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Calibration of the comet assay using ionising radiation

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ABSTRACT

Several trials have attempted to identify sources of inter-laboratory variability in comet assay results, aiming at achieving more equal responses. Ionising radiation induces a defined level of DNA single-strand breaks (per dose/base pairs) and is used as a reference when comparing comet results but relies on accurately determined radiation doses. In this ring test we studied the significance of dose calibrations and comet assay protocol differences, with the object of identifying causes of variability and how to deal with them. Eight participating laboratories, using either x-ray or gamma radiation units, measured dose rates using alanine pellet dosimeters that were subsequently sent to a specialised laboratory for analysis. We found substantial deviations between calibrated and nominal (uncalibrated) dose rates, with up to 46% difference comparing highest and lowest values. Three additional dosimetry systems were employed in some laboratories: thermoluminescence detectors and two aqueous chemical dosimeters. Fricke's and Benzoic Acid dosimetry solutions gave reliable quantitative dose estimations using local equipment. Mononuclear cells from fresh human blood or mammalian cell lines were irradiated locally with calibrated (alanine) radiation doses and analysed for DNA damage using a standardised comet assay protocol and a lab-specific protocol. The dose response of eight laboratories, calculated against calibrated radiation doses, was linear with slope variance CV= 29% with the lab-specific protocol, reduced to CV= 16% with the standard protocol. Variation between laboratories indicate post-irradiation repair differences. Intra-laboratory variation was very low judging from the dose response of 8 donors (CV=4%). Electrophoresis conditions were different in the lab-specific protocols explaining some dose response variations which were reduced by systematic corrections for electrophoresis conditions. The study shows that comet assay data obtained in different laboratories can be compared quantitatively using calibrated radiation doses and that systematic corrections for electrophoresis conditions are useful.

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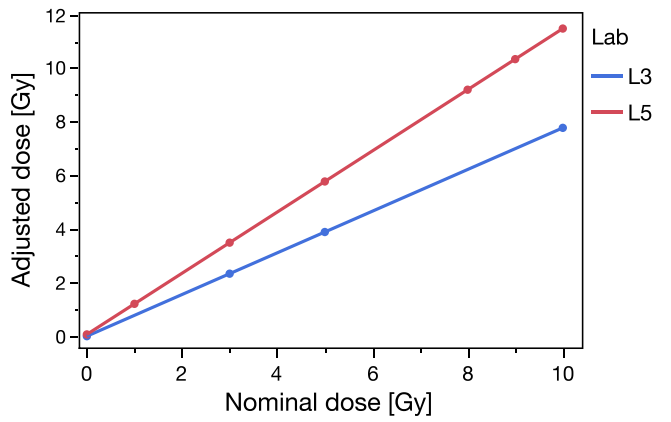

Fig. 1. APD calibration curves. Calibrated doses vs nominal doses. Curves are shown for laboratories with maximum (L5) and minimum (L3) steepness.

Table 1
APD calibration in 8 laboratories (L1-L8). Slopes and intercepts from linear regression of measurements of absorbed radiation dose vs nominal dose, based on four dose levels (0, 1, 5 and 10 Gy) in each laboratory. Regression constants and goodness-of-fit (R^2).

Lab #	Slope	Intercept	R2
L1	0.866	0.001	0.9990
L2	0.792	0.006	0.9998
L3	0.779	-0.018	0.9997
L4	1.006	-0.056	0.9999
L5	1.143	0.048	1.0000
L6	0.884	0.01	0.9998
L7	0.822	0.126	0.9979
L8	0.911	-0.009	0.9999
Mean	0.900	0.013	
STDAV	0.122		
CV (%)	13.5		

1. Introduction

The comet assay (single cell gel electrophoresis) is a sensitive and widely used method to determine the amount of DNA damage in cells. There are considerable variations between experiments and laboratories in comet assay results for cells exposed to a genotoxic treatment. This could reflect the cell type, differences in the exposure, different comet assay protocols, and different scoring procedures. Multiple efforts have been made to identify the parameters most important for the response; such knowledge is a prerequisite for a complete standardisation of a comet assay protocol. In the ECVAG project, several inter-laboratory trials were carried out [1–4]. Among other exercises, cells were grown and exposed in one laboratory, and frozen samples were distributed to partners. These samples were then analysed for DNA damage (both single-strand breaks/alkali-labile sites (=SSBs), and in later studies, also oxidised bases) using protocols that differed to various extents. Analysis of the resulting comet data, which did vary considerably, showed that the protocols could account for some of the variation. A substantial portion was, however, attributed to the laboratory itself, without identification of a clear cause [5]. The variations were reduced when data were corrected using laboratory-specific dose rate calibration curves, i.e. data obtained from analysis of distributed frozen cells previously exposed to defined doses of ionising radiation [1].

Collins and co-workers [6] recommend that comet data should always be compared with the response of irradiated cells. This makes sense since low linear energy transfer (LET) radiation induces lesions at defined rates, close to 1000 SSBs per mammalian cell per Gy (0.3 SSBs per 10^9 Da per Gy [7] or 0.2 $*10^{-6}$ $Bp^{-1}Gy^{-1}$). In this way, absolute lesion rates, rather than the relative fluorescence intensity in the comet tail (=TI%DNA), may ideally be assessed for any treatment. Standardisation of the comet assay protocol should then be less important. In the study of Collins and co-workers, the slopes of dose response curves obtained in different laboratories using X-rays and gamma radiation seemed to vary by up to a factor of three [6]. At that time, we didn't expect such differences in the response of cells exposed to ionising radiation. The observations may be reflected in the large interlaboratory variations later reported by ECVAG and others. However, differences could also be caused by errors in radiation doses and exposure conditions. Furthermore, we hypothesised that, during post-exposure processing, induced breaks may be partly removed since DNA single-strand breaks are repaired quickly, i.e., within minutes if cells are not kept cold [8].

In the light of this we initiated the present calibration trial and ring-study. Our initial emphasis was on the accuracy of the radiation dose and cell exposure conditions. Hence, the doses used in each laboratory were compared using a common calibration procedure. This calibration comprised alanine pellets distributed to eight partner laboratories. After exposure, the alanine pellets were sent to a specialist laboratory in Belgium for analysis. In addition, we explored the usefulness of three other calibration methods two of which could be performed locally. The study demonstrates substantial variations in "true" doses from the radiation units in the participating laboratories, implying that dose calibration should indeed be included to compensate for inaccurate dose estimations when ionising radiation is used as a reference. In addition, the importance of cell exposure conditions and variations in the comet assay protocol were explored, using human peripheral blood cells sampled locally plus two mammalian cell lines. The cell samples were exposed to radiation, and then assayed for DNA damage using the favourite comet assay protocol of each laboratory (=lab-specific protocol) in addition to a standard protocol. By comparing the results, we identified experimental conditions which are likely to be responsible for much of comet assay variations. In conclusion, systematic correction for electrophoresis conditions may be used for improved comparison of results obtained using different protocols.

2. Materials and methods

2.1. Study design

The eight participating laboratories (L1-L8) agreed on a detailed study design (Supplementary file #1), specifying dose calibrations, radiation exposure conditions, as well as the standard comet assay protocol. The study design describes how laboratories should calibrate their radiation unit using up to four different dosimetry systems, of which the first two are non-aqueous and suitable for in vivo measurements with humans [9]: (i) alanine pellet dosimeters (APD); (ii) thermoluminescence detectors (TLD); (iii) Fricke's ferrous sulphate dosimeters (FD), and (iv) benzoic acid dosimeters (BAD) (details in Supplementary file #1). Freshly isolated human peripheral blood mononuclear cells (PBMC), whole blood and/or cultivated mammalian cells (specified for each laboratory in Section 2.3 Chemicals and cells) were exposed to ionising radiation (nominal doses 0 – 15 Gy). Cells were exposed either in suspension or after embedding in agarose gels, to assess the importance of fast repair of DNA damage during sample processing. Cells were processed post-exposure following either a standard comet assay protocol, or a lab-specific protocol. Processed samples were scored for DNA damage by each partner and afterwards sent to L1 for fully automated scoring. Each partner provided a description of the irradiation source (Supplementary file #2), cell exposures, the lab-specific protocol, and any deviations from the standard protocol (Supplementary file #3). Dose response curves (DNA damage versus radiation dose) were constructed based on either calibrated (dose rate measured with APD) or nominal doses.

