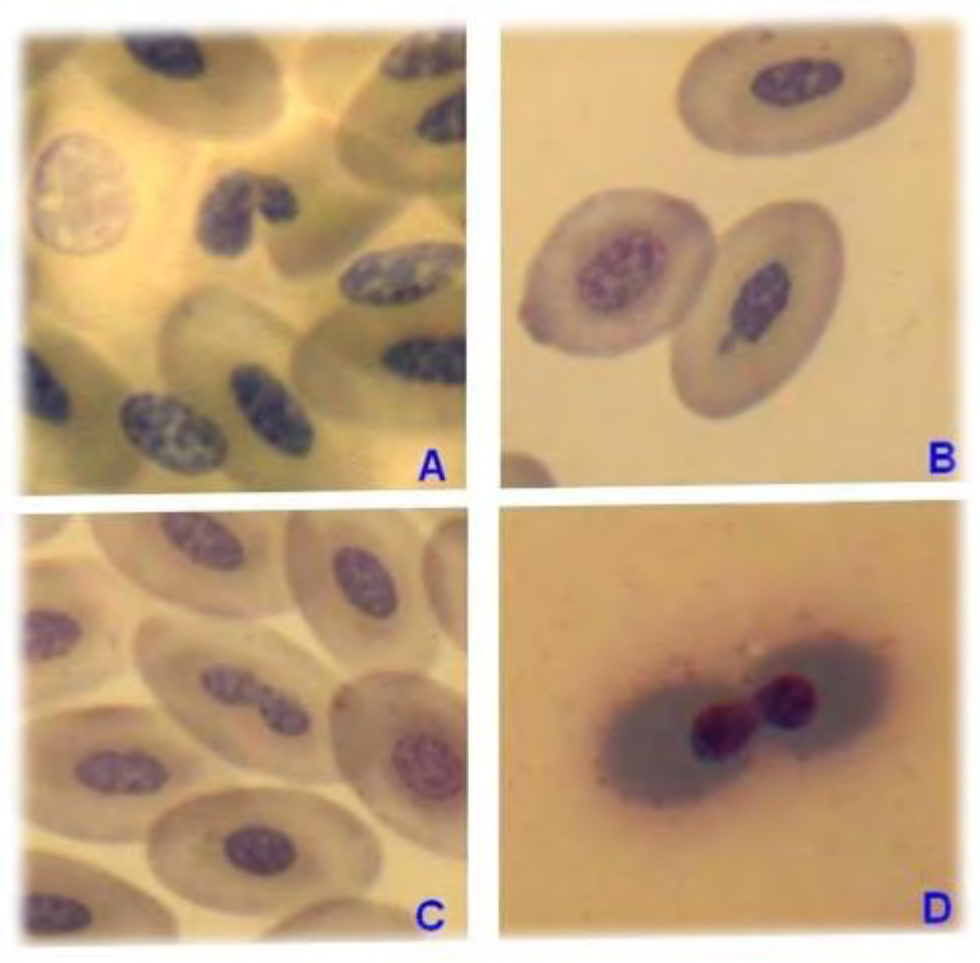

ARTÍCULO ORIGINAL

CytoVision vs conventional scoring, an innovation for the dicentric assay

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ARTÍCULO ORIGINAL

CYTOVISION VERSUS CONVENTIONAL SCORING: AN INNOVATION FOR THE DICENTRIC ASSAY

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RESUMEN

El ensayo dicéntrico es mundialmente aceptado para determinar el valor de dosis de exposición a radiación ionizante en cualquier individuo. El objetivo de este trabajo es presentar una comparación entre la evaluación dicéntrica convencional y la evaluación semiautomática usando un analizador Cyto-Vision. Materiales y Métodos: Los linfocitos humanos se irradiaron a cuatro dosis con radiación gamma, se hicieron cultivos y se analizaron las células en metafase. Las aberraciones cromosómicas se analizaron en el microscopio óptico y en el sistema semiautomatizado de un CytoVision. Las dosis evaluadas de acuerdo con el estadístico Z-Score fueron clasificadas como satisfactorias, y las frecuencias dicéntricas obtenidas con los sistemas convencionales y semiautomatizados se compararon con el programa R. Resultados: Las dosis físicas y estimadas derivadas de nuestra curva de respuesta de dosis dicéntrica concordaron bien; la discrepancia más alta fue de 15%. Conclusión: El estudio demostró que tanto la evaluación convencional como el análisis semiautomático para dicéntricos usando un analizador CytoVision produjeron resultados similares; pero el análisis semiautomático reduce significativamente el tiempo de evaluación.

