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Review

Automatic Dicentric Scoring a Real Option to Be Used in Biological Dosimetry

Aurélie Vaurijoux[1]*, Gaetan Gruel[1], Eric Gregoire[1], Sandrine Roch-Lefevre[1], Pascale Voisin[1], Cécile Martin[1], Philippe Voisin[1], Laurence Roy[1], and Joan-Francesc Barquinero[1, 2]

[1]Institut de Radioprotection et de Sureté Nucléaire (IRSN) Laboratoire de Dosimétrie Biologique (PRP-HOM/SRBE/LDB), BP 17, 92262 Fontenay aux roses cedex, France.
[2]Universtitat Autònoma de Barcelona, Facultat de Biociències, 08193 Cerdanyola del Vallès, Spain.

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In case of a radiological emergency it is essential to assess the possible dose received by possible victims. Several disciplines, such as physical dosimetry, dose reconstruction and biological dosimetry should put together their expertise to respond as fast as possible. Among different methods used in biological dosimetry, "dicentric analysis" is still the most widely used method, as it has the lowest detection limit, is the one that most accurately estimates the dose, and distinguishes between whole- and partial-body irradiations. To score dicentric chromosomes, peripheral blood lymphocytes have to be stimulated to enter the cell cycle and reach metaphase. In addition, skilled scorers should analyze at the microscope complete cells containing 46 centromeres and to recognize dicentrics chromosomes with their corresponding acentric fragment. For this reason, dicentric analysis is time consuming and in case of an accident involving a large number of victims, it should not be possible to respond promptly. One promising improvement of the methodology is to automate dicentric analysis, and nowadays several laboratories of biological dosimetry have the equipment needed to perform automatic dicentric scoring (ADS). Here we present a review of different experiences carried out at the "Institut de Radioprotection et Sûreté Nucléaire" from France to compare ADS in relation to manual scoring (MS) and evaluate the feasibility to introduce ADS as a real option to be used instead of MS. The experience obtained indicates that automatic dicentric scoring is a real alternative and is mature enough to substitute manual scoring.

Key words: biological dosimetry, automatic dicentric scoring, mass casualty event

1. Introduction

In case of radiological emergency involving victims, dose-assessment is a very important input to guide a possible medical treatment. First, an initial triage of potentially exposed people and second, accurate information of the received dose have to be provided as fast as possible. In cases of no physical dosimetry, or when the corresponding values are doubtful or little informative,

*Aurélie Vaurijoux: Institut de Radioprotection et de Sureté Nucléaire (IRSN) Laboratoire de Dosimétrie Biologique (PRP-HOM/SRBE/LDB), BP 17, 92262 Fontenay aux roses cedex, France.
E-mail: address: aurelie.vaurijoux@irsn.fr

the determination of the dose using a biological parameter is of great interest. There are several biological endpoints that can provide information. But in a practical situation when emergency is declared several hours or even days after exposure, or the radiological emergency is too large and involves other critical aspects that makes it difficult to obtain biological samples quickly, biomarkers that persist enough time are desirable. Such biomarkers are based on the radiation induced DNA damage, and the analysis of chromosomal aberrations is still the method of choice[1]. Nowadays, the dicentric assay using peripheral blood lymphocytes is still the best biological method for dose estimation, and among other biological endpoints the dicentric assay is still the technique the most frequently used. One of the limitations is that this technique is labor-intensive and time-consuming. For this reason, dicentric scoring may be a critical step for a mass casualty event, resulting of malicious or accidental exposure to radiation, when the capability of the local laboratory is exceeded. Notably, the recent Fukushima nuclear accident[2, 3], and the enhancing hypothesis of a nuclear crisis or a malevolent act justifies the importance of speeding up the biological dose assessment and population triage as well[4, 5].

