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Induction of Premature Chromosome Condensation (PCC) by Calyculin A for biodosimetry

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ABSTRACT

Background: Premature Chromosome Condensation (PCC) is a method for interphase chromosome analysis in biodosimetry. This study was performed to verify the usefulness of PCC induced by calyculin A in human peripheral blood lymphocytes (pbl) for biological dosimetry and possible construction of dose-response curve.
Materials and Methods: Peripheral blood was obtained from a healthy donor and exposed to various doses (0.25 – 4 Gy) of g-rays. The frequency of simple breaks and dicentrics were scored in G2/M chromosomes of Giemsa stained cells.
Results: Results show that the frequency of simple chromosome breaks appears to increase linearly with dose; while the frequency of dicentrics apparently increases linear-quadratically with the dose.
Conclusion: Induction of chromosome condensation by calyculin A is a powerful biodisimetric method, which provides a high number of spreads for analysis. With the use of this method, it is possible to overcome problems related to low mitotic index or cell-cycle alterations in routine metaphase analysis and low fusion rate in conventional PCC technique.

Keywords: *Biodosimetry, PCC, Calyculin A, human lymphocytes.*

INTRODUCTION

Biological dosimetry of ionizing radiation is generally performed by scoring dicentrics and/or translocations in chromosome from peripheral blood lymphocytes (pbl) at the first mitosis following *in vitro* growth stimulation. It is well known that after irradiation, in this procedure mitotic index is low in some individuals, so the scored mitoses might not be representative of the exposed cell population. For these reasons premature chromosome condensation (PCC) was introduced by Johnson and Rao (1970) following fusion of an interphase with a mitotic cell (Johnson and Rao 1070) 1970). With the use of this method makes it is possible to distinguish individual chromosomes at different stages of the cell cycle. Thus, it has been used to study the formation of chromosomal aberrations shortly after irradiation and the kinetics of chromosome break rejoining (Pantelias and Maillie 1985). This technique is considered as a potent biodosimetric tool (Blakely *et al.* 1995) since it is probably one of the most sensitive methods for analyzing initial chromosomal damage after irradiation (Sipi *et al*. 2000).

At present, PCC can be induced by two methods, cell fusion and using chemicals. In cell fusion method, CHO mitotic cells with high promoting factor (MPF) are fused to interphase cells by polyethylene glycol (PEG) or Sendi virus. After staining, chromosome elements appear as single chromatids and can be discriminated easily from the CHO mitotic chromosomes in human interphase G0/G1 PCC spread

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(IAEA 2001) This procedure is technically difficult and PCC index is generally low and unstable (Durante *et al*. 1998, Prasanna *et al*. 2000). In chemical method, okadaic acid or calyculin A inhibitors for type 1 and 2A protein phophatases are used for PCC induction. These proteins block the MPF during interphase. Therefore they induce only the MPF level which is sufficiently high for induction of premature chromosome condensation in pbl at any time of cell cycle (Durante *et al*. 1998, Kanda *et al.* 1999b). Induction of PCC using chemicals is relatively simple and it is shown that the frequency of PCC induced by calyculin A is 20 times more than induction by okadaic acid (Kanda *et al.* 1999a).

Several studies on chromosomal aberrations induced by high-LET, low-LET radiation, neutrons and charge particles, in lymphocytes, fibroblasts and tumor cells have been done. Calibration (dose-response) curves for biodosimetry using human lymphocytes have constructed by PCC method (Prasanna *et al*. 2000, Vyas *et al.* 1991, Durante *et al*. 1996, George *et al*. 2000, Stronati *et al*. 2001, Kawata *et al*. 2000, Ofuchi *et al*. 1999, Greinert *et al*. 1995, Durante *et al.* 1997, Wu *et al*. 2003). Using this method, radiation damage and radio sensitivity of cancer patients have also been studied (Greinert *et al.* 1995, Yamada *et al.* 1999). It is assumed that PCC induction especially by chemicals (calyculin A) is a powerful method for biodosimetry in case of very high dose irradiation, since in this situation mitotic index is too low. Combining fluorescence *in situ* hybridization (FISH) (chromosome painting) with PCC carries several potential advantages for biodosimetry. PCC allows direct, sensitive analysis of interphase cell and FISH eliminates random scoring error and allows symmetric and asymmetric chromosomal interchanges to be identified as well breaks (Durante *et al*. 1996).

