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Manual Scoring with Shortened 48 h Cytokinesis-Block Micronucleus Assay Feasible for Triage in the Event of a Mass-Casualty Radiation Accident

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The cytokinesis-block micronucleus (CBMN) assay in cytogenetic biodosimetry uses micronucleus (MN) frequency scored in binucleated cells (BNCs) to estimate ionizing radiation dose exposed. Despite the faster and simpler MN scoring, CBMN assay is not commonly recommended in radiation mass-casualty triage as human peripheral blood is typically cultured for 72 h. Furthermore, CBMN assay evaluation in triage often uses high-throughput scoring with expensive and specialized equipment. In this study, we evaluated the feasibility of a low-cost method of manual MN scoring on Giemsa-stained slides in shortened 48 h cultures for triage. Both whole blood and human peripheral blood mononuclear cell cultures were compared for different culture periods and Cyt-B treatment [48 h (24 h at Cyt-B); 72 h (24 h at Cyt-B); 72 h (44 h at Cyt-B)]. Three donors (26-year-old female, 25-year-old male, 29-year-old male) were used for dose-response curve construction with radiation-induced MN/BNC. Another 3 donors (23-year-old female, 34-year-old male, 51-year-old male) were used for triage and conventional dose estimation comparison after 0, 2 and 4 Gy X-ray exposure. Our results showed that despite lower percentage of BNC in 48 h than 72 h cultures, sufficient BNCs were obtained for MN scoring. Triage dose estimates of 48 h cultures were obtained in 8 min in non-exposed donors, and 20 min in 2 or 4 Gy exposed donors with manual MN scoring. One hundred BNCs could be scored for high doses instead of 200 BNCs for triage. Furthermore, observed triage MN distribution could be preliminarily used to differentiate 2 and 4 Gy samples. The number of BNCs scored (triage or conventional) also did not affect dose estimation. Dose estimates in 48 h cultures were also mostly within ±0.5 Gy of actual doses, thus showing the feasibility of manual MN scoring in the shortened CBMN assay for radiological triage applications.

INTRODUCTION

In a radiological mass-casualty accident, fast and reliable triage identification of individuals exposed to ≥2 Gy acute whole-body equivalent dose of radiation from the worried well is essential for immediate medical treatment, as recommended by the U.S. Department of Health and Human Services Radiation Emergency Medical Management (*1*). Multiple biomarkers assessed in human peripheral blood such as γH2AX (*2, 3*), proteins (*4, 5*), gene expression (*6–8*) and miRNA (*9*) have been evaluated for triage as these biomarkers bypass the need for peripheral blood lymphocyte culture to evaluate DNA damage. However, standardized radiological triage guidelines by the International Organization for Standardization (ISO) are currently only available for cytogenetic endpoints scored in cultured peripheral blood lymphocytes. Both dicentric chromosomes and micronuclei (MN) can be used for cytogenetic triage, as seen in ISO 21243 (*10*) and ISO 17099 (*11*), respectively.

For triage assessment, 50 metaphases/30 dicentrics are scored for dicentric chromosome assay (DCA) (*10*), while 200 binucleated cells (BNCs) are scored for cytokinesis-block micronucleus (CBMN) assay (*11, 12*). In contrast, conventional dose assessment requires at least 500 metaphases/100 dicentrics to be scored for DCA (*13*), and 1,000 BNCs to be scored for CBMN assay (*11*) for reliable dose estimation. In manual dicentric scoring for one individual by experienced scorers, conventional triage assessment can take up to 150 min (*10*) or 30 min with the QUICKScan method (*14*). However, MN scoring is much quicker than dicentric scoring as the criteria for MN scoring is much

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simpler than dicentrics and requires no prior knowledge of chromosome aberrations and karyotypes (*15*). Despite the much longer scoring time, DCA is usually preferred over CBMN assay for dose estimation as DCA is the international ''gold standard'' for cytogenetic biodosimetry due to dicentrics being highly radiation specific with a low background frequency (*13, 16*). In contrast, background MN frequency is affected by a variety of factors, including age, sex and lifestyle (*17*). Furthermore, cell culture for DCA can be completed in 48 h, much faster than the conventional CBMN assay culture of 72 h.

