Performance Of The Dicentric Assay In A Recent NATO Exercise Of Established And Emerging Biodosimetry Methods

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Abstract

Accidents involving human exposure to radiation can cause severe health effects which may require extensive medical resources. Particularly in mass-casualty events, the rapid identification and classification of potentially overexposed individuals into medical treatment groups is of prime importance. For this purpose, clinical signs and symptoms and biological dosimetry methods are the two main approaches for assessing radiation exposure in situations where no dosimetry badge was worn.

The dicentric chromosome assay (DCA) is considered the "gold standard" method for biological dosimetry after an acute radiation exposure. However, several novel techniques are emerging which may be faster and have a higher throughput than the DCA and could thus become valuable dosimetric tools in the future.

This comprehensive study was organized under the umbrella of the NATO Research Task Group RTG-033 "Radiation Bioeffects and Countermeasures" in order to compare the performance of the two most validated techniques (DCA, cytokinesis block micronucleus assay) and of two candidate assays (γ-H2AX, gene expression) for biodosimetry. To this end, an inter-laboratory and inter-assay comparison exercise was performed. In a first step, blood samples exposed to known X-ray doses were provided for establishing calibration curves at each laboratory and for each assay. In a second step, ten coded blood samples irradiated with different X-ray doses were distributed among 15 institutions for triage-mode biodosimetry.

This manuscript focuses particularly on the inter-laboratory comparisons of the DCA. Earliest dose estimates were reported only 2.4 d after sample receipt in the respective laboratory. An almost 7-fold difference in dose estimate precision (variance 0.07-0.47) was observed among participating laboratories. In particular, the calibration curve used and the actual dose levels of coded blood samples proved to be of significance in explaining the variances. Additionally, our analysis provided further hints to unused optimization potential of the DCA.

Key words: radiation accident, medical management, biological dosimetry, dicentric, radiation dose assessment,

1. Introduction

Radiation accidents with exposure of human beings can assume huge dimensions with regard to occurring health impairments and required medical resources such as personnel, patient care management and adequate health care facilities. Individuals with little or no exposure, not facing acute health impairments, have to be distinguished from those with mild, moderate or severe exposures in order to ensure the best possible use of medical resources (Walchuk, 2007). Therefore, the evaluation of diagnostic strategies for rapid classification of victims into clinically relevant treatment groups is of prime importance.

For biological dosimetry, a number of cytogenetic and molecular dosimetry techniques with different characteristics are potentially available (IAEA 2011). Projects throughout Europe aim at the harmonization and validation of selected biodosimetry methods and their adaptation to large scale scenarios to finally establish a functional network of cooperating laboratories for biodosimetry based diagnostics enabling mutual assistance (Multibiodose 2010, RENEB 2012). The ultimate goal is to increase the currently limited capacity of biodosimetry for triage purposes, using high throughput approaches that are especially necessary in case of a mass casualty event. Strategies to achieve this goal also include the automation of long-established biodosimetry methods and development of new fast scoring protocols (Multibiodose 2010, Flegal *et al.* 2012).

The dicentric chromosome assay (DCA), a highly standardized and harmonized technique for individual dose assessment after acute whole-body or significant partial-body radiation overexposure, is still the "gold standard" biodosimetry method. The technical performance has been described in great detail in the International Atomic Energy Agency Manual (IAEA 2011), whereas ISO standards provide performance criteria for cytogenetic service laboratories conducting the DCA in its routine or triage mode, ensuring reproducibility and accuracy of the assay (ISO 19238:2004, ISO 21243:2008). At present, much effort is made to establish the software-based automation of dicentric aberration scoring for individual dose assessment in triage situations (Multibiodose 2010, Vaurijoux *et al* 2011) and to explore new avenues for laboratory networking such as web-based telescoring (Multibiodose 2010, Livingston *et al* 2011).

This NATO exercise was organized in order to compare the established cytogenetic biodosimetry tools, DCA and cytokinesis block micronucleus assay, to the novel emerging methods (γ-H2AX , gene expression analysis) with regard to the reliability of dose estimates and the time needed to provide them. All participants were requested to perform the assays as they have been established in the individual laboratory without explicit arrangements concerning methodological details. This manuscript focuses on the ring-trial among six institutions to validate the DCA with regard to triage dose assessment.