In this study, using PCC induced by calyculin A for G2/M chromosome aberrations analysis were tried to construct a dose-response curve for dicentrics and simple chromosome aberrations in pbl following gamma irradiation.

MATERIALS AND METHODES

Blood sampling

Peripheral blood was obtained via venopuncture using a lithium heparinized syringe from a healthy male donor aged 20, with no drug or radiation treatment at least one month prior to experiment. To avoid interindividual differences including nutritional habits and living styles, all experiments were performed using the samples taken from this person.

Irradiation

Whole blood was exposed to 0.25, 0.5, 1, 2, 3 and 4 Gy g-rays generated from a ^{60}Co source (Terateron 789-30, Canada) at room temperature at Novin Medical Radiation Institute. Dose rate was in a range of 163.99-165.131 cGy/min at the time of irradiation.

Lymphocyte culture and slide preparation

0.5 ml heparinised blood was added to a culture vessel containing 4.5 ml RPMI 1640 (Gibco) medium, supplement with 10% FCS (Gibco), 1% PHA (Gibco), 1% L-glutamin and 1% Penicillin/Streptomycin (Gibco) (100 iu/ml Penicillin and 100 µg/ml Streptomycin). Cells in complete medium were incubated in tightly closed culture vessel at 37°C. After 24h 20 µl colcemid (Gibco) (final concentration: 40 ng/ml) was added, the vessel was incubated again for 22 hours. Finally, 5 µl of calyculin A (Sigma) (final concentration: 50 nM) was added and the vessel incubated for 1h at 37°C.

After the incubation of cells, they were transferred to 15 ml centrifuge tube (Falcon) and centrifuged for 5 min at 1000 rpm. Pellets were carefully resuspended in 8 ml of KCl (75 mM), and incubated at 37C° for 20 min. After hypotonic treatment, cells were fixed in 5 ml of fresh,

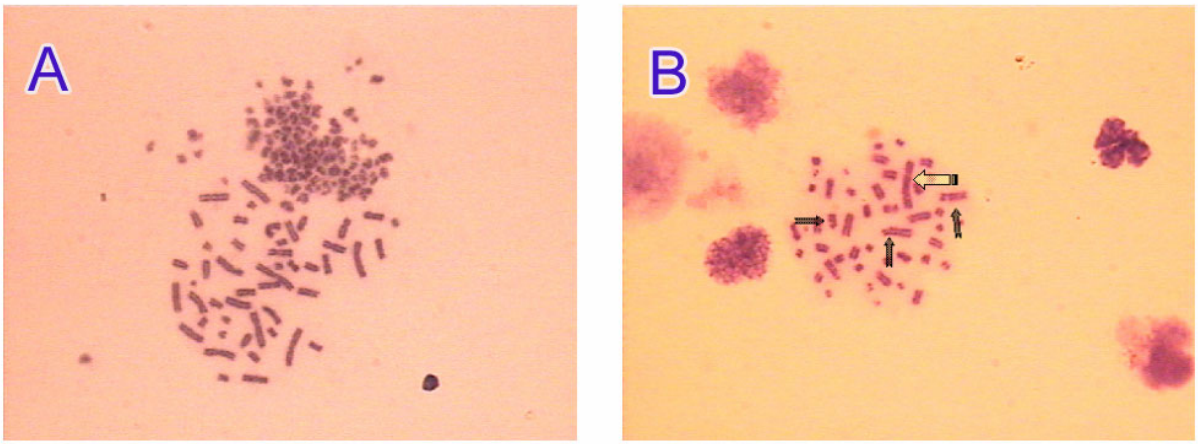

Figure 1. A: G2/M chromosomes; B: Dicentrics & Breaks in G2/M chromosomes

prepared fixative (methanol: acetic acid; 3:1 v/v). After 10 min, cells were washed twice more in fixative.