Since many years the possibility to automate dicentric analysis has been matter of interest. Early developments were mostly focused on automate metaphase detection. These studies were not always focused on biological dosimetry but in clinical uses of cytogenetics[6]. In the late 80's and during 90's a great improvement was done in automate metaphase detection and automation was enlarged to other mutagenic tests[7 - 10]. In parallel efforts to automate chromosome aberration analysis were also done[11]. In spite of some successful results, the absence of commercial interest and the elevated cost reduced the number of laboratories able to test these improvements. More lately a renewed commercial interest has opened a new step, and nowadays commercial software is able to detect metaphase spreads and to automate dicentric scoring (ADS) is being included in many laboratories of biological dosimetry. On the occasion of a NATO meeting we presented the improvements done at the IRSN on ADS[12]. The present review is a revised version of this previous document, with the aim to give an overview of the expertise gained by the IRSN laboratory in the use of ADS during the last past years. Thus, we could conclude that ADS is mature enough, and can replace manual dicentric scoring.

2. Elaboration of dose-effect curves

To validate a biological parameter for biological dosimetry the first step is to fit a correct dose-effect curve, and to evaluate the background level. For this purpose we have established a dose–effect curve for ADS[13]. Irradiation of blood samples and culture conditions are similar to the ones used for manual dicentric scoring. In our case, to construct a dose-effect curve by ADS, blood from healthy volunteers were exposed to Caesium-137 gamma rays at doses of 0.1, 0.2, 0.3, 0.5, 0.7, 0.9, 1, 1.5, 2 and 3 Gy, with a dose-rate of 0.5 $Gy.min^{-1}$. Then whole-blood was cultured using the usual method of the biological dosimetry laboratory from IRSN. Briefly, blood was cultured during 48-50h in presence of BrdU and colcemid was added after 46h culture. Similarly to conventional manual scoring, for ADS fluorescence plus Giemsa technique (FPG) was applied to satin chromosome spreads.

A significant difference exists between the two scoring processes. For manual scoring (MS) it is recommended that dicentrics should be scored only in complete metaphases (46 centromeres) using the classical FPG technique to differentiate first from second metaphases[1, 14]. In MS, this step is the most tedious. In ADS method, a screening of the images acquired at a magnification x 63 is performed in order to images with a number of chromosomes clearly higher or lower than 46, metaphases where the two chromatids are sticked or with twisted chromosomes, and metaphases where centromeric constrictions are not visible. Metaphases in their second or further cell-division are also excluded. After this selection, dicentrics are automatically detected by DCScore software. Finally, dicentrics are validated by an operator to exclude twisted chromosomes, two aligned chromosomes, and other figures detected as dicentrics by the software.

When the results obtained by ADS are compared to the ones obtained by manual scoring, the comparison indicated that for a same irradiation dose, the dicentrics yield obtained by ADS method is systematically lower than by MS method. This leads to lower coefficient values in the linear-quadratic function, and accordingly equations obtained were significantly different: $Y_{MS} = 0.0013 (\pm 0.0010) + 0.0491 (\pm 0.010) \times D + 0.0452 (\pm 0.0077) \times D^2$ for MS, and $Y_{ADS} = 0.0008 (\pm 0.0004) + 0.0070 (\pm 0.0026) \times D + 0.0217 (\pm 0.0016) \times D^2$ for ADS. Particularly, we observed that α coefficient was seven times higher in manual scoring, while the β value was only two times higher. Interestingly, the constant coefficient is quite similar in both methods, which confirms a conservation of the spontaneous background level[15]. By MS the unity where dicentrics are recorded is a complete metaphase, and in ADS the unity is an image with chromosomes, not necessarily with 46 centromeres. One cannot expect that dicentrics are more or less present in complete metaphase, respect incomplete ones or in metaphases with chromosomes coming from another cell. For this reason the observation of lower yields of dicentrics seems to be related to the software algorithm. Probability to