2. Material and Method

2.1. Blood sampling, radiation exposure and distribution to participants

At first, blood samples exposed to known radiation doses were provided to participants for optional production of X-ray calibration data using the same irradiation conditions as for the blind samples.

Human peripheral blood was drawn from one healthy volunteer (male, 29 years) and aliquots of 2-3 ml whole blood filled into heparinized vials using a vacutainer system (Becton Dickinson, Germany). Blood was taken with informed consent and the approval of a local ethics committee.

Samples were irradiated immediately at approximately 37°C using single doses of X-rays with a mean photon energy of 100 keV (240 kV accelerating potential, maximum photon energy: 240 keV; X-ray tube type MB 350/1 in Isovolt 320/10 protection box; Agfa NDT Pantak Seifert GmbH & Co.KG, Ahrensburg Germany) filtered with 7.0 mm Beryllium and a 2.0 mm Aluminium layer. The absorbed dose was measured using a duplex dosimeter (PTW, Freiburg, Germany). The dose-rate was approximately 1 Gy min^{-1} at 13 mA. Applied doses for calibration curve production ranged from 0.25 to 5 Gy, whereas doses for blind samples ranged from 0.1 to 6.4 Gy.

After irradiation, samples were incubated for 2 h at 37°C before shipping samples at room temperature according to United Nation Regulation 650. Temperature profile and potential radiation exposures were monitored by adding temperature loggers (TL30, 3M, Neuss, Germany) and film badges (Helmholtz Zentrum Munich, Germany) to the packages.

2.2. Collection of data and requested information

Two data sheets were provided (I) to report the triage dose estimates ("quick" estimates) of blind samples including number of contributing "scorers" and "checkers" of observed aberrations and (II) to provide the "complete data" with regard to (a) collected interim results (dicentric frequencies and dose estimates after evaluating 20, 30, 40 and 50 cells), (b) calibration data (optional), and (c) details concerning the technical performance of the DCA. Additionally, the time it took between the arrival of the samples at the participating laboratory (FedEx report) and the return of the dose estimates of blind samples to the organizer via email (arrival time stamp) was documented.

Further information about each laboratory was collected as follows using a questionnaire: (a) number of exercises the Institution had participated in prior to the NATO exercise, (b) own judgment on the specialization status of the DCA, (c) time since the group established the method, (d) time since the group started using the DCA for biodosimetry, (e) level of priority that was given to the examination of the NATO samples during daily business.

2.3. Statistical methods

In a preliminary approach the variance of estimated doses relative to the actual doses was calculated per exposure dose or per institution simply by calculating the squared difference of dose estimates to the actual dose and summing it up for all 10 samples when calculating variance per institution. Each set of data was also analyzed by using a linear model in which for estimated dose D_{ei} and true dose D_{ti} applies, $D_{ei} = D_{ti} + \sum_{j=1}^{N} \alpha_j X_{ji} + \varepsilon_i$, as well as for some other variables X_{ji} and estimated parameters α_j. We fitted the model by ordinary least squares. The ε_i are assumed to be identical independently distributed $N(0,\sigma^2)$ random variables. Significant contributions of various additional variables were analyzed by adding them to the model and examining improvements on the fit (F-statistic). Residuals of the fits were examined for heteroscedasticity using a Breusch-Pagan test.

3. Results

3.1. Shipping of samples and return of data

Temperature loggers showed a mean temperature of 20°C and a range of 18-24°C for the duration of transport. None of the included film badges indicated undesired radiation exposure to the samples during transport. The six participants provided "quick" dose estimates within 2.4-6.1 days after sample receipt and remaining data were supplied shortly afterwards. Methodological information on DCA performance with regard to blind sample processing is summarized in table 1, whereas table 2 gives details of the calibration curves used to assess radiation doses of blind samples. Results of the questionnaire and times needed to provide dose estimates by participants (referred to as institutions A-F) are shown in table 3.

