Construction of Calibration Curve for Premature Chromosome Condensation Assay for Dose Assessment

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ABSTRACT

Cytogenetic dosimetry plays an important role in the triage and medical management of affected people in radiological incidents/accidents. Cytogenetic biodosimetry uses different methods to estimate the absorbed dose in the exposed individuals, and each approach has its advantages and disadvantages. Premature chromosome condensation (PCC) assay presents several advantages that hopefully fulfill the gaps identified in the other cytogenetic methods. To introduce this technique into the panel of other cytogenetic methods, a calibration curve for PCC after γ -irradiation was generated for our laboratory.

Key words: γ -irradiation, biological dosimetry, calibration curve, premature chromosome condensation

Introduction

Cytogenetic dosimetry plays an important role in the triage and medical management of radiological casualties. Cytogenetic biodosimetry uses different end-points (unstable and stable aberrations, micronuclei, premature chromosome condensation [PCC] aberrations) to estimate the absorbed dose in the exposed individuals and each method has its advantages and disadvantages.^[1] Dicentric (unstable aberrations) and translocation (stable aberrations) analysis are based on the investigation of chromosome damages in phytohaemagglutinin (PHA) - stimulated lymphocytes after ~48 h of culturing. This led to the estimation of a wide range of doses as well as the influence of dose rate of high and low linear energy transfer (LET) radiation. Dicentric analysis is considered for many years to be the “gold standard” assay for biodosimetry and could be used to detect dicentrics immediately and few weeks after irradiation. Fluorescence *in situ* hybridization-based translocation assay can be used for biodosimetry even decades after exposure. However, the analysis of chromosomal aberrations (i.e., dicentric) in metaphases has several disadvantages, which can cause dose underestimation

especially in cases of high-dose irradiation (>4 Gy) because of mitotic cell death and mitotic cell cycle delay that are operating at this dose range. Moreover, the results of these assays could be received no earlier than 48 h after setting up samples for investigation. Another cytogenetic assay, i.e., micronucleus analysis requires 72 h of cell culturing. However, PCC assay can be performed immediately after irradiation and data can be generated within 3–4 h after setting up the experiment as compared to the other existing assays. Therefore, PCC assay appears to be the most suitable assay for estimating doses of exposure soon after blood sampling. Using PCC assay, it may be possible to accurately detect the effect of low doses (<1 Gy) as well as high doses (>4 Gy) following acute exposure to low and high LET ionizing radiation.^[2] Furthermore, it was proven that PCC has also the potential to discriminate accurately between total and partial body exposures.^[3] All these circumstances led us to consider this technology as a very useful assay for triage people in mass casualty radiation accidents. Therefore, the aim of the investigation is introducing of the technology of PCC - fusion methods into the panel of cytogenetical methods for biodosimetry of radiation exposure in our laboratory.

Materials and Methods

PCC technique is based on fusion G_0 cells with mitotic cells. Mitotic promoting factors from mitotic cells induce chromatin

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condensation of G_0 cells. For biological dosimetry, G_0 human lymphocytes and Chinese hamster ovary (CHO) cells as mitotic cells are used. Polyethylene glycol (PEG) is a reagent that promotes the fusion reaction; therefore, the reaction is performed in the presence PEG. After cell fusion, chromosomes in hybrid cells present 46 single structures (fragments) that could be easily identified under the microscope.

Collection of mitotic Chinese hamster ovary cells

The CHO cells, line K1 (obtained from cells culture depository “BioloT,” Saint-Petersburg, Russia) was used as mitotic cells for PCC assay. CHO cells were cultured in growth medium consisting of RPMI-1640 (BioloT) supplemented with 10% fetal calf serum (BioloT) and 1% gentamicin (BioloT). Mitotic cells were harvested according to shake-off procedure^[4] and stored at -70°C for later use.

Blood sample collection and lymphocytes isolation

Blood samples were collected from 13 apparently healthy donors (8 males and 3 females) by venipuncture in heparinized tubes. Lymphocytes were isolated from whole peripheral blood using ficoll-hypaque (Sigma) according to the protocol of manufacturer. Isolated cells were used for PCC experiments and for establishing the calibration curve.

