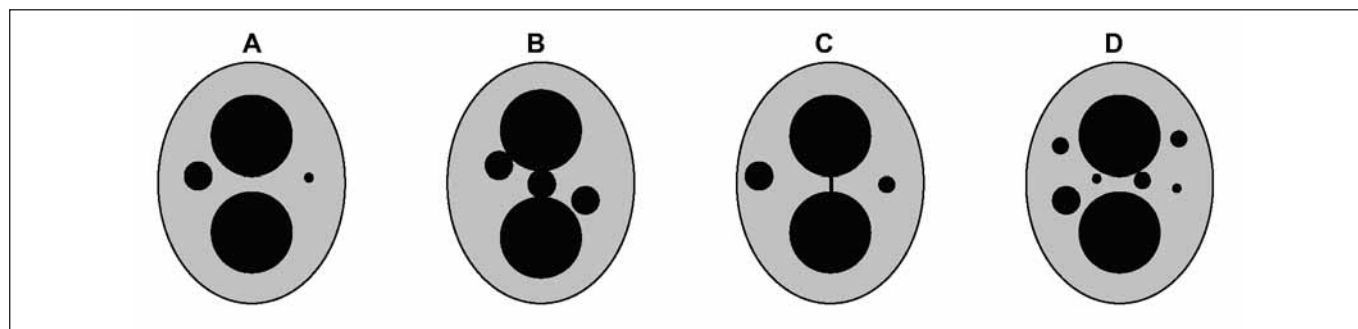


Figure 2: Typical appearance and relative size of MN in BN cells. A: Cell with two micronuclei; B: Micronuclei touching but not overlapping; C: Binucleated cell with nucleoplasmic bridge; D: Binucleated cell with six micronuclei (Redrawn from Fenech, 2003).

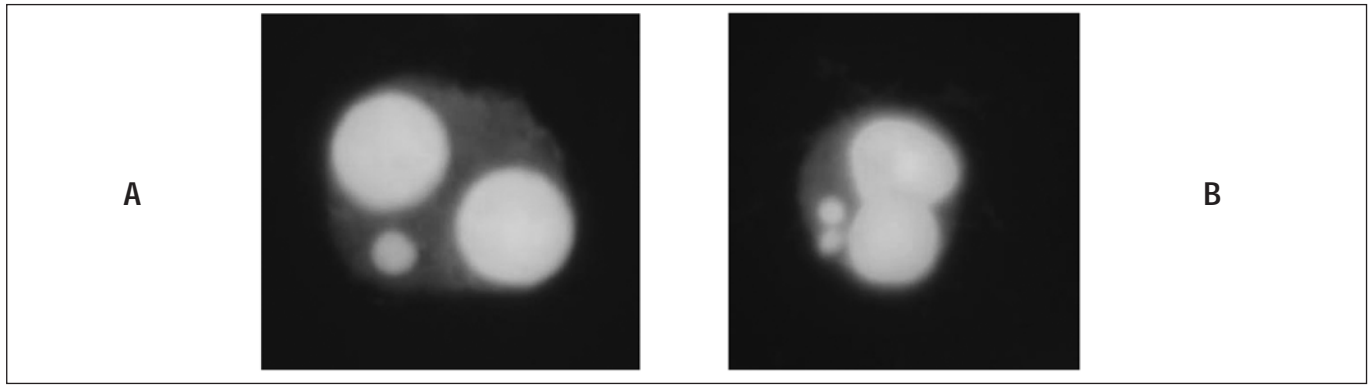


Figure 3: Photomicrographs of BN cells containing one (A) and two (B) micronuclei.

	GAMMAS		NEUTRONS	
	α value (Gy^{-1})	β value (Gy^{-2})	α value (Gy^{-1})	β value (Gy^{-2})
Experiment 1	0	43.71	150.8	6.79
Experiment 2	0	17.57	106.4	0
Experiment 3	12.77	19.80	86.95	10.24
SD	47.8 (All)	15.4 (Exp 2 & 3)	49.7 (All)	12.7 (Exp 2 & 3)
CV	31 (All)	24 (Exp 2 & 3)	32 (All)	9 (Exp 2 & 3)

Table 1: Statistical data on MN frequencies in lymphocytes exposed to gamma and neutron radiation. Alpha and beta values of dose response curves were derived from a linear quadratic model.