institution	set of dose estimates[1]	culture medium	culture time	colcemid incubation	fixation procedure	staining	automated metaphase finding system	scoring of blind samples
A	1	RPMI / 20% FCS	48 h	3 h	automated	FpG	yes	manual
B	1	RPMI / 10 % FCS	48 h	curve: 3 h blind: 24 h	manual	curve: FpG blind: Giemsa	yes	manual
	2	RPMI / 10 % FCS	48 h	24 h	manual	Giemsa	yes	automated
C	1	RPMI / 10% FCS	48 h	3 h	manual	Giemsa	no	manual
	2	RPMI / 10% FCS	48 h	3 h	manual	Giemsa	yes	automated
D	1	MEM / 10 % FCS	48 h	3 h	manual	Giemsa (FPG check: following day)	automated and manual	manual
E	1	RPMI / 10% FCS	48 h	24 h	manual	Giemsa	yes	manual
	2	RPMI / 10% FCS	48 h	24 h	manual	Giemsa	yes	automated
F	1	RPMI/ 15% FCS (no BrdU)	52 h[2]	4 h	manual	Giemsa	yes	manual

Table 1: Summary of DCA performance characteristics (blind samples). [1]First set of dose estimates: „quick" estimates for documentation of time needed, second set: additional one performed optionally. [2]Addition of Cytochalasin B after 24 h culture time (CytB method, IAEA 2011).

institution	set of dose estimates	standard calibration curve			
		c ± SE	α ± SE	β ± SE	origin, radiation quality*, scoring mode
A	1	0.0007 (±0.0002)	0,0432 (±0,0059)	0,0630 (±0,0039)	own, 240 kVp X-ray, manual
B	1	0.0000 (±0.0000)	0.0301 (±0.0068)	0.0480 (±0.0036)	own, Co^{60} γ-ray, manual
	2	0.0019 (±0.0010)	0.0306 (± 0.0056)	0.0206 (±0.0028)	own, based on NATO samples, 240 kVp X-ray, automated
C	1	0.0000 (±0.0000)	0.0185 (±0.0060)	0.0550 (±0.0031)	own, 200 kVp X-ray, manual
	2	0.0008 (±0.0004)	0.0221 (±0.0041)	0.0217 (±0.0022)	own, based on NATO samples, 240 kVp X-ray, automated
D	1	0.0005 (±0.0005)	0.046 (±0.005)	0.065 (±0.003)	own, 250 kVp X-ray, manual
E	1	0.0004 (±0.0023)	0.0374 (±0.0083)	0.0549 (±0.0034)	from literature, Co^{60} γ-ray, manual (Voisin *et al.* 2000)
	2**	0.0000	0.0150	0.0272	own, based on NATO samples, 240 kVp X-ray, automated
F	1	0.0093 (±0.0018)	0.0377 (±0.0097)	0.0682 (±0.0045)	own, 200 kVp X-ray, manual

Table 2: Summary of characteristics and values of coefficients (α, β, c; with standard errors, SE) for all used standard calibration curves. *kVp: kV potential. ** SE not determined

institution	method established since… (months)	method established for biodosimetry purposes since… (months)	# previous exercises	laboratory specialized in biodosimetry	NATO samples processed with	personnel involved in scoring	time (d) required to report "quick" dose estimates
A	60	60	2	yes	priority	1	5.3
B	360	360	5	yes	priority	4	4
C	18	36	0	yes	priority	1	4
D	480	480	6	yes	priority	3	2.4
E	30	30	0	yes	priority	4	2.6
F	120	96	9	yes	priority	2	6.1

Table 3: Details on experience and exercise performance and time to report "quick" dose estimates.

3.2. Standard calibration curves

All participating institutions applied pre-existing standard calibration curves for triage biodosimetry of blind samples. All calibration curves were based on manual dicentric scoring. Three laboratories generated new calibration curves based on automated dicentric scoring of provided calibration samples and reported additional dose estimates also based on automated scoring for comparison purposes. Coefficients (α, β, c) of applied standard calibration curves are listed in table 2. Corresponding curves are shown in figure 1.