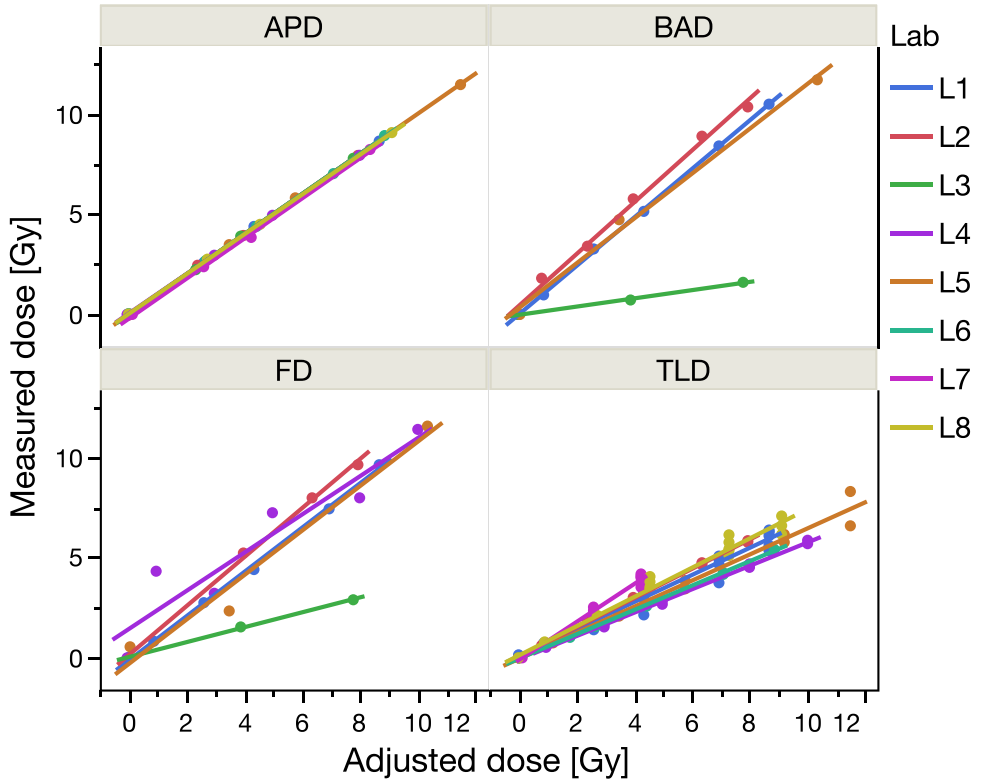

Fig. 2. Dose estimations for BA, FD and TLD dosimetry in individual laboratories, vs adjusted doses determined with APD (laboratory-specific correction factors).

Table 2
Linear regression constants for Benzoic Acid (BAD), Fricke's (FD) and thermoluminescence (TLD) dosimeters, calculated from data in Fig. 2 (one determination per exposure). All constants refer to the APD-calibrated radiation dose. Outliers (asterisks in 2nd column) are excluded from Mean slope and STDEV (of each dosimetry system) in the column to the right.

Method	Lab #	Slope	Intercept	Std Error Slope	Std Error Intercept	Mean slope and STDEV w/o outliers
BAD	L1	1.211	-0.014	0.011	0.057	1.208
BAD	L2	1.294	0.412	0.053	0.240	+ /- 0071
BAD	L3 *	0.205	-0.023	0.012	0.061	
BAD	L5	1.120	0.298	0.089	0.560	
FD	L1	1.104	-0.133	0.023	0.116	1.097
FD	L2	1.222	0.134	0.042	0.229	+ /- 0096
FD	L3 *	0.372	0.038	0.014	0.069	
FD	L4	0.952	1.433	0.173	0.993	
FD	L5	1.108	-0.308	0.203	1.281	
TLD	L1	0.676	0.073	0.021	0.107	0.704
TLD	L2	0.732	0.046	0.006	0.026	+ /- 0112
TLD	L4	0.584	-0.087	0.009	0.057	
TLD	L5	0.651	-0.058	0.029	0.189	
TLD	L6	0.609	-0.049	0.005	0.027	
TLD	L7	0.949	-0.117	0.029	0.087	
TLD	L8	0.730	0.109	0.018	0.093	

2.2. Calibration and radiation dose measurements

Details of the dosimeters and radiation sources are given in Supplementary files #1 and #2, respectively. The radiation sources were either X-rays (5 laboratories), photons from a linear accelerator (1 laboratory), or gamma-rays (2 laboratories, ^{137}Cs and ^{60}Co), using different types of filters as specified in Supplementary file #2. L1 and L8 used the same radiation unit, but dose calibrations and cell isolations/exposures were performed independently. Local nominal doses (i.e., doses and dose rates determined previously by the staff in L1-L8) were used as the basis for exposure of dosimeters and cell samples.

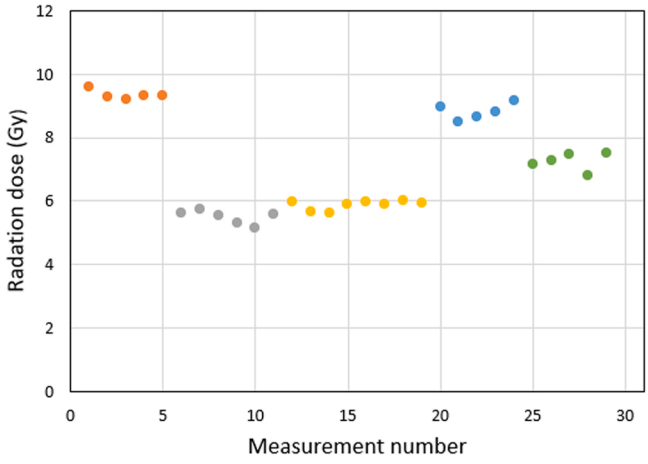

Fig. 3. Radiation field homogeneities. Ordinate axis: uncalibrated dose (Gy). Laboratories (and individual samples) from left to right (mean +/- STDEV, Gy), with same colours: L1 (9.35 +/- 0.13); L2 (5.50 +/- 0.20); L4 (5.88 +/- 0.14); L5 (8.81 +/- 0.23); L7 (7.24 +/- 0.25). 5–8 TLD chips per laboratory. Nominal dose: 10 Gy.