PALABRAS CLAVE

dosimetría biológica,

dicéntrico,

análisis citogenético,

análisis semiautomático

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ABSTRACT

The dicentric assay is the gold standard of biological dosimetry to assess ionization radiation dose from any person. The objective of this work is to present a comparison between conventional dicentric scoring and semi-automatic scoring using a CytoVision analyser. Materials and methods: Human lymphocytes were irradiated with four doses of gamma rays and cultured to metaphase; cells were scored for dicentric aberrations conventionally 'by-eye' and with a semiautomatic CytoVision analyser. Doses estimated according to Z-score were classified as satisfactory, and dicentric frequencies obtained by the conventional and semiautomated systems were compared with the R program. Results: The physical and the estimated doses derived from our own dicentric dose response curve agreed well, the largest discrepancy was 15%. Conclusion: The study established that conventional dicentric scoring and semiautomatic scoring using a CytoVision analyser provided similar results, but the semiautomatic scoring significantly reduced the scoring time.

KEYWORDS

biological dosimetry,

dicentric,

cytogenetic analysis,

semi-automatic analysis

Introduction

The dicentric assay is the gold standard of biological dosimetry and many years of experience have demonstrated its reliability and capability to assess radiation dose from any person potentially exposed to ionization radiation. This assay is considered a gold standard method because it assesses radiation dose interpolating the chromosomal aberrations yielded from a sample of peripheral blood with a dose-effect calibration curve.

This method is based on the misjoining of two broken chromosomes as a result of the interaction of a radiation particle with the DNA; this structure with two centromeres (or even more than two) is called dicentric. In addition, the dicentric has a dose-dependent response, and the slope behavior of gamma emitters such as ^{60}Co, ^{137}Cs, ^{192}Ir are different from that of alpha, beta particles or neutrons, which makes it an excellent biological dosimeter. This information could contribute to guide medical treatment of any exposed person in case of accidental overexposure to ionizing radiation. However, the major disadvantage of the dicentric assay is that it is time consuming, particularly during the scoring process.

With the ever-present potential for large scale radiation accidents, in which many individuals may be exposed to unknown doses of radiation, no single national laboratory could cope with the required rapid response. For such an event, several strategies have been developed to increase the throughput of the time-consuming analysis of the dicentric method. The mutual assistance networks at national (1,2) and regional level (3,4,5), the scoring approaches by reducing the number of cells to be analyzed, as a triage mode (6,7) and/or scoring cells in a less restrictive manner (8,9), the sharing of images electronically or using web site (10,11,12) and the software-based automated or semi-automated scoring (12,13,14) are the most widespread strategies already in place.

The value of software-based automated scoring of dicentric chromosomes for individual dose assessment has been evaluated (13). The procedure of automation with a CytoVision system or any other, is nearly the same: it includes metaphase finding, image capture at high resolution, and detection of dicentric candidates. This is all performed 'hands-off'. Automatically detected dicentric candidates are then displayed to a trained human scorer for final evaluation: either rejection or confirmation. This combination of instrument and human interaction has led to the term semi-automated analysis (9,14). The final development would be to rely on the efficiency of the instrument to identify dicentrics accurately enough so that the human evaluation were unnecessary. The experience obtained through several years indicates that fully automatic dicentric scoring is certainly feasible, and could become mature enough (15), to replace manual scoring.