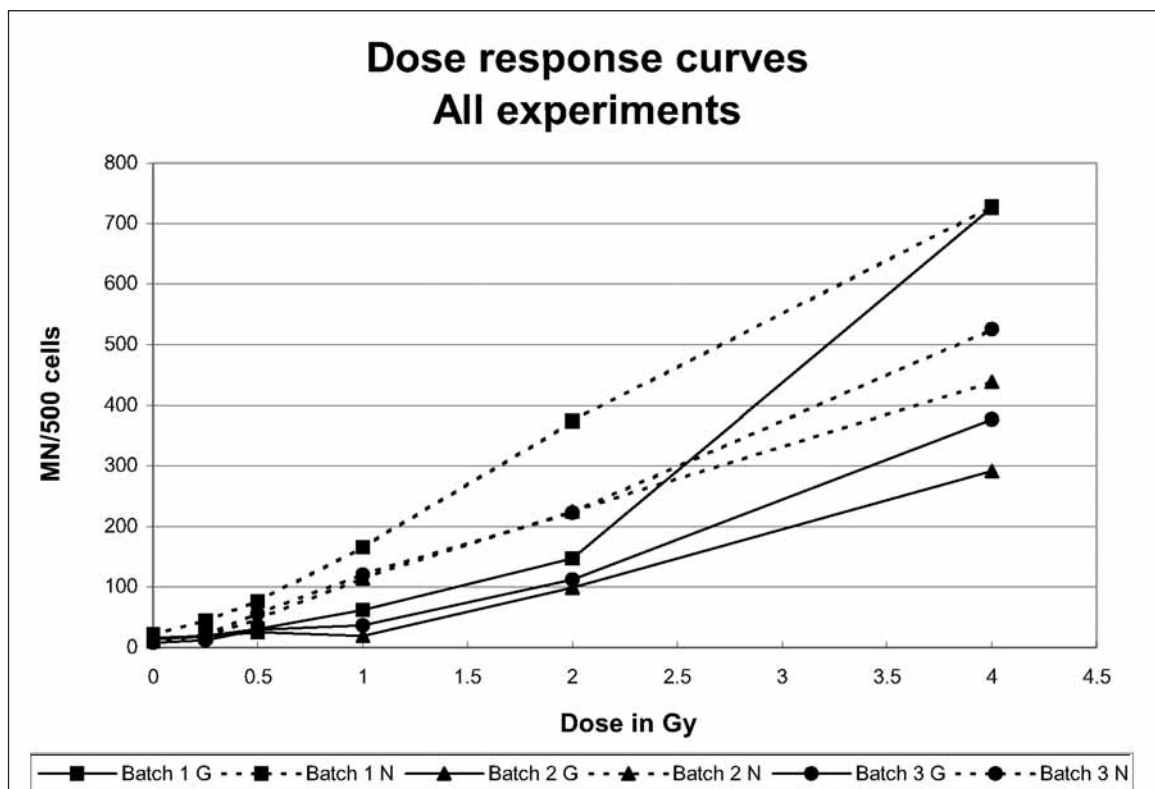


Figure 4: Dose-response curves of micronucleus frequencies of lymphocytes exposed to gamma (G) and neutron (N) radiation. All data points represent the values of one experiment. Control values (0 Gy) have not been subtracted from data points.

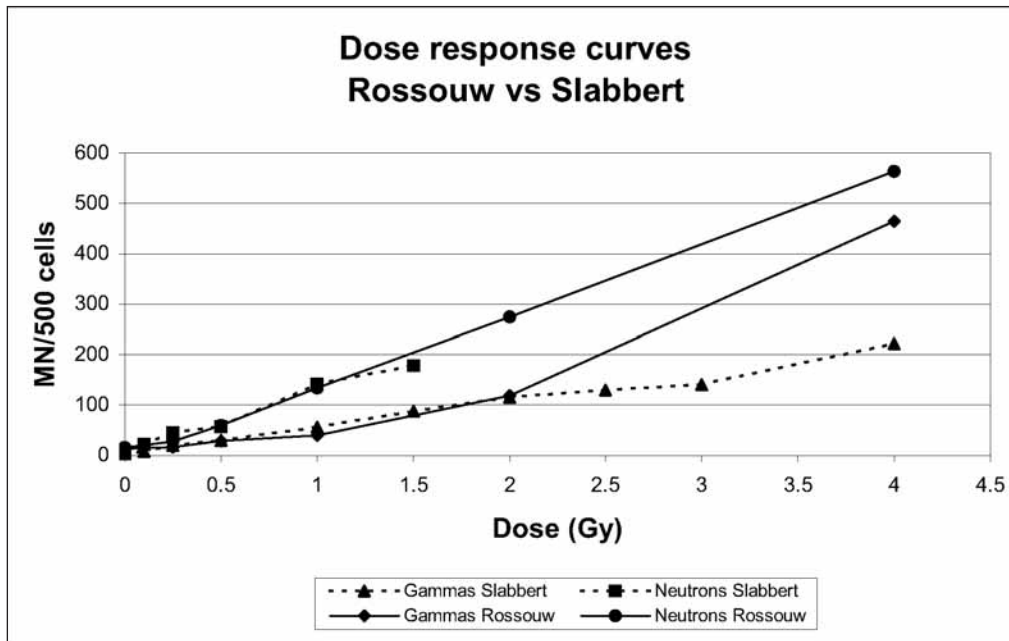


Figure 5: Dose response curves of micronuclei frequencies observed by two study groups. Dashed lines represent the data from Slabbert et al (1993) and solid lines represent data from the present study.