APD used for calibration (of absorbed dose in water) were provided by ZNA-Middelheim, Belgium. Pellets (Harwell, Oxfordshire, UK; ø = 4.8 ± 0.1 mm; h = 2.70 ± 0.01 mm, $\overline{m}$ = 59.8 mg; composition 90.9/9.1% w/w of l-α-alanine/paraffin) were used [10], stacked as 5 pellets in a cylindrical polypropylene tube. The absorbed dose was analysed by means of electron spin resonance (ESR) spectroscopy (Bruker HS0824 at XIOS-NuTec, Diepenbeek, Belgium). These dosimeters show no detectable fading during storage (at RT and RH<40%) for months, and the analysis is non-destructive. The uncertainty at 10 Gy is estimated as 0.76% [10]. The dose detection range is from 1 Gy to more than 10, 000 Gy [11]. The laboratory-specific results from the APD

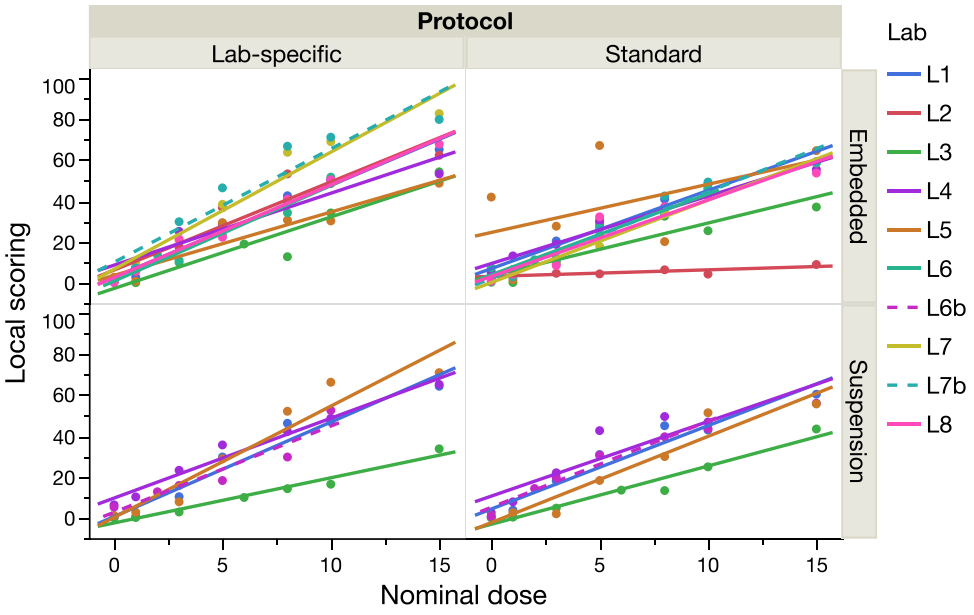

Fig. 4. DNA damage (TI%) results from eight laboratories (L1–L8), local scoring. Data from exposure of cell samples in suspension (lower panels) or embedded in gels (upper panels), to a set of nominal doses of radiation (0 – 15 Gy), and analysis for DNA damage using a lab-specific (left) or the standard (right) protocol. Each data point represents the median or mean TI% (of 50–100 comets) of one irradiated sample. Full lines: PBMC (L1-L3 and L5-L8); mononuclear cells in whole blood (L4). Dotted lines: L6b CHO-K1; L7b, HeLa.

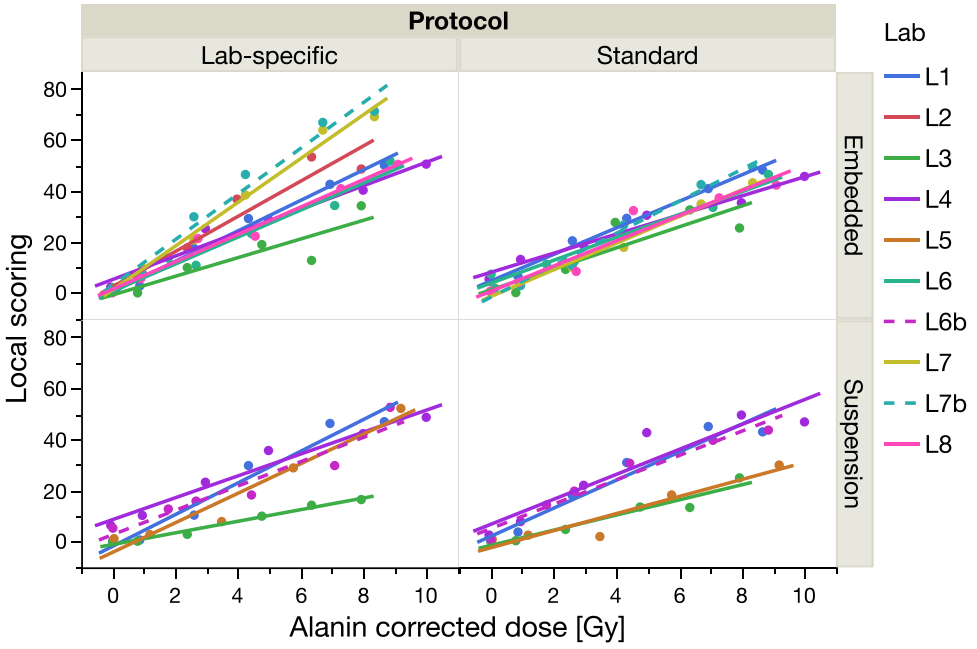

Fig. 5. DNA damage (TI%) results in eight laboratories (L1–L8), local scoring. Data from exposure of cell samples in suspension (lower panels) or embedded in gels (upper panels), as a function of calibrated radiation doses (0–10 Gy). See legend of Fig. 4 for further specifications.

measurements were used for retrospective conversion of the nominal doses to calibrated dose values.

TLD: Detector chips were of size 3.2 mm x 3.2 mm x 0.9 mm (LiF:Mg, Ti) and were analysed by SCK·CEN (Belgium), in a Harshaw 5500 reader (ThermoFisher, Belgium).

FD: 10^{-3} M Fe^{++} ($FeSO_4{\cdot}7H_2O$ or $Fe(NH_4)_2(SO_4)_2{\cdot}6H_2O$), 10^{-3} M NaCl, 0.249 M H_2SO_4. The ferrous salt was dissolved in water and sulphuric acid was added dropwise to avoid heating. Absorbance of ionising radiation in an air-saturated aqueous solution leads to oxidation of ferrous ions into ferric ions, which is measured as the dose-dependent linear increase in optical absorbance. With 1 cm cuvette: Absorbed dose (Gy) = 275xOD (305 nm) [12,13].

BAD: 10^{-3} M $FeSO_4$, 10^{-3} M C_6H_5COOH, 0.5 M H_2SO_4. The optical absorbance at 260 nm is linearly related to the absorbed dose. With 1 cm cuvette (quartz, since UVC light is used): Absorbed dose (Gy) = 69.4xOD (260 nm) [12].

Due to the logistics of the trial, APD and TLD were irradiated in L1-L8 at different times before being sent to L1 in Oslo for temporary storage. All APD and LTD samples were analysed in laboratories in Belgium during a few weeks, at times up to 1.5 years after exposure. The chemical dosimeters (i.e., BAD and FD) were analysed locally in L1-L8 shortly after exposure.

2.3. Chemicals and cells

All chemicals were analytical grade, but from different suppliers in the various laboratories.

PBMC were prepared by L1-L8 from blood samples obtained via

Table 3
Slopes and intercepts from linear regression of DNA damage (TI%, local scoring) as a function of calibrated radiation dose. Protocol: Lab-specific or Standard assay protocol. Cell sample: Irradiation of cells in Suspension or Embedded in agarose. Lack of data (no data) from a particular lab is indicated by "n.d." Outlier data excluded for technical reasons were not included in the statistical analysis and are indicated as "ni".