The biological dosimetry laboratory at the Instituto Nacional de Investigaciones Nucleares (ININ) is the only one in Mexico, and it recently acquired a CytoVision (Leica, Wetzlar, Germany) semiautomated system for dicentrics analysis. Before using it as a routine instrument, it was necessary to define the scoring criteria, the frequency and morphology of dicentric candidates identified by the software and to establish dose response curves and estimate

test doses. It was necessary to demonstrate that using the instrument it was possible to perform dose estimation as accurately as, or better than, the dose estimates performed by a human seated at a conventional light microscope.

Here we present a comparison between conventional dicentric scoring and semiautomatic scoring using a CytoVision analyser. Samples prepared in our laboratory and obtained in an international intercomparison exercise were used.

Materials and methods

Blood sample irradiation and culture. Whole blood was ^{60}Co gamma irradiated *in vitro* free in air at room temperature at the irradiation facilities of the ININ using a Gamma Cell 220 machine. Doses of 2.7 and 0.85 Gy were delivered at a dose rate of 0.689 Gy/min (41.36 Gy/h). Blood was collected using conventional vacutainer system with litium heparin, from a healthy female donor (20 y), after obtaining informed consent, and it was processed for the standard dicentric assay (16,17,18). For gamma exposure, whole-blood was contained in 15 ml conventional conical centrifuge PS tubes. After irradiation, the cells were given a two-hour recovery period.

Whole-blood was cultured using the usual method for biological dosimetry laboratory as described by the International Atomic Energy Agency (IAEA) (16). In brief, routine phytohaemagglutinin (PHA) (Gibco, Grand Island, NY) stimulated 48h blood cultures in Minimum Essential Medium (MEM) (Gibco) including bromodeoxyuridine, antibiotics (Sigma, Germany), 10% fetal calf serum (Hyclone, S Logan UT) and colcemid (Gibco) added at 45h. Lymphocyte metaphases were harvested by the standard method (16) after a KCl (0.075 M) hypotonic shock for 7 min at 37°C and fixed three times in methanol/acetic acid. Microscope slides were stained with fluorescence plus Giemsa and first division metaphases scored for unstable chromosome aberrations; dicentrics, centric rings, and excess acentric fragments.

Irradiated blood samples distributed by the RENEB. As part of the Latin American Biological Dosimetry Net (LBDNet) contribution to an international intercomparison exercise, blood samples distributed by the Realizing the European Network in Biodosimetry (RENEB) network were available to our laboratory (19). The blood had been irradiated with ^{137}Cs gamma rays *in vitro* in a water bath at 37°C using a HWN D2000 machine from Wälischmiller Engineering Gmb H, Markdorf Germany. Doses of 2.8 and 0.84 Gy were delivered at a dose rate of 0.495 Gy/min. The blood was cultured in one of the LBDNet labs. Unstained slides were received at ININ to be scored.

Scoring criteria. First division metaphases were scored for unstable chromosome aberrations; dicentrics (Dic), centric rings (R), dicentrics plus rings (Dic + R), and excess acentric fragments (Ace). The scoring processes were nearly the same for both manual (MS) or semi-automatic dicentric scoring with a CytoVision (SADS-CV). The classical scoring process (16) recommends that dicentrics should be scored only in complete metaphases (46 centromeres) using the FPG technique to differentiate first from second division metaphases. The SADS-CV method (13) uses 10X magnification, for firstly screening the slides to detect metaphases and to place them in an image file. This is followed by a second automatic screening process that identifies and counts the number of chromosomes; candidate dicentrics are then presented to an operator to validate the dicentrics and exclude twisted chromosomes, two aligned or overlapping chromosomes, and any other false positive figures which are not dicentrics.

Semi-automatic dicentric scoring using Cyto-Vision (SADS-CV). The present paper reports, for the first time in Mexico, the use of the Cyto-Vision instrument for biological dosimetry. This automated system follows closely the SADS method outlined above and is presented here as SADS-CV. Slide screening is performed at low magnification and an image of every candidate object is stored in a file together with its location in the x, y, and z axes. The quality of every image is automatically evaluated and color coded; green for good metaphases, red for doubtful images unlikely to be metaphases, and white for undefined objects likely to be rubbish. At this point, the operator rapidly selects on the screen each object discarding the rubbish, obviously incomplete clusters of chromosomes and metaphases with poor definition such as twisting of the chromosomes or wide separation of sister chromatids. The remaining objects are transferred to a file known as 'image capture'. Next, the metaphases are enlarged on the screen and detailed analysis starts. It is possible to simply look at each image, recognize that it is more-or-less complete, and identify any aberrations present. This would be done following the 'Quickscan' protocol described by Flegal *et al*. (8). However, for the purposes of this paper and for direct comparison with classical manual scoring where all 46 centromeres have to be present, the operator counted every centromere using a software clicking tool and concurrently looked for dicentrics, rings, and acentrics.