3.1 Inter-experimental variability

Standard deviations and coefficients of variation of all three experiments are displayed in Table 1. Micronucleus frequencies in experiment 1 were consistently higher than in the other two experiments resulting in CVs ranging from 9 to 55% for gammas and 21 to 51% for neutrons. If experiment 1 was excluded from the calculation of CVs, the values decreased to 9 to 44% for gammas and 0.5 to 18% for neutrons. The average NDI for experiment 1 (1.72) was also 32% higher than the average NDI of 1.30 for the other two experiments.

3.2 Dose-response

Both radiation qualities induced micronucleus formation in lymphocytes in a dose-responsive manner (Figure 4), ranging on average from 12 micronuclei per 500 cells in unirradiated samples to 335 in gamma- and 482 in neutron-irradiated samples. Neutron data produced linear dose-response curves, while gamma data displayed linear-quadratic curves.

Dose response curves represented in Figure 5 display the agreement between the present study and the data obtained by Slabbert et al. 1993 upon which this protocol was based.

Alpha values for neutron-exposed samples ranged from 86.95 to 150.8 and were consistently higher than gamma-exposed samples, which ranged from 0 to 12.77. An alpha value of zero was assigned when the estimate was not significantly different from zero and inter-experimental variability was too high.

3.3 Dispersion of micronuclei

Over the three experiments micronucleus frequencies were over-dispersed (>1.96) in 5 dose points for gammas and 10 for neutrons and under-dispersed in 5 dose points for gammas and 4 for neutrons. The average relative variances (σ^2/y) ranged from 1.05 to 1.12 across both radiation qualities with no significant difference in the values for gammas and neutrons.

4. DISCUSSION

From the alpha and beta values it is clear that neutron radiation induced a linear dose response while gamma radiation induced a linear-quadratic dose response. The low alpha values observed in the gamma curve, confirms the low-LET quality of gamma radiation with most of the induced damage of the repairable type. The two zero-values observed in two experiments were possibly due to large inter-experimental variability. The neutron beam produced higher alpha values in accordance with the more densely ionisation pattern of high-LET radiation where less cells are hit but with more lethal consequences.

High inter-experimental variability was observed between experiment 1 and the other two experiments; the influence of different batches of PHA could have contributed to this observation. It was conspicuous that experiment 1, apart from higher micronucleus frequencies, also produced a NDI that was 32% higher than those from the other two batches. This suggests

the enhancement of cell division in batch 1 compared to batches 2 and 3, therefore the possibility that the quality of PHA could influence the formation of micronuclei should be investigated. The high frequency of micronuclei in the 4 Gy gamma-irradiated samples in experiment 1 may have influenced other statistical data.

Data obtained in this study correlates well with the data from the radiobiology laboratory at iThemba LABS upon which protocol this study was based (Figure 5) (Slabbert et al., 1993). Data points for both gammas and neutrons below 2 Gy was in good agreement with each other, but the high 4 Gy-value (Figure 4) observed in the present study is as a result of the higher than expected number of micronuclei frequencies observed in batch 1.

The dispersion of micronuclei was not significantly different for gammas and neutrons and produced μ values of close to one as expected for gamma-exposed cells.

The assay was not sensitive enough to detect significant radiation damage below 0.5 Gy in either of the two radiation qualities.

5. CONCLUSION

The CBMN assay is the gold standard as an *ex vivo* measure of chromosome breakage and loss and has been standardised by the HUMN project with respect to methodological variables and scoring criteria (Fenech et al., 2003). Despite the use of these criteria and an established experimental protocol in this study, the inter-experimental variability between three experiments was unusually high. It has been suggested that different batches of PHA could induce different micronucleus frequencies; this observation should be investigated thoroughly before any attempt is made to employ the assay in its current format as biological indicator of radiation damage.

The assay was sensitive enough to distinguish between low- and high-LET radiation doses above 0.5 Gy and therefore the potential exists to use the assay as biomarker of radiation damage for radiation doses above 0.5 Gy. Lymphocytes responded to both radiation qualities in a dose-responsive manner.

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