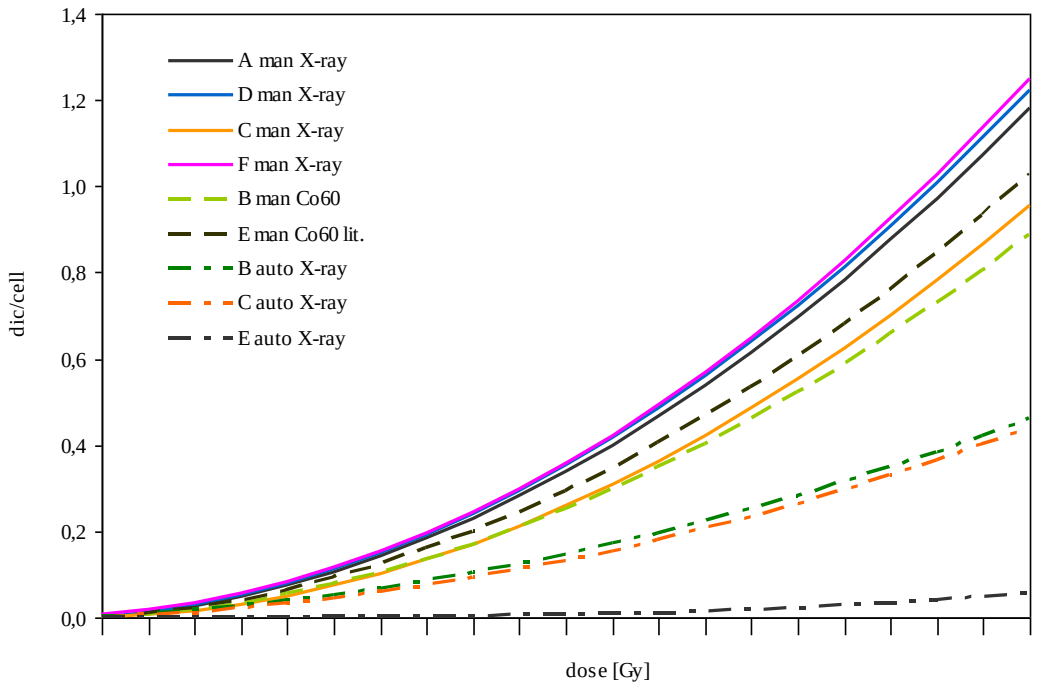

Figure 1: Comparison of dose-response calibration curves used for estimating doses by manual and automated scoring. man: manual scoring, auto: automated scoring, lit: taken from literature.

3.3. Validity of "quick" dose estimates based on scoring of 50 cells and inter-laboratory comparison

In order to compare the accuracy of dose estimates among laboratories and methods used we examined whether individual dose estimates were within the accepted uncertainty +- 0.5 Gy of the actual dose (Lloyd et al 2000). In particular samples 9 and 10 irradiated with the highest doses did show deviations of more than 0.5 Gy in ≥ 50% of the supplied dose estimates (table 4). Consistent with this effect, an increased variance (two-fold and higher) of these samples was observed. Up to 4 dose estimates not falling into the +- 0.5 Gy out of the overall 10 dose estimates were observed when estimates were based on a Co-60 calibration curve (labs B and E). Again, deviations were in particular associated with higher dose estimates (table 4).

We then examined the contribution of various additional variables to explain the variance observed. This was analyzed by adding the variables to our linear model and examining improvements on the fit. Variables significantly improving the linear regression of observed vs. actual dose were the laboratory (p-value = 0.003), the calibration curve used (p-value = 0.0005), the number of checkers (p-value = 0.02) and the time for reporting dose estimates (p-value = 0.02).