Statistical analysis. For each dose and scoring method data set, the dicentrics distribution per cell was evaluated using the Dose Estimate Software (20). The frequency confidence interval assuming a Poisson distribution of aberrations in the cells was calculated. Since the mean of aberrations per cell exceeded 0.1, the asymptotic *U* test was used to test whether dispersions of aberrations or the frequency of undamaged cells can be described by a Poisson distribution. If the distribution of aberrations follows a Poisson statistical law, there is only a 5% likelihood that the *U* value exceeds 1.96. The counts of aberrations from the different analysis methods, SADS-CV and MS were compared by the Poisson exact test applying the R program (21). The z score is calculated by subtracting the physical dose to the estimated dose and dividing the result by the standard deviation(s). The s value is obtained dividing the maximum permissible error of 30%, the physical dose, by a factor of 3.0. Consequently, an s value of 10%, the physical dose, is applied in the calculation of the z-score. The z-score allows classification of results as satisfactory ($|z| \leq 2$), questionable ($2 < |z| < 3$), and unsatisfactory ($|z| \geq 3$).

Dose estimation. The Dose Estimate Software was used for dose calculation (20). The laboratory's linear-quadratic dose effect curve for ^{60}Co rays was used for dose estimation $Y = 0.00074 \pm 0.0009 + 0.026 \pm 0.008 \times D + 0.053 \pm 0.004 \times D2$, where Y is the frequency of dicentrics expected after an exposure to a dose (D).

Results

Table 1 shows results for the material irradiated at ININ (22) and supplied by the RENEB network. The doses simulating whole-body exposure were chosen because those below 1Gy, in a real accidental overexposure, would be unlikely to induce any early effects, whilst the high dose would produce mild to moderate prodromal effects. The number of dicentrics and rings, cells scored, and their distribution, the dispersion index (σ^2/Y) and U value are reported. The counts of aberrations from the different analysis methods, SADS-CV and MS showed no statistical differences according to the Poisson exact test applying the R program. The dose estimated using both methods were satisfactorily according to the Z score. The largest difference between estimated and physical dose was 15%.

Discussion

Dose estimations. The mission of the biological dosimetry service in case of radiation emergency is to provide as fast as possible and with the minor uncertainties the dose received by the irradiated persons. An arbitrary acceptance criterion for dose estimation has been used before for comparisons and to detect inconsistent results. According to this criterion dose estimations within 20 to 30% or the physical dose have been considered as satisfactory (23,24). In the present work, the estimated dose fell within this acceptance criterion, the largest discrepancy being 15%. The LBDNet (25) used another statistical approach to examining results from intercomparison exercises recommended by the International Organization for Standarization (ISO 5725-5:1998 and ISO 13528:2005) (17,18). Using this method, all the results obtained by MS or SADS-CV classify as satisfactory. Thus, there is no difference in dose estimation using these two methods of data acquisition (MS or SADS-CV). In addition, samples prepared elsewhere and analyzed by SADS-CV can be as reliable as those prepared at the same lab. The dose estimation using the combination of slides provided by another lab plus SADS-CV gives confidence for the laboratory to participate in collaborative networking in case of a mass casualty event.

All the results obtained provide evidence of the capability of the lab to provide accurate dose assessment in case of radiation emergency using SADS-CV.

CytoVision instrument in the routine work. Having acquired a CytoVision instrument, it was necessary to undertake commissioning work before it could be adopted into the routine work of the laboratory, in particular for dose response calibrations and dicentric analysis for biological dosimetry. Whilst other instruments such as Metafer (12,19,26) have been evaluated, no laboratory has, to date, evaluated a CytoVision system for this application.