Samples irradiation

Isolated lymphocytes were transferred into 25 ml tissue culture flasks, supplemented with 6 ml RPMI-1640 medium with 20% fetal calf serum. Samples were irradiated using a ^{60}Co source at room temperature at doses of 0.25, 0.5, 1, 2, 3, 4, 5, and 6 Gy at dose rate of 1.2 Gy/min. Lymphocytes were used for PCC analysis 24 h after irradiation.

Induction of premature chromosome condensation

Induction of PCC was performed according to the International Atomic Energy Agency (IAEA) technical recommendation concerning PCC technology.^[1,4] Hybrid cells were observed under a bright light microscope at $\times 1000$ magnification [Figure 1]. Chromosome fragments were counted. PCC index was determined as a percentage of interphase cells showing prematurely condense chromosomes.

Statistics

Dose-effect relationship was fitted to a linear model using Dose estimation software.^[5]

Ethics

Ethics Committee permission for investigation was received. Informed consent from research participants was obtained.

Results and Discussion

Ideally, advanced biodosimetry laboratory should have a panel of different cytogenetic methods to perform biological dosimetry for different situations: For risk estimation for occupationally or accidental exposed individuals or for general public. Introduction of PCC assay will strengthen the panel of cytogenetic methods and expand the capability of the laboratory in the field of biodosimetry. It is recommended that each laboratory is required to establish its own reference curve. Keeping these points in view,

we set out to implement PCC technique in our laboratory and to develop a calibration curve for PCC induced chromosome alterations.

Premature chromosome condensation index estimation

The IAEA technical recommendation concerning PCC technology was followed for the experiments.^[1] Preliminary results demonstrated that fusion yields between lymphocytes and mitotic CHO cells are generally low so it was difficult to obtain a sufficient number of hybrid cells in some cases. According to the literature, PCC index could be increased by changing interphase-mitotic cells ratio.^[5] It was suggested that 1:5 ratio (CHO cells – lymphocytes) is optimal to receive the highest level of PCC. Our experiments revealed PCC index of 3.6% for this ratio. Other ratio (1:4 and 1:3) demonstrated higher frequency of hybrid cells [Table 1]. The highest level of PCC metaphases was induced at 1:3 ratio (1×10^6 CHO cells and 3×10^6 lymphocytes) and this ratio was used for the next experiments.

Number of cells examined

The next important question is the number of PCC-cells that should be analyzed to receive statistically relevant results. Chambrette *et al.*^[2] conducted experimental and statistical analyses to determine the required number of observed cells to obtain an acceptable precision for a biological dosimetry assay. To investigate this precision, the excess PCC fragment yield has been established by examining 10, 25, 50, 100, 150, 200, 250 hybrid cells for radiation doses up to 4 Gy. It was shown that confidence limits decrease as expected when more cells are analyzed, but tend to reach a plateau when the number of cells exceeds 150. While it was suggested that 30–50 lymphocytes may be scored for each experimental point,^[5] Blakely *et al.*^[3] scored 41–81 PCC cells were counted and Lamadrid Boada *et al.*^[6] analyzed about fifty cells

Figure 1: Giemsa stained premature chromosome condensation s in human lymphocytes: (a) Demonstrating 46 single chromatid chromosomes in a nonirradiated lymphocyte; (b) 50 premature chromosome condensation fragments can be visualized in an irradiated lymphocyte

Table 1: The results of premature chromosome condensation index estimation

Lymphocytes: CHO ratio	PCC index (%)
1:5	3.6
1:4	4.0
1:3	9.7-10.3

PCC: Premature chromosome condensation, CHO: Chinese hamster ovary

per point Karachristou *et al.*^[7] performed an experiment for the assessment of the minimum number of cells required to detect and discriminate between exposed and unexposed individuals at different dose levels for cases of mass casualties and with respect to the speed of analysis blood samples were irradiated at four different doses i.e. 1.0, 2.0, 3.5, 7.0 Gy and PCC fragments were scored in 10, 20, and 30 cells. Results demonstrated that the analysis of only ten PCC spreads gives satisfactory dose estimates and comparable results to those obtained from the analysis of thirty spreads, even for the exposure of 1 Gy.^[7] Therefore, based on the published data, fifty cells per person and per point were counted in our investigation.