sample no.	1	2	3	4	5	6	7	8	9	10	variance (s^2)
	actual dose [Gy]										
institution_cell no_ scoring_cal curve:	**0**	**0.1**	**0.7**	**1.4**	**2**	**2.2**	**2.6**	**3**	**4.2**	**6.4**	
F_50cell_man_X *	0.0	0.6	0.9	1.6	1.9	2.3	3.0	3.3	4.2	6.4	0.07
A_50cell_man_X*	0.3	0.5	1.1	1.3	2.3	2.8	3.0	3.5	4.3	5.9	0.16
D_50cell_man_X*	0.5	0.7	1.2	0.9	2.3	2.1	2.9	2.5	4.5	5.3	0.30
B_50cell_man_Co*	0.0	0.0	0.9	1.5	1.9	2.6	3.2	3.8	5.0	7.4	0.31
C_50cell_man_X*	0.0	0.0	1.3	1.8	3.0	2.8	3.0	3.1	5.2	nd**	0.38
E_50cell_man_Co*	0.0	0.0	0.8	0.9	1.6	2.6	2.1	2.5	3.0	5.1	0.47
variance (s^2) per sample	**0.1**	**0.1**	**0.1**	**0.1**	**0.2**	**0.2**	**0.2**	**0.2**	**0.5**	**9.0**	
Additional sets of dose estimates :											
B_200cell_aut_X	0.0	0.1	0.9	1.4	1.9	2.4	3.2	2.9	4.5	6.5	
C_varcell_aut_X	0.2	0.1	0.9	1.3	2.3	2.0	2.7	2.6	4.1	5.6	
E_varcell_aut_X	0.0	0.0	0.8	1.3	2.1	2.8	2.1	2.6	3.6	4.8	

Table 4: "Quick" dose estimates based on manual scoring of 50 metaphase spreads (top, highlighted in grey) and dose estimates based on automated dicentric scoring (varcell: variable cell number). Comparison of variances between actual doses (shown in increased order) and dose estimates. Dose estimates not falling into the ± 0.5 Gy uncertainty interval are underlined.
Preliminary calculations on the variance per laboratory (last column) and per dose point were performed as the squared difference of dose estimates to the actual dose and summed up for all 10 samples/no. of samples (variance of lab-contribution across samples) or for all 9 lab-contributions/by no. of lab contributions (variance per sample across labs). *: "quick" dose estimates, **: nd: not determined based on 50 cells, but on 30 cells: 7.3 Gy.

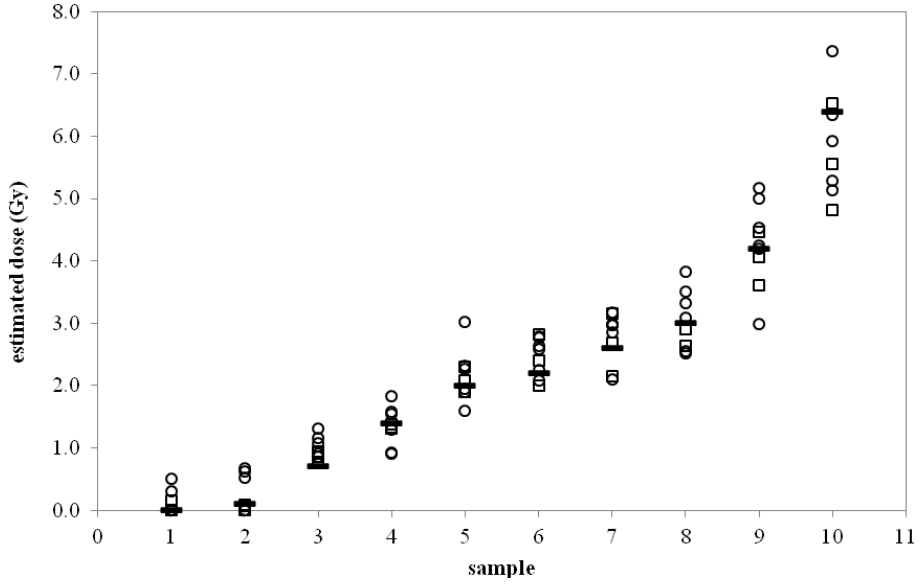

Figure 2: "Quick" dose estimates based on manual scoring of 50 metaphase spreads (○), dose estimates based on automated dicentric scoring of a variable cell number (□) and the actual doses (▬).

Discussion

The DCA is considered to represent the gold standard cytogenetic dosimetry method to be used as a reference method to validate new potential diagnostic tools.

In this manuscript we focus on the inter-laboratory comparison to validate the DCA by determination of the accuracy of radiation dose prediction taking into account a variety of variables such as experience, specialization for biodosimetry and methodological characteristics. As the DCA forms a common methodological platform for national, regional and global biodosimetry networks to enhance the response capacity in case of a large-scale radiological incident (IAEA 2011, Christie *et al.* 2010), this study also contributes to the further validation of the DCA for network biodosimetry applied in large-scale radiological incidents. To maintain such an assistance network, periodically organized ring trials between biodosimetry service laboratories are recommended to ensure the accuracy and reliability of their results (ISO 19238:2004, Wilkins *et al* 2008, Beinke *et al* 2011).

