Volume 12, No 3 | International Journal of Radiation Research, July 2014

Cytokinesis blocked micronucleus (CB-MN) assay for biodosimetry of high dose accidental exposure

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► ***Original article***

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Received: June 2013
Accepted: Oct. 2013

Int. J. Radiat. Res., July 2014; 12(3): 211-215

ABSTRACT

***Background*:** Most of the cytogenetic dosimetry, except Premature Chromosome Condensation assay, saturates at 6 Gy. Present studies are aimed to assess feasibility of simple CB-MN assay for prescreening of high dose accidental exposures. ***Materials and Methods*:** Human peripheral blood lymphocytes from two donors were gamma irradiated to different doses ranging from 0-15Gy, cultured for 72 h. Cytochalasin-B was added to the cultures at 44 h. Slides were analyzed for frequency of Mononucleated cells (MNC), ratio of Tri/Quadra nucleated cells, micronuclei (MN) yield in MN positive cells, mitotic bridges and apoptosis. ***Results*:** At 5 Gy 67% Mononucleated cells, 0.22% apoptotic cells and 1.76% mitotic bridges were observed. Micronuclei yield in MN positive binucleated cells was found to be 1.43±0.04. At 8Gy 96.63% Mononucleated cells, 22.64% apoptotic cells and 3.8% mitotic bridges were observed. Micronuclei yield in MN positive binucleated cells was found to be 2.18±0.18. At 15 Gy, 99.6% of the cells were Mononucleated and 0.38% was binucleated and MN yield was 2.9 ±0. The total number of MN in MN positive cells increases with increasing radiation doses. ***Conclusion*:** All the three indicators number of MN in all binucleated cells (BNC) , number of MN /MN positive binucleated cells and the nuclear division index (NDI) can serve as good biomarkers for biodosimetry at high doses. Although the frequency of mitotic bridges and apoptotic bodies increases with dose, there is no consistent difference between various doses. Thus the CBMN + NDI assay can be a biodosimetry method at high doses.

Keywords: *CB, MN assay, biodosimetry, high dose exposures.*

INTRODUCTION

Accidental overexposure to radiation causes a variety of radiation injuries. The extent and intensity of the exposure strongly influence subsequent medical treatment. Physical information about dose is rarely available in these situations. Since last four decades dose estimation is based on the frequency of unstable dicentric aberrations in peripheral blood lymphocytes of the exposed person. In case of accidental situation where dose is greater than LD 50/60 value i.e. 5 to 6 Gy, for whole body exposure, the conventional dicentrics assay fails to provide accurate dose estimation. This can be attributed to the disappearance of lymphocytes in circulating blood system, G2 block and mitotic delay. It is still most specific and sensitive method to determine radiation dose in the range of 100 mGy - 6Gy [1]. However, in the recent years a multi-parametric approach to investigate over exposures, using other assays like micronucleus, translocations, premature chromosome condensation and gamma-H2AX assays, has been proposed [2,3,4].

The cytokinesis blocked micronucleus (CBMN) assay in blood lymphocytes developed by Fenech and Morely in 1985[5-7] is a very reliable

technique to evaluate *in-vivo* occupational, medical and accidental exposures of individuals and also it is an *in-vitro* test to assess radiosensitivity [8]. Micronucleus (MN) originates from chromosome fragments or whole chromosomes left in cytoplasm during cell division. From the frequency of MN in binucleated cells the radiation dose received by the person can be evaluated. In case of radiation accident where a large number of persons are exposed, Cytokinesis Block Micronuclei (CBMN) assay can be an alternative method for dose estimation, due to its advantage of easy and quick analysis.

The objective of the present study is to establish dose response relationship between radiation dose and frequencies of MN, and also percentage of mono, bi and poly-nucleated cells, nucleoplasmic bridges and apoptotic bodies in gamma irradiated peripheral blood lymphocytes exposed to high doses (5-15 Gy).

MATERIALS AND METHODS

Subjects and irradiation of blood samples

Blood samples from two healthy donors aged 24 and 29 years were used for experiments. The samples were irradiated with Co-60 gamma rays in a Gamma Chamber supplied by BRIT, BARC at dose rate of 2.93Gy/min. The doses employed were 0, 5, 8 and 15Gy. The blood samples were maintained at 37^{0}C during and 2h post irradiation to allow repair. The blood samples were collected from voluntary donors who were informed about study.

Reagents

RPMI medium, fetal calf serum, purchased from Gibco, Phytohaemagglutinin (PHA) and cytochalasin B purchased from Sigma Chemicals were used for the experiments.

Cell culture and slide preparation

Whole blood cultures were set up with RPMI 1640 medium supplemented with 2mM L-glutamine and 15% fetal bovine serum and PHA at a concentration of 10µg/ml and incubated at 37^{o}C for 72 hrs. Cytochalasin-B was added to the cultures at 44h to have a final concentration of 5µg/ml to block cytokinesis. After 72 hrs cells were harvested, treated with 125 mM KCl and fixed with 5-7 ml of saline fixative solution consisting of 15 parts of 1:4, acetic acid: methanol and 13 parts of 0.9% saline. Again the cells were centrifuged and fixed in mixture of methanol acetic acid 4:1 [9, 10]. Slides were stained with 5% Giemsa stain in phosphate buffer.