Lab #	Protocol	Cell sample	Cell type	Intercept	Slope
L1	Lab-specific	Suspension	PBMC	-1.90	6.21
L2	Lab-specific	Suspension	PBMC	n.d.	n.d.
L3	Lab-specific	Suspension	PBMC	-1.19	2.27
L4	Lab-specific	Suspension	Whole blood	8.53	4.29
L5	Lab-specific	Suspension	PBMC	-4.26	5.78
L6	Lab-specific	Suspension	PBMC	n.d.	n.d.
L6b	Lab-specific	Suspension	HeLa	2.64	4.77
L7	Lab-specific	Suspension	PBMC	n.d.	n.d.
L7B	Lab-specific	Suspension	CHO-K1	n.d.	n.d.
L8	Lab-specific	Suspension	PBMC	n.d.	n.d.
L1	Lab-specific	Embedded	PBMC	0.40	5.97
L2	Lab-specific	Embedded	PBMC	2.37	6.90
L3	Lab-specific	Embedded	PBMC	-0.81	3.65
L4	Lab-specific	Embedded	Whole blood	5.22	4.59
L5	Lab-specific	Embedded	PBMC	n.d.	n.d.
L6	Lab-specific	Embedded	PBMC	0.71	5.29
L6B	Lab-specific	Embedded	HeLa	n.d.	n.d.
L7	Lab-specific	Embedded	PBMC	1.14	8.58
L7b	Lab-specific	Embedded	CHO-K1	2.93	8.95
L8	Lab-specific	Embedded	PBMC	1.41	5.37
L1	Standard	Suspension	PBMC	2.03	5.49
L2	Standard	Suspension	PBMC	n.d.	n.d.
L3	Standard	Suspension	PBMC	-1.52	2.96
L4	Standard	Suspension	PBMC	6.72	4.88
L5	Standard	Suspension	PBMC	-2.45	3.34
L6	Standard	Suspension	PBMC	n.d.	n.d.
L6b	Standard	Suspension	HeLa	5.04	4.77
L7	Standard	Suspension	PBMC	n.d.	n.d.
L7B	Standard	Suspension	CHO-K1	n.d.	n.d.
L8	Standard	Suspension	PBMC	n.d.	n.d.
L1	Standard	Embedded	PBMC	4.65	5.19
L2	Standard	Embedded	PBMC	n.i.	n.i.
L3	Standard	Embedded	PBMC	1.17	4.11
L4	Standard	Embedded	PBMC	8.04	3.74
L5	Standard	Embedded	PBMC	n.d.	n.d.
L6	Standard	Embedded	PBMC	3.80	4.47
L6B	Standard	Embedded	HeLa	n.d.	n.d.
L7	Standard	Embedded	PBMC	-1.42	5.21
L7B	Standard	Embedded	CHO-K1	-1.73	6.26
L8	Standard	Embedded	PBMC	0.62	4.93
			MEAN	**1.69**	**5.12**
			STDAV.P	**3.28**	**1.50**
			CV (%)	**194.43**	**29.34**

venepuncture of volunteers, using Lymphoprep (Axis-Shield; local supplier) and standard procedures. The fraction of mononuclear cells was washed and diluted to 1 million cells/mL in PBS. One laboratory (L4) used both PBMC and whole blood. In two laboratories, mammalian cells (L6, HeLa; L7, CHO-K1) were cultivated using standard procedures, and diluted for exposure as with PBMC.

2.4. Irradiation of dosimeters and cell samples

Radiation doses (0, 1, 3, 5, 8, 10 and 15 Gy, denoted nominal doses) were used for calibration and cell exposure in L1-L8. Doses were chosen based on historic dosimetry in each laboratory (Supplementary file #2). All dosimeter samples were positioned at a fixed distance from the source and in the central beam. The FD and BAD solutions were prepared by each laboratory and irradiated in volumes 1.5–3 mL in microcentrifuge tubes or glass scintillation vials. Irradiations took place at the same temperature as the cell samples (i.e., ice-cold). Five laboratories (L1, L2, L4, L5, L7), estimated the radiation field homogeneity of their radiation units, by placing five to ten TLD chips in circles at varying distance from the central beam, and exposure to 10 Gy (nominal dose) at room temperature. Cell samples, in suspension or gel-embedded, were positioned in the central beam and exposed with the specified nominal doses, while on ice. Cell suspension samples were irradiated with nominal doses in 1.5 mL microcentrifuge tubes kept on ice and thereafter processed for comet analysis using lab-specific protocols (i.e., samples were transferred to ice-cold lysis solution within 30 min post-exposure). For irradiation of cells already embedded in gels, samples were mixed with low melting point (LMP) agarose and added to a substrate (GelBond® polyester film [14] or a glass slide). After irradiation on ice, these samples were put directly into lysis solution, i.e., within seconds after exposure.

The exposures of both dosimeters and cells took place using only one parallel sample per dose in each laboratory.

2.5. Comet assay protocols

2.5.1. Standard protocol

Details are shown in Supplementary file #1: Cells were mixed 1:10 with LMP agarose (final concentration 0.68%) and added to the substrate (GelBond polyester film or glass slide pre-coated with agarose) at defined volumes (4 and 60 μL, respectively). The polyester films or slides were placed in the lysis solution (2.5 M NaCl, 0.1 M EDTA, 10 mM Tris, pH 10, 1% Triton X-100) for 1 h at 4 °C. Alkaline treatment (unwinding of DNA) was in 0.3 M NaOH, 1 mM EDTA, pH> 13.3, for 40 min at 4 °C, followed by electrophoresis for 20 min at an Electric Field Strength (EFS) of 0.8 V/cm (on the platform of a horizontal electrophoresis tank). Slides were then washed for 10 min in neutralisation buffer (PBS, 0.4 M Tris, pH 7.5) and another 5 min in water at room temperature, before fixation in absolute ethanol for 1.5 hr. Slides/films were then air dried and stored until scoring.

The comet assay conditions actually used in each laboratory are listed in Supplementary file #3. The participating partners used protocols which deviated at different points from the recommended standard protocol, as specified.

2.5.2. Lab-specific protocols

In addition to the standard protocols, laboratories carried out the comet assay on exposed cells also using their own lab-specific protocol. Major deviations from the standard protocol relate to the final agarose concentration, gel sample and substrate (glass/plastic, layout, and sample volumes), electrophoresis (tank dimensions, voltage/currents, time, and temperature), and post-electrophoresis treatments before scoring. The protocol details are specified in Supplementary file #3.

Table 4
Linear regression of dose response curves. Mean slopes (+/- standard deviation and CV) for all laboratories, calculated separately after grouping for protocol and cell sample type. Data from Table 3 (calibrated doses) and from parallel data for nominal radiation doses.

	Nominal dose				*Calibrated dose*			
	Lab-specific protocol		Standard protocol		Lab-specific protocol		Standard protocol	
	Suspension	Embedded	Suspension	Embedded	Suspension	Embedded	Suspension	Embedded
MEAN	4.52	5.26	4.23	4.20	4.66	6.16	4.29	4.85
STDAV.P	1.67	1.33	0.97	0.56	1.38	1.74	0.97	0.77
CV(%)	36.94	25.25	23.06	13.44	29.55	28.31	22.57	15.94

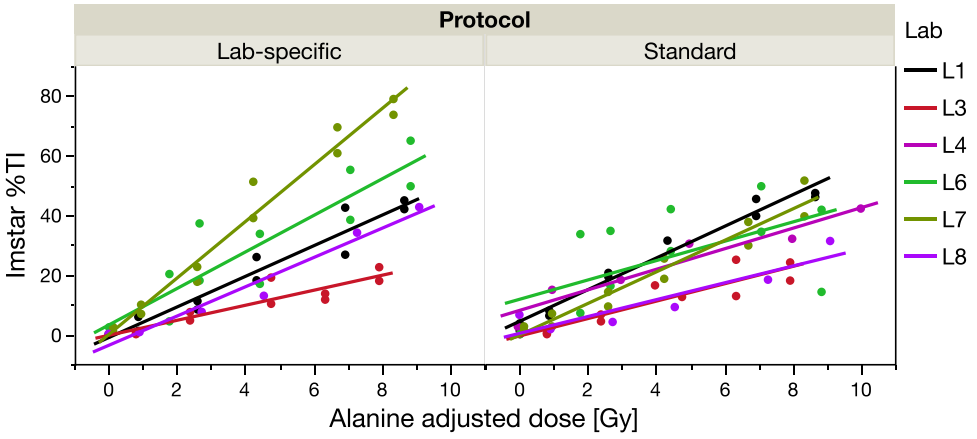

Fig. 6. Scoring with IMSTAR, centralised. TI% results for laboratories L1-L8 (incomplete). Data from exposure of samples using a lab-specific (left) or a standard (right) protocol. Some curves (see text) are excluded from the statistical analyses (Table 5). Each data point represents the median or mean TI% (of approximately 100 comets) of one irradiated cell sample.