As the SADS-CV procedure introduces several new experimental variables, necessary to investigate whether the technique produces comparable results between biological materials generated by this laboratory with material provided by another laboratory. It was also important that this verification was carried out by the same skilled laboratory staff.

Automatic detection of dicentric chromosomes started in the 1980s (27). At that time the scoring criteria were very restrictive, only complete cells with 46 centromeres were accepted for analysis, mainly because the software developed at that time was not able to fulfil all requirements for analysis as it has been developed over years of conventional microscopy (9). In particular, the chromosome count to verify 46 (or more) objects in the metaphase was not reliable, and chromosomes, which are very close, overlapping or twisted, would be frequently misclassified as a false positive dicentric.

Deliv. Dose (Gy)	Syst.	Cells	Dic + R	Ace	Dicentric Distribution					y	σ^2/y	U	Estimated Dose (Gy)	z	Diff. (%)
					0	1	2	3	4						
Co 60 Gamma irradiation (ININ samples)															
0	SAD S-CV	1000	1	3	999	1	0	0	0	0.001± 0.001	1	0	0	0	0
	MS	1000	1	10	999	1	0	0	0	0.001± 0.001	1	0	0	0	0
0.85	SAD S-CV	1000	56/1	68	946	52	2	0	0	0.056± 0.01	0.97	0.37	0.81± 0.1	-0.47	5
	MS	2000	101/1	62	1902	95	3	0	0	0.05± 0.005	1.01	0.23	0.76± 0.1	-1.06	10
2.7	SAD S-CV	1000	437/27	345	670	243	71	12	4	0.44± 0.02	1.16	3.66	2.6± 0.1	-0.37	4
	MS	2000	863/51	394	1344	479	151	22	4	0.43± 0.02	1.13	4.04	2.6± 0.1	-0.37	4
Cs 137 Gamma irradiation (RENEB samples)															
0.84	SAD S-CV	385	29/2	40	356	29				0.075± 0.01	0.93	-1.03	0.97± 0.12	1.55	13
	MS	2000	155/15	115	1853	140	6	1	0	0.077± 0.01	1.04	1.24	0.98± 0.07	1.67	15
2.8	SAD S-CV	254	92/6	85	173	72	7	2		0.36± 0.03	0.92	-0.86	2.4± 0.14	-1.43	14
	MS	2000	858/51	380	1323	522	132	20	3	0.43± 0.02	1.06	1.93	2.6± 0.1	-0.714	7

Table 1. Dicentrics analysis using two different systems (Syst.): Semi-automatic analysis using Cyto Vision (SADS-CV) vs conventional manual scoring (MS). The comparisons of yields (y) between the two systems were performed with Poisson exact test, for all doses tested p>0.05. The σ2/y represents the variance (σ2) of dicentric (Dic) plus ring (R) counts divided by the yield (y) of the dicentric plus ring counts. The values of z score between -2 and 2 represent satisfactory results. Differences (Diff.) between delivered (Deliv.) and estimated dose below 30% represent satisfactory results. *Assuming an assigned σ of 10% of the physical dose.

This evaluation has shown that under the classic parameters of analysis, *i.e*. the presence of 46 centromeres in the image, with both samples prepared specifically for this exercise and samples from another laboratory, acceptably close agreement has been obtained. Nevertheless, the RENEB samples were much more difficult to score. The total number of cells shown in table 1 is lower compared to the material whose entire process was done in the ININ. The most probable reason for this result is the time elapsed between the preparation of the slides and the moment of reading. This material was originally prepared for another study, as mentioned before, and it was used in this study 12 months later. It is known that for the SADS analysis, it is necessary to have top quality material. Nevertheless, using Cyto-Vision, it was possible to analyze a suitable number of cells for dose estimation in these samples.