miss a dicentric by the ADS method remains important because a lack of adaptability of software algorithm between slides in a same experiment. If dicentrics are too small, too dark, with two centromeres too close, or involve acrocentric chromosomes they may be not detected by the software. These different points explain the differences observed between the dose-effect curves of the two methods. An important point to consider is that initially undetected dicentrics should not be added during the validation step of the ADS. In fact, during dicentric validation process only images where the algorithm detected a dicentric are checked. So to add undetected dicentrics in these images will produce a bias that would affect the final dicentric cell distribution because images with no detected dicentrics are not checked (if they were checked there is no need of ADS). Hence this bias will affect the possibility to detect partial body irradiation on the basis of the expected Poisson distribution of dicentrics among cells. It is noteworthy to mention that after ADS, Khi-square and u-test have been performed on the dicentric distribution and compliance with Poisson law was observed in all doses except doses 0.3 Gy. Indicating the ADS will show the same suitability in detecting partial-body exposures as MS.

3. Dose heterogeneity

In case of an accident, the interpolation of the observed yield of dicentrics to a pre-established dose effect curve gives an estimation of the average of the dose absorbed by the body, so called "whole-body dose". However, in many cases the radiation is not absorbed homogeneously, and the detection of heterogeneity may be crucial to guide medical treatment. For this reason the second step is to check if this biological indicator, in our case dicentrics detected automatically, can correctly assess a heterogeneous overexposure, and, when possible, quantify the irradiated partial-body fraction. It is assumed that after low-LET irradiation the dicentric cell distribution among cells follows a Poisson distribution, with the mean equal to the variance. After a partial body irradiation the observed cells with 0 dicentrics are a mixture of non-irradiated cells (the background frequency of dicentrics is 1-2 per 1000) and irradiated cells without dicentrics. This mixture can be detected as an overdispersion of the Poisson distribution, the ratio between the variance and the mean (dispersion index) higher than 1. To test if an observed dicentric cell distribution is in agreement with the Poisson distribution the u-test is widely used in the "biological dosimetry community"[16]. This test is a normalized unit of the dispersion index, and values higher than +1.96 indicate overdispersion with 95% of confidence. Applying the zero-inflated Poisson distribution, the frequency of dicentrics in the irradiated cells, as well as the number of cells analyzed that were irradiated can be estimated, and hence it is possible to estimate a "partial body" dose[1, 17].

To test if ADS is suitable to detect partial body exposure, the same way as MS, peripheral blood from three different healthy volunteers was irradiated with 2 Gy of ^{137}Cs γ-rays at a dose rate of 0.5 Gy/min[18]. The samples were incubated for 2 hours at 37°C before mixing. To simulate partial exposures, irradiated blood was mixed with unirradiated blood before culturing, by the following ratios: 0%, 5%, 25%, 50%, 60%, 75%, 90% and 100%. Metaphases were analysed in 2 ways, MS in 500 complete metaphase cells (containing 46 centromeres), and ADS in 2000 to 6000 images of cells in metaphase. The Papworth u-test showed overdispersion ($u > 1.96$) in the blood mixtures containing from 5% to 75% of 2 Gy irradiated blood. For the overdispersed samples, a correction of the dose received by the irradiated fraction was calculated using the zero-inflated Poisson distribution. In 4 among the 6 ratios of mixed blood, the theoretical dose of 2 Gy was included in the 95% confidence interval of the re-evaluated doses. Globally, the estimated doses to the irradiated fraction using the contaminated Poisson method were always higher than the theoretical ones. Additionally, the body-fraction (F) extrapolated from the proportion of irradiated blood in the blood mixture were calculated. It is interesting to note that for all the referenced F's lower than 50%, the corresponding estimated F's were also lower than 50%. In the same way, for all the referenced F's greater than 50%, the corresponding estimated F's were also greater than 50%. However, the precision of the estimated F's including the 95% confidence intervals was better by using a reference lethal dose for lymphocytes of $D_0 = 3.5$ Gy or 3.8 Gy[19, 20] compared to the lethal dose $D_0 = 2.7$ Gy[21].