Table 5
Linear regression constants from re-scoring with IMSTAR. Averaged slopes and coefficients of variance (CV). The comet assay protocols were either a lab-specific protocol or a standard protocol. Radiation doses used for exposure were either uncalibrated (nominal; left) or calibrated with alanine pellets (right).

	Nominal doses		Calibrated doses	
	Lab-specific protocol	Standard protocol	Lab-specific protocol	Standard protocol
Mean	4.85	3.43	5.74	3.98
STDAV	1.88	0.99	2.27	1.18
CV [%]	38.8	28.8	39.55	29.59

2.6. Scoring

Samples on glass slides and GelBond films were scored (at least 50 cells per gel) locally, by any system routinely used in the participating laboratories, mostly semi-automated image analysis systems (Supplementary file #3). TI%DNA median values (means, if the median value was not provided) were used as the measure of DNA damage (SSBs, i.e. DNA strand breaks and alkali-labile sites). L5 used visual scoring (5 categories of damage (0−4)); these data were converted into TI%DNA [15,16]. L4 used automated scoring (MetaSystems Comet-Imager, Germany); L7 used Metasystems in addition to semiautomated scoring. After local scoring slides were sent to L1 for centralised scoring, using fully automated scoring with the IMSTAR Pathfinder™ (Paris, France) and in addition semi-automated scoring (Perceptives Comet IV). For about half of the samples, this re-scoring was not carried out as planned for capacity and technical reasons.

2.7. Statistics

2.7.1. Dosimetry data

Data from doses measured with APD were plotted against nominal doses, for each laboratory. Calibration curves were analysed by linear regression, from which slopes and intercepts (correction factors) were used allowing conversion of all nominal doses to calibrated ones. The

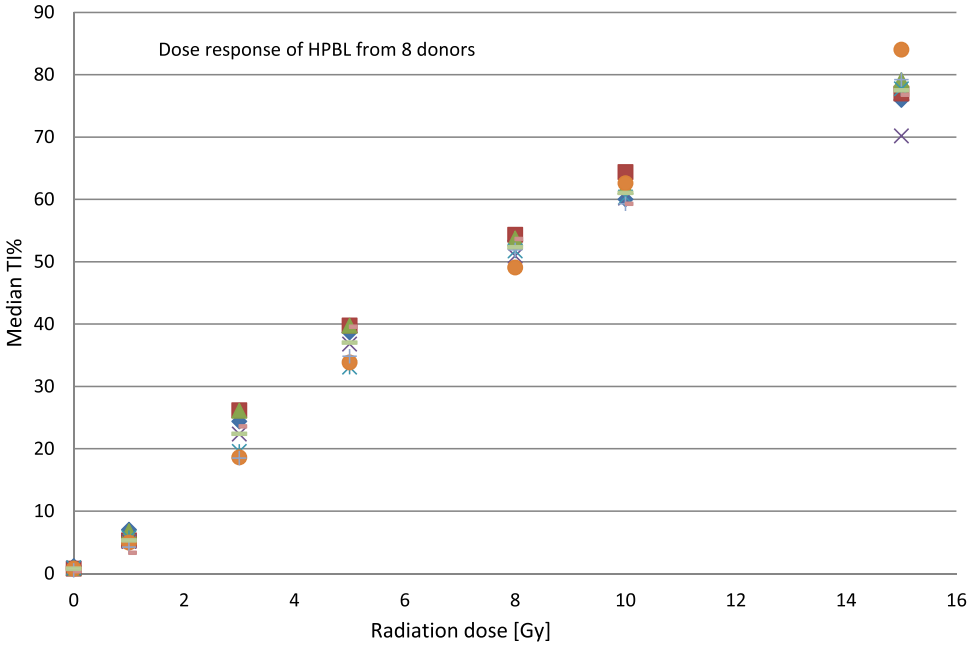

Fig. 7. DNA damage as a function of radiation dose, in PBMC from 8 donors (one cell sample per dose). Analysis in one comet assay experiment. The coefficient of variance of slope values (0–10 Gy) was 4%.

Table 6
Correction factors (dimensionless) for electrophoresis conditions [electric potential (A), time (B) and agarose concentration (C)]. Reference values (= 1.00) are 0.8 V/cm, 20 min, and 0.68% agarose, for A, B and C, respectively. The factors are different for lab-specific protocols. They differ from 1.00 also for laboratories using the standard protocol parameters but deviating from one or more of the reference values.

Lab#	Protocol:LS (lab-specific) S (standard)	Electric field strength (V/cm) A	Electrophoresis time (min) B	Agarose concentration (%) C
L1	LS	0.77	1.00	1.00
L2	LS	0.55	1.00	1.00
L3	LS	1.06	1.00	0.89
L4	LS	0.56	1.00	1.09
L5	LS	0.82	1.00	1.00
L6	LS	0.75	0.67	1.02
L7	LS	0.51	0.67	1.42
L8	LS	0.57	0.67	1.26
L1	S	0.88	1.00	1.00
L2	S	1.72	1.00	0.86
L3	S	1.22	1.00	1.00
L4	S	0.56	1.00	1.00
L5	S	0.82	1.00	1.00
L6	S	0.75	0.67	1.02
L7	S	0.51	0.67	1.42
L8	S	0.88	1.00	1.00

dose measurements for TLD, FD and BAD were also analysed by linear regression but as a function of the calibrated doses obtained with APD. Correction factors (slopes and intercepts) for each dosimetry system were obtained, specific for each laboratory (some laboratories did not report data for all three systems).

2.7.2. DNA damage

Raw data on median DNA damage values (TI%) (or mean, if the median was not reported) were plotted against radiation doses (nominal or calibrated values as specified). Dose responses were analysed by linear regression. Statistical significance of differences between groups means was tested using Student's t-test.

2.7.3. Correction factors for electrophoresis

Corrections were included to account for the protocol- and laboratory-specific characteristics of the electrophoresis, which have major impacts on the TI% level (and ultimately the dose response slopes). The effects of correction factors on the dose response slopes for each laboratory were studied in a linear mixed model (using Restricted Maximum Likelihood since we were not interested in the random effects). *Lab* was the random effect, *APD* (corrected dose value) was the independent variable, and *TI%* was the outcome. This model was applied to the five different TI% estimates (obtained with local scoring): (i) before correction, and after correction for (ii) agarose concentration, (iii) electrophoresis time, (iv) electric potential (V/cm); and (iv) all three correction factors combined. The best correction factor was the one with the lowest percentage of the total variance attributed to Laboratory number.

Statistical analyses were performed using JMP^{P} pro16.1 (SAS Institute, Cary, NC, USA) and Microsoft Excel 365 MSO.

3. Results

Ideally, calibration of a radiation source in a biology laboratory should be carried out using methods which do not rely on costly equipment or external calibration services. In this trial we used APD (alanine pellet) dosimetry which is considered to be a gold standard for measuring absorbed doses. Since APD pellets must be obtained from a supplier and shipped to a central institution for analysis, we also investigated three other alternative dosimetry systems.