Notably, for this study no specified agreements concerning DCA performance were made in order to allow each laboratory to conduct the assay according to its established procedures. Furthermore, the time of specialization for biodosimetry as well as the practical experience of participating laboratories (prior ring trial participation) ranged from 2.5 to 40 years and from zero to 9 years, respectively.

The first step of the study was the provision of radiation exposed reference blood samples to allow the optional establishment of a calibration curve, because it is recommended that any laboratory performing the DCA has to establish its own dose-response data for dicentric induction in order to minimize uncertainties in dose assessment (Wilkins *et al* 2008). However, only three laboratories made use of the provided samples to generate calibration data, namely by employing automated dicentric scoring with the DCScore software module (Metasystems, Altlussheim, Germany) to finally compare estimated doses with the conventional manual scoring of dicentric frequencies. Automated dicentric scoring is obviously considered to be still in the development and validation stage, which explains why the "quick" dose estimates from five of the six participants were based on manual scoring (table 4).

For the "quick" dose estimates, each laboratory applied a pre-existing own calibration curve (labs A, B, C, D, and F) or a calibration curve produced elsewhere and published in the literature (lab E). Two of these six calibration curves were not generated with X-rays, but with Co^{60} gamma radiation. The other four were based on X-rays of slightly different accelerating potential (200 – 250 kVp) and filtration characteristics (with or without a half-value layer of copper) have been used. The values of the coefficients α, β, and c, and thus the slopes of the curves differ in a certain range (figure 1, table 1). These differences have been expected due to laboratory specificities of pre-established calibration data (type of radiation, number of evaluated dose points and scored cells per dose point) and DCA performance (reagents, equipment, method, scoring). Nevertheless, all calibration data on which the calibration curves of the laboratories were based on, manual as well as automated scoring, indicated a good fit to the linear-quadratic model as expected due to the typical model of dicentric induction by low LET radiation (figure 1; IAEA 2011). Additionally, figure 1 clearly shows a systematic difference between automated and manual calibration curves, reflecting the ~50% dicentric detection rate of DCScore relative to manual scoring, which is consistent with published data (Bayley et al 1991, Finnon and Lloyd 1992).

The second step in our study was to document the time duration after sample receipt until the triage dose ("quick") estimates for blind samples were delivered to the organizer and to determine and compare the accuracy of dose prediction by triage biodosimetry among the laboratories. Transportation time ranged from minutes (organizing laboratory) to not longer than 26 h. Temperature logger and dosimeters did not indicate any irregularities such as extreme temperature fluctuations or radiation exposure during transit.

Dose assessment by DCA based on scoring of 50 cells or until 30 dicentrics have been observed is an accepted triage strategy to provide a first rough estimate and to categorize potentially overexposed individuals into broad 1.0 Gy categories (Lloyd *et al* 2000). Although no agreement was made concerning the cell number to be scored and the mode of scoring (manual/automated) all "quick" estimates were based on manual scoring of 50 metaphase spreads demonstrating that, presently, the manual scoring is still favored for reliable dose assessment.

The earliest set of "quick" estimates was delivered 2.4 d after sample receipt from laboratory D assisted by three contributors for aberration scoring (table 3). This laboratory is one of the experienced ones in biodosimetry, because it has acquired experience within this specialized field over 40 years including six previous inter-laboratory comparisons. Due to the fact that the cell culture lasts already 48 h, the following metaphase fixation, staining and scoring procedures are evidently highly optimized and take as little time as possible. The second fastest results took no more than 5 h more (lab E). The last results were returned 6.1 d after sample receipt with two scorers involved, despite that the laboratory has already participated in 9 comparison studies and the DCA is established for biodosimetry for eight years. Obviously, unused potential for optimization in four of the six laboratories (labs A, B, C, and F) exists and should be utilized to speed up their procedures for dose prediction by DCA.