The results showed that ADS can detect heterogeneity in samples within 5% to 75% of blood irradiated with 2 Gy. Using MS, the results showed the detection of heterogeneity in samples within 12.5% to 87.5% of blood irradiated with 2 Gy[20]. It is then interesting to note that, in our experimental conditions, heterogeneous exposure can be detected for a lower threshold with automatic scoring than manual scoring. The probability of detecting an aberrant metaphase increase with the number of "metaphases" scored. Knowing that we analyze by ADS the equivalent of around twelve times more metaphases than by MS, we hypothesize that ADS could have an advantage over MS in the detection of low levels of heterogeneity. In addition, we hypothesize that the scoring of complete (or incomplete) metaphases in a factor of secondary importance in the detection of heterogeneity provided that the initial sorting of images to be analysed has been properly performed.

Overall, the evaluation of the ability of the method

to detect partial body exposures showed that ADS is a credible alternative for biological dosimetry expertise. However, the *in vitro* experiments have shown that the quantification of the irradiated fraction of the body by ADS is perfectible.

4. Triage

The third step to demonstrate that a biological indicator is relevant for biological dosimetry is to check its response capacity for a population triage in an emergency situation. For this reason an emergency situation was simulated[22], and all ongoing experiments related to research projects were stopped, to mobilize all laboratory staff on simulated emergency.

From a unique blood bag, aliquots were coded and irradiated at 0, 0.3, 0.8, 1.5, 2.4, 3.7, and 4.8 Gy of ^{137}Cs γ-rays at a dose rate of 0.5 Gy·min^{-1}. After irradiation, samples were incubated for 2 hours at 37°C. A total of 34 whole-body and 16 partial body irradiations were simulated. Partial-body irradiations were obtained by mixing irradiated (25, 33 and 50%) with non-irradiated blood. For each sample, four cultures were carried out according routine laboratory procedures. Staining based on the fluorescence plus Giemsa (FPG) technique was then carried out. The analysis was done simultaneously using several microscopes equipped with a scanning stage for 8 slides, linked to a 2-axis stepping motor. Metaphase capture and dicentric detection was as indicated above.

According to the critical situation, a biological dosimetry triage allows to complement clinical triage by categorizing potentially exposed victims in non-exposed (0-<0.5 Gy), mild (0.5-<1 Gy), mild to moderate (1-<2 Gy), moderate (2-<3.5 Gy), and severe (≥3.5 Gy). We evaluated if the doses estimated from the first slide by ADS fall between the ± 0.5 Gy interval of the real dose (taken into account that for manual scoring a basis of 20-50 cells, an error on the estimate dose of ± 0.5 Gy is accepted[5, 23]. For all samples we have also analyzed the slides by ADS reaching at least 1000 cells. In addition, to discriminate between non-exposed samples and samples exposed at doses lower than 0.5 Gy, and to identify partial body exposures[24], the automatic scoring was extended to 3000 cells. In respect to the concept of an emergency simulation, and accordingly to the method used during dose-effect curve elaboration, if the operator observed dicentrics not detected by the software, these dicentrics were not considered. In the same way, a tricentric detected as a dicentric was considered as a dicentric. Similarly to manual scoring, it was also assumed for ADS that the distinction between whole- and partial body irradiation was obtained by checking if the dicentric distribution among cells followed a Poisson distribution.