3.1. Radiation dose measurements

Absorbed doses as measured with APD were used to construct calibrated curves, i.e., the measured dose as a function of the nominal dose. Fig. 1 shows APD calibration curves for two of the participating laboratories representing the minimum (L3) and the maximum (L5) slope; these values differ by 47%. Linear regression parameters are listed for all participants (L1-L8) in Table 1. The eight calibration curves are strictly linear in the nominal dose range 0 – 15 Gy, with low intercepts and R^2 close to 1.00. The high linearity and precision imply that the exposure time in all radiation units is an accurate estimate of the relative dose, and the dose rate is constant with time. However, there are major deviations in the calibrated vs the nominal dose rates within each laboratory. These differences are important when comparing the levels of DNA damage reported by L1-L8. The errors can be corrected for by applying the laboratory specific radiation dose correction factors (Table 1). The deviations represent systematic and not stochastic errors (not normally distributed although their mean was close to 1). The APD calibration constants show no apparent association with the type of radiation (X-rays or monoenergetic gamma rays, with nominal dose rates in the range 1–6 Gy/min; Supplementary file #2).

Linear regression constants for the other dosimetry systems, BAD, FD and TLD, were calculated from the data in Fig. 2, vs the APD-calibrated doses (Table 2). Few of the participating laboratories could complete all the dosimetry measurements. Specifically, for BAD dosimetry (3 labs out of 4, excluding the outlier L3) the mean linear slope is 1.21 + /- 0.07, CV= 5.9%. BAD dosimetry is more sensitive than FD at low doses (higher UV extinction coefficient per Gy); however, spectrophotometric absorbance is measured with UVC light (260 nm) rather than UVB (305 nm for FD) which may be a slight challenge. For FD (4 labs out of 5 in total) the mean slope was 1.10 + /- 0.10, CV= 8.7% (excluding one outlier, L3). The TLD (7 labs) gave, unexpectedly, consistently much lower values (mean 0.70 +/- 0.11, CV=16.0%). We have no explanation for this, other than possibly the low values might represent a reduced reading due to storage of TLD samples for several months before shipment for analysis (no correction was made for fading which may be significant for TLD). The TLD calibration values seem reliable as relative measurements, i.e., they can be used for comparison between and within laboratories, although Table 2 suggests that comparison of absolute dose values requires additional dosimetry (FD or APD). [The TLD data are given as exposure levels (not absorbed dose) by the laboratory performing the readings, but these values are not much different from absorbed radiation doses in water, with the types of radiation used here (Filip Vanhavere, personal communication, 2021).].

The small physical size of TLD chips makes them ideal for analysis of radiation fields. Inaccurate absorbed doses may be caused by heterogeneities in the exposure field. To test this, strips were placed at various distances (several centimetres) from the central beam in the radiation unit. Among the five laboratories reporting such measurements, the maximum difference between the position-dependent relative doses (one measurement per position) varied between 4.2% and 10.8% (Fig. 3), which are likely to reflect measurement imprecision rather than real radiation field differences. Cell samples are routinely irradiated close to the central beam (maximum dose rate). In these experimental setups, the inaccuracy of the absorbed dose due to field variations is considered as less than 5%.

3.2. Comet data for irradiated cells

3.2.1. Comet assay protocol and exposure conditions

The ring trial protocol (Supplementary file #1) comprises a multitude of comet assay experimental conditions and parameters, specific for each laboratory and for the standard and the lab-specific protocol (i.e., irradiation of cell suspensions and cells embedded in gels, electrophoresis conditions, fluorochromes used for staining, semi-automated and automated scoring, local scoring and centralised scoring). Several of the

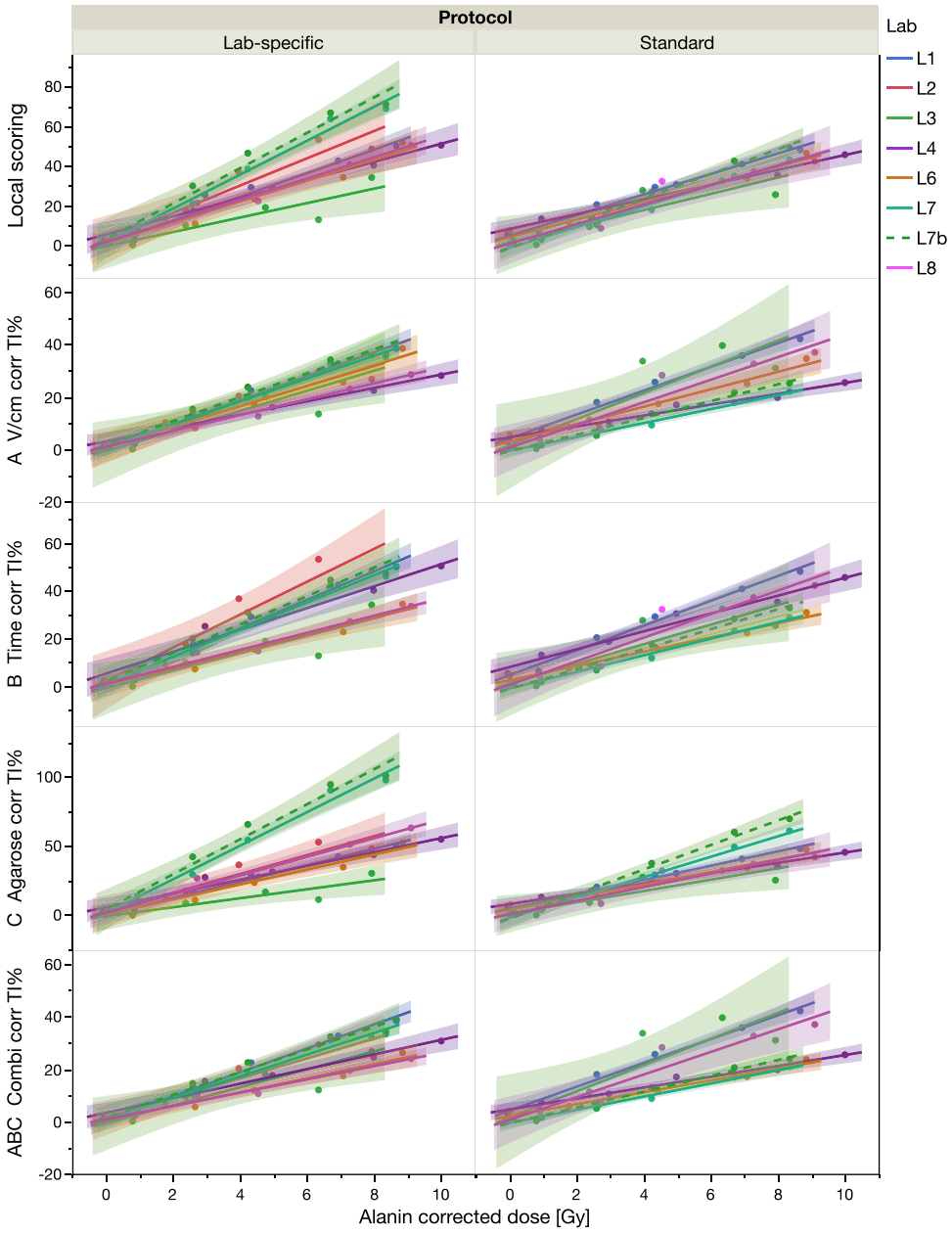

Fig. 8. Corrected and uncorrected dose response curves for irradiated embedded cells. Laboratory-specific electrophoresis parameters were used to correct the slopes of dose response curves [TI% (local scoring), as a function of calibrated doses of ionising radiation]. Comet assay data obtained with (left) Lab-specific protocols, or (right) the Standard protocol. Linear regression lines are shown with shaded confidence intervals. Corrected curves (with correction factors from Table 6) represent, from top to bottom: No correction; A (V/cm); B (time); C (agarose); A*B*C combined.

conditions specified in the standard protocol were not met by all partners; the conditions actually used are listed in Supplementary files #2 and #3. There are few data for each category of conditions. Statistical analysis could therefore not be undertaken for all protocol specific parameters.