To increase the feasibility of CBMN assay over DCA in radiological triage, previous efforts were made to reduce CBMN assay culture time to 70 h (*18*), 64 h (*18, 19*), 60 h (*20*), 54 h (*18, 20, 21*), 48 h (*20, 22*) and 36 h (*20*). Similarly, technological advancements for high-throughput assessment of MN were also developed for faster scoring and multiple samples. Semi-automated and automated MN scoring systems were developed for Giemsa (*23, 24*), DAPI (*25–27*), DAPI/Fast Green (*28*) and PI (*24*)-stained cells on microscope slides. For direct imaging with fixed cell solutions, imaging flow cytometry coupled with Rapid Automated Biodosimetry Technology (RABiT) was also developed to handle multiple low volume blood samples (*29*). The RABiT system can also be used for direct cell culture and fixation, cell imaging and automated MN scoring of cells from multiple donors on glass-bottomed microplates (*21*). However, these reagents and equipment used are highly specialized, relatively expensive and may not be commonly found in all cytogenetic laboratories. As such, we assessed the feasibility of the low-cost method of manual MN scoring with light microscopy on Giemsa-stained slides in 48 h CBMN cultures for triage in this study.

Furthermore, triage MN scoring is often performed only in whole blood (WB) cultures (*12, 18, 19, 21–27, 29*). Despite ISO recommending both cultures of WB and peripheral blood mononuclear cells (PBMCs) isolated from WB for CBMN assay as the target cells analyzed are assumed to be phytohemagglutinin (PHA)-stimulated T lymphocytes, plasma and other cellular components present in WB but absent in PBMCs could influence DNA damage induction and repair. As reported in our previous studies (*30, 31*) and other studies (*32, 33*), differences in CBMN parameters were seen between WB and PBMC cultures. To the best of our knowledge, this study is the first to compare triage MN scoring for WB and PBMC cultures. In addition, as PBMC separation from WB is preferred for γH2AX (*3*), a multi-parametric approach for triage assessment with γH2AX and CBMN assay could also be performed with PBMCs directly isolated with density centrifugation.

In this study, we shortened the culture period of CBMN assay from 72 h to 48 h and evaluated various parameters with manual scoring of Giemsa-stained cells as a low-cost alternative. In the first part, cell proliferation indicators of nuclear division index (NDI) and percentage of BNC in all cells (%BNC), conventional and triage MN frequency (MN in 500/1,000 BNCs, MN in 100/200 BNCs) and time taken for triage MN scoring were compared in 0, 2 and 4 Gy WB and PBMC cultures from 3 donors in three conditions, varying in culture period and time of cytochalasin B (Cyt-B) addition [48 h culture (24 h at Cyt-B), 72 h culture (24 h at Cyt-B), 72 h culture (44 h at Cyt-B)]. In the second part, dose-response curves (DRCs) using radiation-induced MN/BNC from another 3 donors were constructed for WB and PBMCs in the shortened [48 h culture (24 h at Cyt-B)] and conventional [72 h culture (44 h at Cyt-B)] CBMN assay. Dose estimation was performed using radiation-induced MN frequencies after triage and conventional scoring from the first part of the study.

MATERIALS AND METHODS

Blood Collection and Irradiation Conditions

Three healthy donors (26-year-old female, 34-year-old male, 51-year-old male) were used for 48 and 72 h CBMN assays in the first part, while another 3 healthy donors (23-year-old female, 25-year-old male, 29-year-old male) were used for DRC construction in the second part of the study. Peripheral blood was collected in 6 ml lithium-heparin tubes (BD, Franklin Lakes, NJ) with their informed consent. The informed consent form was approved by the Committee of Medical Ethics in Hirosaki University Graduate School of Health Sciences (Approval number: 2012-278). Among all the donors, only the 51M donor is a smoker.

X-ray dose-rate was first calibrated with either 6 ml lithium-heparin tubes or 5 ml round-bottom polystyrene tubes containing water in an angled tube rack. Blood in lithium-heparin tubes was then directly irradiated with 1 Gy/min X ray for respective doses (150 kVp, 20 mA, 0.5 mm Al + 0.3 mm Cu filter; MBR-1520R-3, Hitachi Power Solutions, Tokyo, Japan). For 0 Gy blood, tubes were placed in the X-ray generator without irradiation. Monitoring of cumulative radiation dose was performed with a 0.3 cm^3 semiflex ionization chamber (TN31013, PTW, Freiburg, Germany) connected to a dosimeter (MZ-BD-3, Type 153, Hitachi Medical Corporation, Tokyo, Japan). The thimble chamber and dosimeter were calibrated annually by the Japan Quality Assurance Organization, satisfying national standard traceability and ISO/IEC 17025 requirements

Blood was then incubated in a 37°C water bath for 2 h postirradiation for DNA repair. In WB cultures, blood was directly used. In PBMC cultures, PBMCs were first isolated with Histopaque 1077 (Sigma-Aldrich, St. Louis, MO) before use, according to our previously published protocol (*30, 31*).