During the emergency exercise a total of 275 slides were processed for automatic dicentric scoring. All the 0 Gy samples were well classified as "None" after the analysis of the first slide corresponding to a mean of 460 cells scored. However, in all cases the 95% confidence limits of the first estimated doses did not permit to eliminate an exposure to a dose higher than 0.5 Gy. To remove the possibility of such exposure it was necessary to analyze 1500 to 2000 cells. For the dose of 0.3 Gy, all cases were classified as "None" after the analysis of the first slide (mean of 316 cells analyzed). In this case, to eliminate the possibility of a whole-body dose equal or greater than 1 Gy, it was necessary to analyze more than 800 cells. Additionally to consider them as exposed (0 Gy excluded from 95% confidence limits of estimated doses) it was necessary to analyze more than 2500 cells. All samples irradiated at 0.7 Gy were correctly classified as "Mild" from the first analyzed slide, with a mean of 524 cells analyzed. For the 1.5 Gy irradiated samples, the first analysis of about 220 cells permits to classify correctly all cases as "Mild to Moderate". However, to reduce the uncertainties in order to exclude doses higher than 2 Gy, it was necessary to analyze more than 1000 cells. With about 220 cells initially analyzed for samples irradiated at 2.4 Gy, only one case was misclassified as "Mild to Moderate" but with an estimated dose of 1.89 Gy. All cases corresponding to irradiations of 3.7 and 4.8 Gy were considered as "Severe" after the analysis of the first slide with a mean of 309 and 269 cells analysed respectively.

For simulated partial irradiations, at 0.7 and 1.5 Gy, some cases were detected as partial after the analysis of the first slide but even in this situation, the final partial dose estimations were overestimated. For 2.4 Gy samples, none of the simulated partial irradiations were detected as partial after the analysis of about 1450 cells, and it was necessary to analyze about 3000 cells to classify them correctly. For simulated partial body irradiations at 3.7 and 4.8 Gy, all simulated partial exposures were correctly detected after the analysis of the first slide, with a mean number of analyzed cells of 851.

From the previous annual crisis exercises performed at IRSN, it was determined that a manual scoring of 50 cells takes 1 hour per individual which is also the time to score 1000 cells automatically. From statistical point of view, the detection threshold for manual scoring of dicentric is 0.5 Gy for 50 cells and it is 0.2 Gy for 500 cells. The detection limit for automatic scoring is 0.3 Gy for 1000 cells and 0.2 Gy for 3000 cells.

In the emergency exercise carried out at the IRSN, using the data obtained from the first slide (with a mean of 334 cells analysed) it was possible to classify correctly 33 of the 34 whole-body simulations. An element to define the quality of triage is to consider a range of ± 0.5 Gy for doses below 2.5 Gy and a range ± 1 Gy for doses above 3 Gy. If one applies this criterion, only one of the

36 cases could be considered as wrong after automatic scoring. An important feature to take into account in a population triage is the uncertainty associated with the dose estimation. If a large uncertainty is not so critical at low doses, because few consequences in terms of radiation damage, the precision of dose estimates must be better at higher doses, and particularly around the DL50 in humans. Nevertheless, at low doses, it is also important to discriminate a positive dose for a better follow-up of victims. This confirms the advantage of using automatic detection which permits to have a better precision of dose estimation than manual scoring. Another important feature is the possibility, when the overexposure is averred, to point out the heterogeneity of exposure and to quantify the proportion of irradiated body. It is well established from previous experiments and open literature than it is difficult to detect heterogeneity especially for doses lower than 2.5 Gy based upon 50 cells manually scored [4, 25]. It was unexpected to observe similar difficulty by using automatic detection and a number of cells scored quite higher. In fact, the observation of 1000-1500 equivalent cells is mainly required to have a good chance to correctly detect dose heterogeneity by ADS, whatever the doses.

5. ADS in Dakar accident

In August 2006, an Iridium-192 source was discovered in the ejection duct of gammagraphy equipment in Abidjan. This source stem from gammagraphy equipment first located in Dakar. Indeed, a technical incident kept the source to being returned to its protected storage container and it remained several weeks in the ejection duct. The ejection duct was transported to Abidjan where the source was finally discovered. Many people were potentially in contact with the unprotected source, in both Dakar and Abidjan. Four individuals hospitalized at the military hospital of Percy (Clamart, France) were analysed at first, and later 59 individuals in contact more or less directly with source were analysed[26].