For the lab-specific protocols, 6 laboratories reported results for cells in suspension and 10 for embedded cells. For the standard protocol, 5 laboratories reported results for cells in suspension and 8 for embedded cells. For the two laboratories using both PBMC and cell lines, samples were irradiated independently producing data designated L6b and L7b. The latter results were included with PBMC and whole blood in downstream analysis, since the dose response did not differ from that of the blood cells (this has also been reported in a similar calibration study performed in one laboratory [17]).

Original comet assay raw data for laboratories are shown in Fig. 4, with TI% as a function of nominal (uncalibrated) radiation doses (0 – 15 Gy). The data in Fig. 4, obtained with local scoring of comets, show highly variable dose responses for both the lab-specific and the standard protocol. Linear slopes range between 0.19 and 7.36 [TI% per Gy]. Intra-laboratory data points show large deviations from the linear regression line which is not unexpected since there was only one cell sample per exposure.

Fig. 5 shows TI% as a function of radiation doses calibrated with APD for each laboratory. At least with the standard protocol and gel-embedded cells, the dose response curves are very similar for the participating laboratories. For experiments with embedded cells, two laboratories reported variable or almost flat curves due to technical problems; these data are not shown in Fig. 5 and they were excluded from further analysis (indicated by "n.i." in Table 3). For the comet assay in general, TI% values saturate and level off at high radiation doses (see also Fig. 7). Consequently, the linear regression constants in Table 3 (slopes and intercepts) are calculated with calibrated radiation doses in the range 0–10 Gy. Table 3 also specifies the cell type used in each case. Data not reported are marked with "n.d.".

Means and standard deviations of the linear regressions are summarised in Table 4, calculated after grouping according to *protocol* (lab-

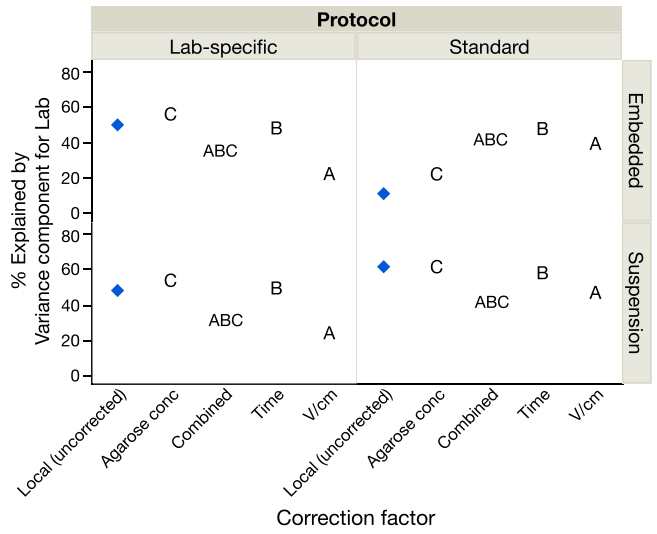

Fig. 9. Graphic illustration of analysis of correction factors using a linear mixed model. Left panels: Lab-specific protocol, right panels: Standard protocol. Upper panels: Embedded cell exposure, lower panels: Suspended cell exposure. The corrections factors are indicated in the x-axis (cfr Legend to Fig. 8). Specific correction factors A, B, C and the combination ABC are shown on the X–axis. Symbols are, from left to right: Local uncorrected (rectangle); Agarose conc (C); Combined (ABC); Time (B); V/cm (A). For each symbol, the ordinate axis shows the estimated percentage of the variance attributed to the laboratory, for a specific correction factor.

specific or standard) and *irradiated sample* (suspension or embedded). The mean interlaboratory slope values are not much different using calibrated rather than nominal dose values; however, the variance is considerably lower comparing the standard and the lab-specific protocols. There were laboratory-specific differences in results obtained using the two different exposure conditions, i.e., cells irradiated in suspension versus embedded in agarose; in particular this applies to the standard protocol. However, only three labs (L1, L3 and L4) could be compared in this respect using the data in Table 3, due to incomplete data sets from the other laboratories. For L1 there was little or no relative difference in the response of suspended and embedded cells (1.04 and 1.06, for the lab-specific and the standard protocol, respectively); similar data for L4 were 0.93 and 1.30. For L3, however, the ratio was markedly lower for both protocols (0.62 and 0.72, respectively). These data suggest substantial DNA repair in L3, also implying that routines for post-exposure treatments may be quite different in different labs.

3.2.2. *Scoring systems*

The importance of scoring methods has been given attention [18]. Standardisation of scoring is likely to reduce variation [4]. In the present ring test, each laboratory analysed its own samples using any dye, method, or equipment, with either semi-automated (L1, L2, L3, L6, L7, L8), automated (L4 and L7) or visual scoring (L5). There are indications that light intensity and lens magnification may affect results even for the same semi-automated system (G. Brunborg, preliminary data presented at ICAW, Porto, 2018). A second analysis of all samples was planned to be carried out in the coordinating laboratory L1, using a fully automated scoring system (IMSTAR Pathfinder) and a standard Perceptive system. However, several laboratories did not submit samples for all protocols/exposure conditions or samples had dried out before they could be scored; ultimately fewer than 50% of the samples were received and could be re-scored successfully by L1. There was clearly an increase in variation when these samples were re-scored (Fig. 6).

However, the regression slopes from the IMSTAR scoring were not much different from local scoring data (Table 5 vs. Table 4), although R^2 values (not shown) are higher. Inter-laboratory variations are also higher, which is probably due to incomplete data sets.

3.2.3. *Individual cell response to radiation*

Fig. 7 shows results from an experiment in which blood was sampled from 8 donors of both genders and varying age (20 – 68 yr.); PBMC samples were irradiated and analysed for DNA damage by L1 (lab-specific protocol). No significant difference in slopes was observed. Inter-laboratory variations in comet assay levels for PBMC are often reported; historically they might inappropriately be interpreted as real differences due to biological variations between donors associated with lifestyles, dietary habits, or environmental and occupational exposures. However, as discussed in [19], variations due to such factors are relatively modest. The highly similar dose rates found in one laboratory (Fig. 7) suggest that major inter-laboratory differences of PBMC often reported are likely to reflect assay conditions rather than donor differences.

3.2.4. *Correction factors for electrophoresis*

Electrophoresis parameters are known to determine the apparent DNA damage level visualised as TI% values in the comet assay. TI% is linearly correlated with the electric field strength (V/cm) and electrophoresis time, and inversely correlated with the agarose concentration [20–23]. The electrophoresis parameters specified in our ring test were planned to be equal for the standard protocol but expected to vary for the lab-specific protocols. However, in reality there were major deviations also from the standard recommendations, in particular pertaining to the electric field strength. The reported laboratory-specific parameters were used to calculate a set of correction factors shown in Table 6. As an illustration, when comparing results from lab X and Y, if Y used 2 V/cm and X used 1 V/cm, the TI% values of Y should be divided by a factor of 2 (and similar for differences in electrophoresis time and agarose concentration).

The laboratory specific slopes in Table 3 were multiplied by the correction factors in Table 6 (A, B, C, or A*B*C), to generate a revised set of slopes. The resulting dose response curves shown in Fig. 8, for embedded cell irradiations, show that the dose responses do vary considerably in response to the electrophoresis conditions. More importantly, it appears that systematic laboratory-specific corrections are justified.

We analysed the statistical implications of introducing the correction factor using a linear mixed model as explained in Materials and Methods. The model was applied to the dose response curves shown in Fig. 8 for embedded cells and also to the data for cells in suspension. When the slopes for all labs are similar the variance component of “the lab” (random effect) is smaller compared to when the slopes are more different, with results illustrated in Fig. 9. The best correction factor is the one with the lowest percentage of the total variance attributed to “the lab”. The most striking change is seen with factor A (V/cm) correction in the lab-specific protocol for embedded cells (upper left). Correction with A reduces the likelihood of “the lab” being responsible for slope variations, from approximately 50 to 20%. With the standard protocol, effects of correction factors are absent for embedded cells. Applying the correction factors to the data for suspended cells shows a clear reduction in the variance percentage due to “the lab”, both for the lab-specific and the standard protocols, in particular for A (V/cm) (lower panels in Fig. 9).