Postirradiation time

Earlier reports demonstrated that variability could influence the results of PCC assays and this should be taken into account while planning the experiments. It was shown that the initial yield of PCC fragments soon after irradiation was high, but drop quickly even at 3 h postirradiation and reached plateau at 6–8 h and stayed stable up to 48 h.^[7,8] The rejoining kinetics of excess PCC fragments were highlighted in earlier publications^[4,9,10] and suggested that PCC assay is sensitive to the delay time between exposure and analysis. Therefore, experimental investigation should be performed in more realistic condition to the accident situation taking into account the kinetics of PCC fragments. Therefore, 24 h repair period was used for the generation of calibration curve in the present study.

Establishment of background frequency of chromosome aberrations for premature chromosome condensation

We used blood from 11 control donors in our study with 8 males and 3 females. Seven samples had more than 46 PCC fragments in some cells; therefore, the mean frequency of PCC fragments was estimated as 46.04 ± 0.001 .

Validation of the technology

To determine the technology at the practice experiment with donor blood irradiation was performed, samples of blood were irradiated by X-ray in a dose of 1 Gy. Two analyses - PCC fragments and unstable chromosome aberrations were set up simultaneously. The frequency of dicentric and PCC fragments were estimated. The dicentric frequency let to reconstruct the dose of irradiation that was in a good agreement with initial dose of irradiation and number of PCC fragments (50.6 ± 0.02) correspond to the published data for dose used in our experimental condition. Therefore, our results suggest that the PCC technique established in our laboratory works properly and it allowed performing the next step of our project calibration curves for γ -irradiation generation and the validation of the PCC assay.

Generation of calibration curve

Peripheral blood from two healthy individuals was used for the development of calibration curve. Isolated lymphocytes were irradiated at different doses ranging from 0.25, 0.5, 1, 2, 3, 4, 5, and 6 Gy. Yield of PCC fragments was analyzed using Dose estimation program. Frequency PCC fragments in lymphocytes exposed are presented in Table 2. The dose-response relationship was better adjusted to a linear model and corresponded to the

equitation $Y = 0.249 (\pm 0.089) + 1.930 (\pm 0.084) D$, where Y is the number of PCC fragments per cell, D is the dose of irradiation (Gy) [Figure 2]. The linear dose-effect relationship obtained in this investigation is in agreement with the results of other investigations.^[2,11,12]

Therefore, the calibration curve generated using PCC fragments in our laboratory allows us to perform dose estimation in cases of gamma-ray high doses exposure. In the recent years, PCC method has attracted much attention in laboratories because of its potential use in biological dosimetry. Several modifications of this method such as analysis of PCC-ring fragments,^[8,9] analysis PCC-dicentric chromosomes using peptide nucleic acid centromere and telomere probes^[10,11] were recently introduced into practice, and such improvements have increased the capability of this technique in estimating the dose more precisely. Therefore, the introduction of PCC fusion assay as a platform for other PCC-based techniques is the first step toward further strengthening of the biodosimetry laboratories worldwide.

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Figure 2: Dose response relationship for the yield of premature chromosome condensation fragments after γ -irradiation (weighted $\chi^2 = 28.63$, degrees of freedom = 6, P value for goodness of fit = 0.66, P values for coefficients (Z-test): $P_A = 0.03$, $P_{\alpha} = 0.0000$, Correlation coefficient [r] = 0.99)

Table 2: Frequency and distribution analysis of premature chromosome condensation fragments after γ -irradiation

Dose (Gy)	Number of cells	Number of PCC fragments	Yield	SE yield
0.25	100	79	0.79	0.089
0.5	100	117	1.17	0.108
1	100	223	2.23	0.149
2	100	338	3.38	0.184
3	100	619	6.19	0.249
4	100	888	8.88	0.298
5	100	1014	10.10	0.318
6	100	1118	11.20	0.334

PCC: Premature chromosome condensation, SE: Secondary electron

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Conflicts of interest

There are no conflicts of interest.

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