For the 4 most irradiated individuals biology dosimetry assessment was done by the manual scoring of 500 cells. For the 59 other individuals, an initial triage (phase 1) was performed by manually scoring 50 cells. Following triage results, individuals were merged into three classes of radiation exposure by comparison with laboratory reference population: class 0, no significant dose; class 1, a dose measured but without statistical significance and class 2 a significantly detected dose. After the initial triage more precise doses (phase 2) were determined by scoring between 250 metaphases for individuals from class 0 and 500 metaphases for individuals from class 1 and 2. For the 59 individuals non severely exposed, the triage results were obtained in 1 week by 6 operators, and the extension to 250 to 500 cells was performed in 3 weeks by 6 operators. For the triage a mean of 1 hour was needed to evaluate 50 cells.

The triage results allowed classifying 46 individuals in class 0, 11 in class 1, and 2 individuals in class 2. However, the classification obtained after the analysis of 250–500 cells resulted in classifying 21 individuals in class 0, 33 in class 1 and 5 individuals in class 2. The comparison indicates a 54.2% of agreement between both classifications (32 on 59 individuals). It is interesting to point out that 45.8% (27 on 59 individuals) of data obtained after manual scoring of 50 cells (phase 1), lead to underestimation of individual categorization by comparison with the phase 2.

After the conventional analysis by manual scoring and for comparisons, automatic dicentric detection was performed on 46 individuals including the 4 most irradiated individuals[13]. To avoid a sampling mistake, a slide was always fully analysed with ADS. The range of the number of analysed cells was then from 570 to 1102. By ADS the mean time of analysis is about 1 hour per 1000 cells. Using the same classification as indicated above 14 individuals were classified in class 0, 24 individuals in class 1, and 8 individuals in class 2. Comparing this classification to the one obtained after to analyse manually 500 cells an agreement of 95.7% is observed (44 on 46 individuals), and only a 4.3% (2 on 46 individuals) of the data obtained by ADS lead to an underestimation. Additionally the conversion of dicentric yields in radiation doses using automatic and manual dose-effect curve respectively, shown that each estimated dose by ADS method is included in the 95% confidence interval of the reference dose obtained by manually scoring of 250 to 500 cells.

The Dakar accident has been the first occasion for testing in real situation the efficiency of ADS method for population triage. Officially, the population triage was performed by using conventional cytogenetics by manual scoring. Practically, we have compared the results obtained on the 46 individuals exposed in the Dakar/Abidjan accident by the two methods. The results indicated that in a real accident, the analysis of 500-1000 cells by ADS gives a more precise classification of the victims within a similar time of analysis than manual scoring.

6. Conclusion

This overview clearly highlights the interest of using ADS for biological dosimetry by conventional cytogenetics, both for individual expertise and population triage. A drawback of the conventional dicentric assay is that it is a tedious and time consuming method, particularly during the microscope analysis. The ADS

significantly reduces the scoring time and the observation fatigue. However, ADS method does not reduce so far the requirement of a skilled operator, even if this experience is not employed at the same level. For general purposes, ADS results are quite similar (and sometimes better) to manual scoring results, in the dose range useful for human radiation protection and overexposure detection. The recommended experimental techniques for producing metaphases are not different in both methods, but more cells are required for ADS and the spreading quality is more critical. The more serious limitation in a systematic use of ADS in place of manual scoring is its capability to discriminate heterogeneous exposure. However, this point can be improved by increasing the number of cells analyzed.

Although in this revision only studies done at the IRSN has been described in detail, it is noteworthy to mention that nowadays biological dosimetry laboratories are harmonizing their protocols, in order to collaborate in case of a large emergency overflowing national. Among different strategies, ADS appears as an early and robust method to implement[27, 28].

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