In a further attempt we tried to find out about parameters explaining the observed almost 7-fold differences in dose estimate precision (0.07-0.47) observed in our study. This was surprising since the DCA is considered to be the most established and validated biodosimetry method. Interestingly, associations with the laboratory, the calibration curve used and actual dose levels of blind samples proved to be of significance in explaining the variance using different parameters (variance of dose estimates, fit into ±0.5 Gy intervals accepted for triage, contribution to linear regression model). With the questionnaire distributed to the contributors (table 3) we tried to elucidate whether the association detected might be explained by e.g. experience of laboratories (previous inter-comparisons, time method has been established), but all characteristics related to that remained insignificant. Whether other aspects such as other experience (e.g. true cases in biodosimetry), personnel characteristics (e.g. motivation, accuracy) or in fact the degree of following recommended guidelines (technical manual of IAEA 2011) and the establishment of a quality assurance program within the laboratory (e.g. ISO 17025;2005, ISO 19238:2004) might help explain the variances remains speculative and difficult to quantify.

Overall, most of the "quick" dose estimates of blind samples were in good agreement with the physically applied radiation doses for all six laboratories (figure 2). Each laboratory was able to estimate the actual radiation dose for at least five up to ten blind samples within the ± 0.5 Gy interval accepted for triage (table 4). The accuracy of dose prediction was actually limited concerning the 4.2 Gy and 6.4 Gy samples. This can be explained by the fact that for low LET radiation calibration curves usually are based on data collected in the dose range up to 5.0 Gy, because beyond this dose there is evidence of saturation of the dicentric yield which will lead to a distortion of the β coefficient, and thus to a less accurate dose estimation (Lloyd *et al.* 1983). This is further reinforced by the fact that the variances of the dose estimates for the 4.2 Gy and 6.4 Gy samples were higher than those in the lower dose range (table 4).

Dose estimates of the two laboratories with Co^{60} gamma calibration curve showed variances of 0.31 and 0.47 (table 4, lab B and E). It is well established that dicentric induction for these two low LET radiations are different particularly at low doses. Additionally, it is known, that X-ray curves show a higher RBE than gamma curves, which has consequences in this inter-comparison for the accuracies of dose estimations at doses points higher than 2 Gy. This strengthens the recommendation that every laboratory intending to carry out dose estimations based on DCA should produce its own calibration data for a range of different radiation qualities that may be encountered in radiation accidents.

In order to find out about methodological differences, we restricted our exercise on the analysis of methodological variances in dose estimates (tables 1 and 3). Hence, this study cannot provide information about other sources of variance such as inter-individual variability of dicentric induction (one donor was used throughout the whole exercise).

In this manuscript a crude analysis of variance of dose estimates per institution was applied which includes (undesired) differences in detection sensitivity of samples exposed with different radiation doses. We are currently working on a more sophisticated statistical approach to address this issue. Our linear models currently do not adjust for co-linearity which will be done next. All results have to be interpreted cautiously because of the limited sample size.

With regard to the aim to classify victims of a radiation incident into clinically relevant treatment groups it has to be noted that the rapid triage dicentric assay is acknowledged to be approximate, lacking the precision of a full assay based on evaluation of many more metaphases. This is well illustrated by the ranges of values shown in table 4. Even so, none of the dose estimates would

have led to an error in placing a patient into an incorrect treatment group. This exercise has therefore fulfilled the primary purpose of rapid early assessment.

Conclusion

Although the DCA is considered to be the most established and validated diagnostic biodosimetry method, there is some potential for optimization of the procedures with regard to the time needed to provide triage dose estimates as well as to improve the precision of DCA based dosimetry. Compliance with already known important factors (e.g. choice of calibration curve) and further identification of associated laboratory conditions (e.g. methodological procedures, quality assurance) followed by implementation of optimized protocols is strongly recommended.

Acknowledgement

We are very grateful for the extremely efficient and thoughtful technical and organizational work performed by Cornelia Grothe and Sven Senf (radiation exposure, shipment) and Julia Hartmann (venipuncture). This work was supported by the German Ministry of Defense. Parts of this study were funded by the NIHR Centre for Research in Health Protection. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. The views expressed in this publication are those of the authors and not necessarily those of the funding bodies

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