4. Discussion

This ring-study was undertaken in an attempt to explain major differences in the slope of comet assay dose response curves obtained in different laboratories [6]. Initial planning took place in Warsaw during the International Comet Assay Workshop (ICAW) in 2005. The protocol was developed during the following 4–5 years. Some participants left the core group, whereas others joined; eight partners completed the experiments during a short period more than ten years ago. No

publications have appeared in the intervening years comparing and calibrating radiation doses in different laboratories, in the context of the comet assay or other genotoxicity tests.

Our original objective was quite simple: to compare radiation doses using established radiation dosimetry. The project eventually became more ambitious, with the inclusion of multiple experimental factors thought to be related to comet assay variations. The ultimate goal was then to explain some of the inter-laboratory variations in comet assay results that had been identified in the ECVAG projects. Such variations posed –and still pose– a major challenge for the comet assay used in human biomonitoring studies. It is obvious that comparing data from different sources is greatly facilitated by standardisation of protocols and inclusion of suitable reference material and calibration routines.

In the past, analysis of comet assay data has typically been based on classification of DNA damage into tertile frequency of descriptors [24]. Positive controls and internal standards [25] should give higher convergence of data from different sources and are likely to result in better, at least easier, statistics.

For the first part of our ring-study, the radiation dose calibrations, different methods were used including measurement of the absorbed dose in a central laboratory. Calibration curves were found to be highly linear (with exposure time) in all labs. An important conclusion is that local calibration of radiation doses can indeed be carried out, with reasonable accuracy, in any laboratory (also by biologists!) using simple and low-cost alternatives. Calibration of radiation doses has the potential to correct misclassification of the true exposure. The reported inconsistencies in comet assay results when using different sources of ionising radiation [6] can be explained to a large extent by the results of the present ring test. Our data (Fig. 1) suggest that dose response curves from two laboratories could differ by as much as 46% due to inaccurately measured radiation doses. Calibrated doses of ionising radiation are used to calculate absolute lesion frequencies in cells treated with any agent. Publishing comet data in this form is strongly recommended [26] but does not make much sense if the radiation dose is incorrect. Lisa Giovanelli and co-workers [17] have described in detail how DNA damage measured with the comet assay may be quantified using ionising radiation. In the present SI of Mutation Research, measurement of absolute frequencies of oxidatively damaged DNA is reviewed by Peter Møller [19].

The second, and major part of this trial – comet assay of irradiated cells – showed a considerable spread of data, between and also within laboratories. This is in part due to the design of the project, which specified only one sample for each radiation dose. This reduced the number of samples which had to be scored, but inevitably led to stochastic errors. The inter-laboratory differences in data for dose response, expressed with linear regression statistics, gave considerable variance values for all laboratories and exposure conditions (CV = 44%), somewhat reduced after calibration of radiation doses to 29% (Table 3). These data are results from the participating laboratories using any protocol. By using a more standardised and equal protocol, the variance is further reduced. In particular, the data for embedded cells exposed to calibrated doses of ionising radiation (Table 4) are more homogenous than reported in previous ring trials, and this is promising for comet assay standardisation. We observed major lab-specific difference in the response of cells in suspension versus embedded cells, and this is an indication that post-exposure treatments may be quite different in different laboratory.

For other experimental parameters characteristic for the lab-specific and standard protocols, there were no apparent effects (lysis conditions, substrate (glass slides or plastic), unwinding time in alkaline, electric current during electrophoresis, fluorochrome, scoring), in line with comet assay literature [27]. This is also the case for the temperature during electrophoresis, within certain limits [28].

Centralised fully automated scoring gave, somewhat surprisingly, a large spread of data, but the mean of the slopes was quite similar to local scoring with other methods (Table 5) [18]. Semi-automated and visual scoring are potentially susceptible to operator bias due to insufficient emphasis on blinding procedures. When scoring a dense population of comets, there will always be a wide distribution of tail lengths. An automated system (and probably also an operator) may more frequently select the undamaged comets, cancelling (and not measuring) more damaged and hence often overlapping nucleoids. This type of selection bias therefore relates to all types of scoring and is likely to depend on the cell density in the sample.

Judging from our ring trial, with eight laboratories, standardisation of the comet assay protocol, even to the limited degree achieved in this trial, gives a significantly reduced variation in dose responses. Furthermore, dose calibrations reduce inter-laboratory variations in absolute DNA lesion frequencies; without calibration, errors up to 50% are quite likely. Moreover, the present study demonstrates the superiority of low LET ionising radiation as a genotoxic agent in studies on inter-laboratory variation in DNA damage levels. As an illustration, in a recent ring trial involving a chemical agent (potassium bromate) to induce Fpg-sensitive DNA damage [29], the participating laboratories used the same cells and a semi-standardised comet assay protocol. Linear dose response relationships were achieved in all laboratories, but there was a ten-fold difference between the lowest and highest slopes (CV = 57%, mean of 9 labs), compared to the more modest heterogeneity in the present trial using ionising radiation.

It is likely that the inter-laboratory variations could have been lower, had the conditions in the standard protocol been completely adhered to. After the experiments in this ring-test had been completed and reported, it was realised that the reported values for V/cm in some cases deviated considerably from calculated values, suggesting that power supply problems might have gone unnoticed. In addition, measuring the electric potential in a liquid is, after all, not straightforward [23].

The electrophoresis conditions (V/cm, time and agarose) are known to be important and we have previously recommended to control and report such parameters [22,23]. Any deviation from strict standard conditions should trigger a quantitative correction since the effects of altered electrophoresis conditions are significant. The results of the linear mixed model analysis (Figs. 8 and 9) suggest that systematic corrections should indeed be made when relevant since they lead to less variation between laboratories. We consider the value 4.85 + /- 0.77 [TI % per Gy] (Table 4) as a standard dose response for mammalian cells (embedded cell radiation; electrophoresis 0.8 V/cm, 20 minutes, 0.68% agarose). This slope value could be used as a reference curve to calculate DNA lesion frequencies. Such curves should be achievable in any laboratory with access to a calibrated radiation unit. Human white blood cells are readily available. If a radiation source is not present, there may be other possibilities, such as distribution of lyophilised irradiated PBMC from a central laboratory. Another attractive possibility would be the use of irradiated reference cells which could be taken through the comet assay after mixing with sample cells, acting as a true internal control. Two different approaches have been described for specific scoring of cells in such mixtures [25,30].

To summarise, we have investigated the causes of variation in comet assay results between laboratories. It is important to adhere to a standard protocol, but (moderate) deviations in agarose concentration, time and voltage gradient of electrophoresis, as well as scoring method, can be allowed for by applying correction factors. Conversion of TI% to actual break frequencies depends on calibration with a source of calibrated ionising radiation.

Conflict of interest

All authors declare no conflict of interest.

Data availability

Data will be made available on request.

Acknowledgements

The article is part of a Special Issue which is a tribute to Professor Andrew Collins. It may be surprising that Andrew is among the authors, but it is also quite appropriate since he played such an important role in conceiving our ring trial. The communicating author (GB) has known Andrew for almost 30 years and has had the pleasure to meet him often both socially and for science, during his many years at the University of Oslo. Andrew knows so many people in the scientific comet world, and together we resemble a big family!

The contribution of Bob Schaeken PhD (Deskundige Medische Stralingsfysica, Ziekenhuis Netwerk Antwerpen, dept. Radiotherapie, Lindendreef 1, B-2020 Antwerpen, Belgium) with analysis of TLD samples is greatly acknowledged.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.mrgentox.2022.503560.

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