Analysis of the slides

Micronuclei were scored in binucleated cells under light microscope with magnification of 40x as described by Fenech and Morely [3]. The frequency of mononucleated (MNC), binucleated (BNC), tri nucleated or tetra nucleated (PNC) cells were analyzed (figure 1). Cells with nucleoplasmic bridges and apoptotic bodies were also scored [11, 12]. The yield of MN in binucleated (binucleated cells with and without micronuclei) and total number of MN in MN positive binucleated cells (binucleated cells with micronuclei only) were calculated. Simultaneously the number of cells with 1N, 2N and >2N were recorded. The NDI were calculated by using the formula as given below:

NDI = (1N1 + 2N2 ×2 + >2N × 4/Na
Where:
1 N= no of mononucleated cells
N2 = no of binucleated cells >2N = no of tri- or tetra nucleated cells
Na = total no of cells (N1+ N2 + N3 + N4).

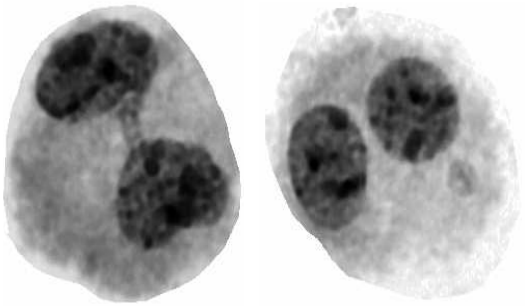

Figure 1. Black and white photomicrographs of giemsa stained Mitotic bridge and binunucleated cell with micronuclei taken at 100x magnification.

RESULTS

The number of mononucleated cells (1N), binucleated cells (2N) tri or tetra nucleated (>2N) and the nuclear division index (NDI) observed at various doses is given in table 1. As radiation dose increases, frequency of MNCs significantly increases whereas percentage of binucleated and poly nucleated cells decreases in both samples. At 15Gy, PNC was found to be 0% for both samples. The dose response data obtained from two donors is given in table 2. The average yield of MN at 0 Gy from two donors is about 3/1000 (table 2). The frequency of MN and cells with MN increased with the radiation dose. The dose response curve for the yield of radiation induced MN in total binucleated cells and in micronucleated binucleate cells alone is given in figures 2 and 3 respectively.

For sample A the yield of MN increased from (0.61 ±0.36) at 5Gy to 3.0 ± 1.22 at 15Gy. For sample B the corresponding values are 1.03 at 5Gy to 2.9 at 15 Gy. The frequency of MN in MN positive binucleated cells alone also increases with dose from 1.43 ± at 5 Gy to 3 ± 1.22 at 15 Gy for sample A and for sample B the corresponding values are 1.72 ± 0.07 and 2.9 ± 0.44 respectively.

Other relevant biomarkers like nucleoplasmic bridges (NPBs) and cell undergoing apoptosis etc were also assessed at different dose points. NPBs and apoptotic bodies increase with radiation dose but the increase was not consistent.

Table1. Percentage of Mononucleated, Binucleated and Poly-nucleated cells with different radiation doses.

Dose (Gy)	% Mononucleated cells (MNC)		% Binucleated cells (BNC)		% of Poly-nucleated cells (PNC)		NDI	
	Donor A	Donor B	Donor A	Donor B	Donor A	Donor B	Donor A	Donor B
0	37.2	20	49.6	48.6	13.2	31.4	1.81	2.30
5	67.00	59.65	25	31.87	6.60	8.46	1.41	1.53
8	96.62	89.21	3.35	9.69	0.33	0.852	1.04	1.12
15	99.62	98.60	0.37	1.30	0	0	1.003	1.01

Table 2. Frequency of Micronuclei in binucleated cells and Micronuclei positive binucleated cells.

Dose (Gy)	MN yield in BNC		MN yield in MN positive BNC		Frequency of Apoptotic bodies		Frequency of Nucleoplasmic Bridges	
	Sample A	Sample B	Sample A	Sample B	Sample A	Sample B	Sample A	Sample B
0	0.003±0.0017	0.003±0.002	1± 0.57	1± 0.57	0	0	0	0
5	0.61±0.036	1.03±0.042	1.43±0.09	1.72±0.07	0.002±0.002	0.024±0.01	0.004 ± 0.003	0.0071± 0.004
8	1.81±0.184	2.26±0.130	2.18±0.22	2.31±0.13	0.226 ±0.065	0.023 ± 0.01	0	0.0225±0.013
15	3.0±1.22	2.9±0.44	3.0±1.22	2.9±0.44	0	0.4 ± 0.16	0	0

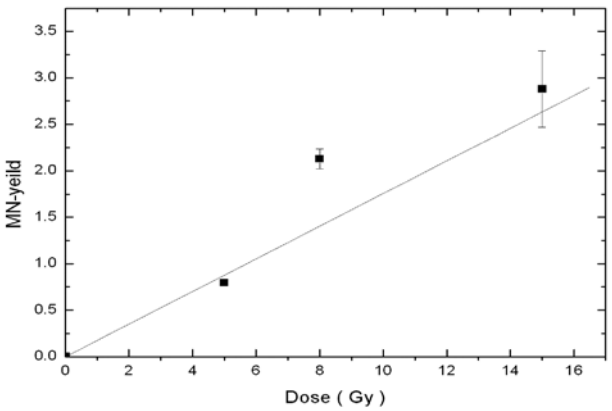

Figure 2. Dose response curve of the yield of Micronuclei in binucleated human peripheral blood lymphocytes exposed to gamma rays.