Cells were dropped on pre-cleaned cold and humid slides. Slides were stained in 10% Giemsa for 8 min, and observed with a light microscope (×100).

Cell cycle stage evaluation

Spreads displaying 46 univalent chromosomes or 92 chromosomes (shortly after cell division) were classified as G1. In S phase, the spreads had a typical pulverized form, G2 and M phase presented two chromatids in each chromosomes. Because a clear distinction between G2 and M was sometimes difficult, all bivalent chromosomes were classified in the G2/M category (figure.1). 2000 stimulated lymphocytes were scored for PCC Index and for every data point 200-500 G2/M cells were scored for dicentric chromosomes and simple chromosome aberrations.

The significance of any inter–dose differences in the number of dicentrics and simple chromosome aberrations was statistically evaluated by *t–test* and chi-square test.

RESULTS

Determination of PCC Index and stress

PCC index was determined with percentage of PCC in the total number of scored cells, i.e.

$$\text{PCC index} = \frac{\text{No. of PCC spreads} \times 100}{\text{No. Scored cells}}$$

PCC indices in (0-4) Gy ranged (39.6 – 57.15). PCC index in different phases of the cell

Table 1. The percentage of cells condensed in different phases of the cell cycle following treatment with calyculin A.

donor	age	sex	Radiation dose (Gy)	Scored cells	G1 %	S %	G2/M %	PCC %
A	20	male	0	2000	8.95	19.4	18.65	46.64
			0.25	2000	6.8	13.74	29.38	49.92
			0.5	2000	15.3	14.85	24.7	54.85
			1	2000	8.9	13.75	28.5	51.17
			2	2000	7.45	14.65	35.9	58
			3	2000	4.9	22.95	29.3	57.15
			4	2000	6.65	12.55	20.4	39.6

Table 2. Frequency of dicentrics and simple chromosome aberrations in G2/M phases at different radiation doses following treatment of calyculin A.

donor	age	sex	Sample / dose	Radiation dose (Gy)	Scored cells	Dicentric / cell (F±SD)	Scored cells	Simple aberration / cell (F±SD)
A	20	male	2	0	500	0	500	0.038±0.003
			2	0.25	500	0.064±0.027	200	0.055±0.021
			2	0.5	500	0.12±0.02	200	0.07±0.014
			2	1	250	0.18±0.04	200	0.11±0.014
			2	2	250	0.29±0.04	224	0.19±0.07
			2	3	250	0.48±0.08	300	0.26±0.052
			2	4	250	0.76±0.067	300	0.32±0.005

cycle is shown in table 1. Five hundreds of PCC in control samples were analyzed to determine PCC stress. Only 19 fragments were scored (0.038 ± 0.003 fragments/ cell) (table 2).

Dose- response curves

After scoring 200-500 G2/M cells, the frequency of dicentrics and simple chromosome aberrations (breaks and fragments) were calculated per cell. As seen in table 2, the frequency of gamma rays induced dicentrics is increasing with increasing radiation dose from 0.25-4 Gy, which is significantly different from control values for all doses ($p<0.001$). This was also the case for simple breaks, statistically different from controls ($p<0.05$). Dose-response curves constructed based on dicentrics and simple chromosome aberrations per cell, show a linear quadratic pattern for dicentrics (figure 2) and linear for simple breaks (figure 3).