SADS-CV dicentric analysis reduce scoring time. In previous works, it has been reported that it is not necessary to count the 46 centromeres using SADS. The number of pieces can be estimated to be around 46 elements. However, it has been observed that, using this approach, partial exposure can be misclassified (15). Therefore, in this study, only metaphases with 46 centromeres were selected for both methods, SADS-CV and MS, and despite this extra step in the SADS-CV protocol, the software enabled the evaluation of the image at high magnification still to be more rapid than for MS (Fig 1). The main advantage of an SADS-CV dicentric analysis is the reduction of scoring time (22). As we recorded using MS, 500 metaphases were analyzed by one operator in 25 h; meanwhile with SADS-CV, 1000 cells were analyzed in 10 h of operator time plus ~1.5 h machine-only time (Fig. 1).

International collaboration and emergency response. This study has used blood samples obtained locally and also from a European laboratory. This is a useful indication that, as other authors have suggested, collaboration is possible between biological dosimetry laboratories (1,2,3,4,

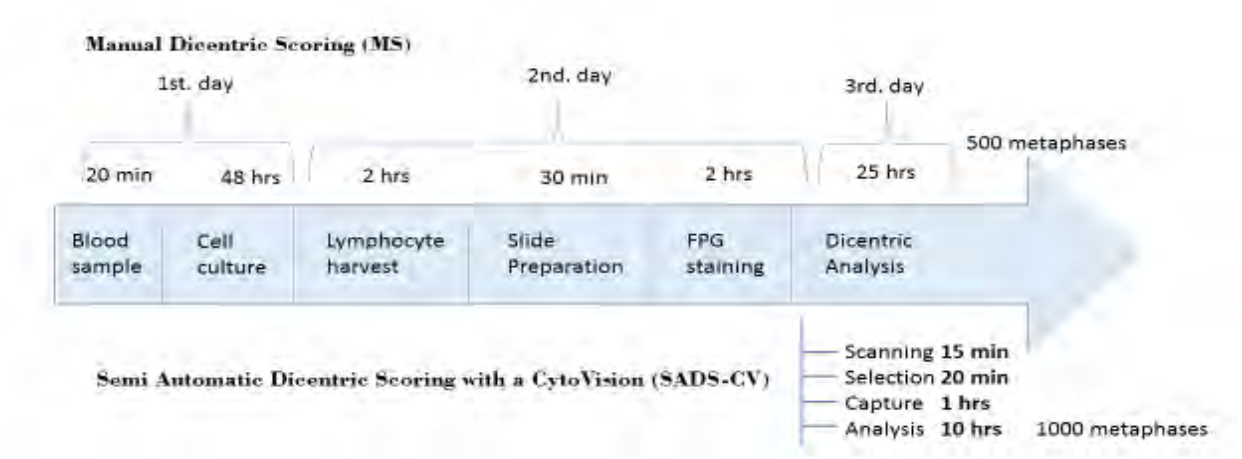

Figure 1. Timeline for dicentric analysis using manual scoring (MS) and semi-automatic dicentric scoring (SADS-CV) (22). 500 metaphase analysis can take 25 hours using MS, but whit SABS-CV 1000 metaphase will take 10 hours, which shows considerable time savings (22).

25,26,29,30). This is essential in the case of a large-scale radiation accident, in which a considerable number of individuals may have been exposed to unknown doses of radiation and need to be assessed rapidly. The material that can be exchanged could be heparinized blood samples, cultured lymphocytes in fixative, stained/unstained microscope slides or files of captured electronic images for operator verification of candidate dicentrics. All these means of international collaboration can speed up the provision of dose estimates so that they can be made available to clinicians during the critical early stages when large numbers of patients need to be managed.

Conclusion

Using an optical microscope (MS) for metaphase assessment gives much more clearer dicentric images than a SADS-CV. The microscope allows adjusting the optics of the metaphase, while SADS-CV is a still image, without option to be adjusted. The results with any of the systems are nearly the same, there is not difference on establishing the dose value from a blood sample irradiated. However, SADS-CV reduces the analysis time of dicentrics, which is the main advantage of this method. Using SADS-CV, 1000 metaphases can be ready after 10 h of operator time; while with MS, it takes 50 h for the same number of cells. The ININ biological dosimetry laboratory is now ready to incorporate the SADS-CV scoring to support national or international emergency response.

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