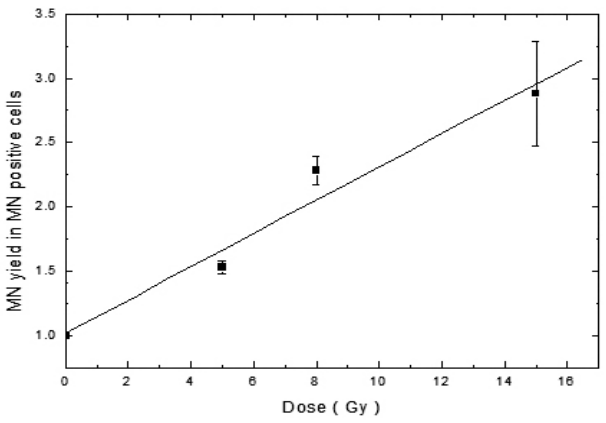

Figure 3. Dose response curve for the induction of MN in micronucleated binucleate blood lymphocyte exposed to ^{60}Co-gamma rays.

DISCUSSION

Chromosomal aberration analysis is being used for biological dose estimation for five decades and it is the most reliable radiation specific indicator at present. CBMN assay is another bio-dosimeter for dose estimation. Since 1990, it has been employed in biodosimetry of several accidents and the estimates were in accordance with CA, physical or clinical dosimetry [13]. An alternate method of assessing chromosomal damage is the frequency of Micronuclei in binucleated cells in cultured blood lymphocytes. The yield of MN at 0 Gy in the present study is 3/1000BN cells. The spontaneous frequency is shown to be variable [9, 14]. However the variable background frequency of MN ranging from 2- 36 per 1000BN cells has limited the use of this assay for biodosimetry when radiation doses are low. Limited data is available at high doses. The nuclear division index and the frequency of mononucleated cells show good dose response at high doses. In an *in-vivo* study reported by Yao *et al.* [15] in China accident victims, there was a good agreement between CBMN + NDI assay, ESR dosimetry and dicentrics assay proving its potential use in accidental situations. MN studies conducted in peripheral blood lymphocytes of different groups of patients treated with partial body radiotherapy and those undergoing radio-iodine treatment have shown that the dose estimates by MN assay is sensitive enough and can detect and quantify average whole body doses [16, 17,18].

In the present study, the dose response for high dose range used can be represented by a linear relationship

Y= 0.003 + 0.18 D, where Y is the yield of micronuclei in BN cells, D is the radiation dose in Gy, the R^2 value is 0.96. Similar observations are made by other authors at high doses [15, 19]. The frequency of MN in MN positive BN cells also showed a linear relationship represented by

Y= 1.017 + 0.13 D and R^2 = 0.96

Our earlier study with low doses (up to 4Gy) indicated a linear quadratic relationship in the low dose region [9,20] In the present study, an increased number of mononucleated cells and a decreased yield of binucleated, tri-tetra nucleated cells were observed with increasing radiation doses indicating a good relationship of NDI with dose. Thus, % of MNCs, BNC and PNC can be used as dose indicators at high doses. Similar study reported by Yao et *al*. [15] also showed a reduced yield of binucleated cells at doses above 10Gy.

The NPBs has been suggested as a potential biodosimeter due to low control values and their relation to dicentric chromosomes [16]. The dicentric analysis in metaphase spreads helps to detect radiation doses as low as 50 mGy when 1000 spreads are analyzed. The scoring of NPBs resulting from non-disjunction of one or more dicentric chromosomes whose centromeres are pulled to opposite poles of the BN cells, provide a direct evidence of genomic instability [17,18]. Scoring of NPBs in the CBMN assay makes it more reliable for biodosimetry due to low background frequency, unaffected by gender, direct measure of asymmetrical chromosome rearrangement, and dose related increase in response after radiation exposure up to 4 Gy [19]. However, in the present study, there is no consistent difference in the frequency of NPBs between different samples, since the cells are not dividing at high doses.

CONCLUSION

The present study shows that all the three, MN in BN cells, MN in MN positive binucleated cells and the nuclear division index are good biomarkers for dosimetry at high radiation doses. Although the frequency of mitotic bridges and apoptotic bodies showed some increase with dose, no consistent pattern was observed with two different samples and with increasing doses. Thus the CBMN + NDI assay is another biodosimeter for dose estimation. More *in-vivo* studies are needed for validation.

ACKNOWLEDGDMENT

The authors gratefully acknowledge the technical assistance provided by P.J. Tondelekar, S.C .Jagtap and UB. Thorat.

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