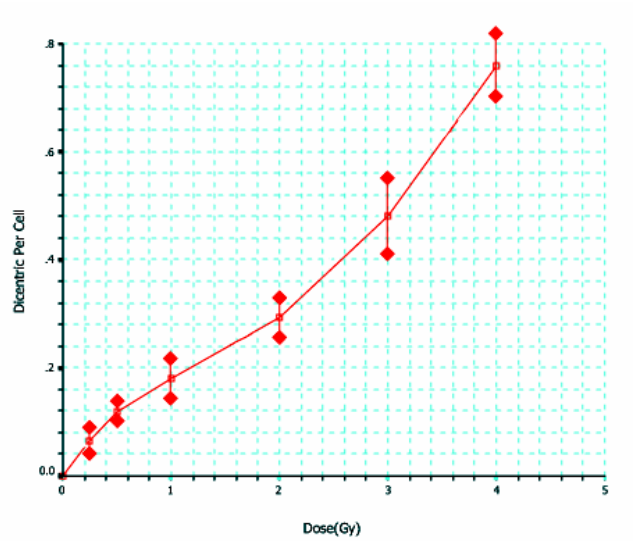

Figure 2. Dose-response curve for induction of dicentrics in G2/M chromosomes.

DISCUSSION

In this study when cells were treated with calyculin A, 39-58% of pbl showed clearly condensed chromosomes. This is in accord with the pervious reports using this drug for chromosome condensation (30-60%) (Durante *et al*. 1998). Relatively low frequency of aberration was seen in control samples (0.038 ± 0.003) but radiation induced both simple breaks and dicentrics with all dose ranges used in this study (table 2).

As seen in figure 2, dose-response curves for dicentrics is linear-quadratic with $P<0.001$ and for simple chromosome aberrations is linear with $P>0.05$. Pervious studies on PCC

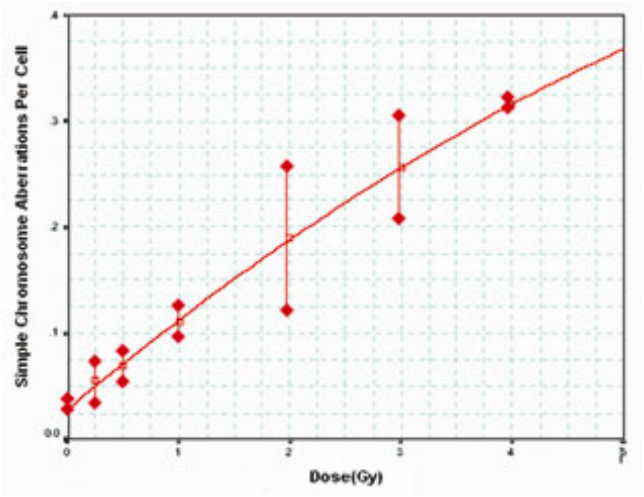

Figure 3. Dose-response curve for induction of simple chromosome aberrations in G2/M chromosomes.

(induction by fusion) showed that the frequency of breaks appears to increase linearly with the dose while the frequency of dicentrics appears to increase up to 1 Gy and linear-quadratically at higher doses (Vyas *et al*. 1991, Greinert *et al*. 1995). Dose-response curve constructed using PCC induced by calyculin A for simple type exchanges were linear-quadratic, which is consistent with the previous reports (Stronati *et al*. 2001).

In conclusion, PCC induced by calyculin A in cells blocked in the first mitosis by prolonged colcemid treatment is a simple powerful method for interphase-metaphase chromosome analysis. Linear dose-response curves for chromosome aberrations shows that, this method is suitable for biodosimetry. PCC index is much higher than mitotic index. It provides a high number of spreads for analysis. In Giemsa stained cells, chromosomes in different phases of the cell cycle, breaks, fragments and rings are easily observed. Analysis of G1 chromosome aberrations is impossible. Scoring dicentrics by c-banding and reciprocal exchanges (dicentrics and translocations) in fluorescence *in situ* hybridization (FISH) is easier and more reliable.

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