CBMN Culture, Harvest and Fixation for WB and PBMCs

Complete medium (CM), used in WB and PBMC cultures, was prepared with RPMI 1640 (Thermo Fisher Scientific, Waltham, MA), 20% heat-inactivated FBS (Sigma-Aldrich) and 1× kanamycin sulfate (Thermo Fisher Scientific). 1:10 WB: CM (0.5 ml WB, 4.5 ml CM) and 1:5 PBMCs: CM (1 ml WB-equivalent PBMCs, 4 ml CM) were cultured in loosely capped 15 ml conical centrifuge tubes. A final concentration of 180 μg/ml PHA HA-15 (Remel Europe, Dartford, UK) was added to stimulate T lymphocyte proliferation. Duplicate cultures were prepared only for the first part of the study.

In the first part, 0, 2 and 4 Gy WB and PBMCs from 3 donors were cultured in three different conditions, differing in culture period and time of Cyt-B addition (48 h culture [Cyt-B at 24 h], 72 h culture [Cyt-B at 24 h], 72 h culture [Cyt-B at 44 h]). A final concentration of 4.5 or 6 μg/ml Cyt-B (Sigma-Aldrich) was added to PBMC and WB cultures, respectively, as the different Cyt-B concentrations are required to obtain optimal BNCs in each type of cell culture (*34*).

In the second part, DRCs were constructed with peripheral blood from another 3 donors irradiated with X rays at 1 Gy/min, at ten doses of 0, 0.15, 0.3, 0.5, 0.75, 1, 1.5, 2, 3, 4 Gy. Due to a high number of dose points, blood initially collected in lithium-heparin tubes was distributed to smaller volumes in 5 ml polystyrene tubes for irradiation. The type of cell culture (WB vs. PBMC) and type of CBMN assay (shortened 48 h culture [24 h at Cyt-B] vs. conventional 72 h culture [44 h at Cyt-B]) were compared.

After the end of cell culture, WB was harvested with the modified IAEA 2011 + 1% formaldehyde (Sigma-Aldrich) protocol while PBMCs were harvested with the protocol developed by the Chromosome Research Group in Hirosaki University, as described previously (*30, 31*). In WB harvest, cells were treated with cold 75 mM KCl, one round of 11:10:1 Ringer's solution: methanol: acetic acid + 1% formaldehyde and two rounds of 10:1 methanol: acetic acid, and centrifuged at 180 *g*, 10 min. Ringer's solution was prepared by dissolving 4.5 g NaCl, 0.21 g KCl and 0.12 g $CaCl_2$ in 500 ml distilled water (*13*). In PBMC harvest, cells were treated with cold 125 mM KCl + 1% formaldehyde, one round of 13:12:6 0.9% NaCl: methanol: acetic acid and two rounds of 4:1 methanol: acetic acid, and centrifuged at 800*g*, 25s.

CBMN Cell Spreading and Giemsa Staining for WB and PBMCs

High humidity spreading was performed with cells from PBMC cultures on microscope slides placed on a moist Kimwipe™. Cells from WB cultures were spread directly on benchtop as fixed WB cells were more susceptible to cell rupture in higher humidity (*30*). A minimum of 2 two-spot slides were prepared for each culture.

PBMCs were diluted in 300 and 500 µl fixative in 48 and 72 h cultures, respectively, while WB were diluted in 500 and 800 µl fixative for 48 and 72 h cultures, respectively. Cells were diluted differently as the cell pellet was larger in longer cultures and in WB cultures. Final cell volume was the same among all doses in the same culture condition. 15 µl was dropped in each spot.

After drying, slides were stained with 5% Giemsa (Merck Millipore, Burlington, MA) in pH 6.8 Gurr Buffer (Thermo Fisher Scientific) for 12 min and mounted with malinol (Muto Pure Chemicals, Tokyo, Japan).

Analysis of CBMN Endpoints (NDI, %BNC, Conventional and Triage MN Scoring)

CBMN endpoint analysis was manually performed by a single experienced scorer. Cells were scored with Olympus CX31 and CX33 (Olympus Co., Tokyo, Japan) at 400× magnification for NDI, %BNC and MN frequency. For NDI and %BNC, a minimum of 125 cells with intact cytoplasmic and nuclear membrane were scored per spot for a total of 500 cells. Equations for NDI (*35*) and %BNC are provided below. M_1, M_2, M_3, and M_4 indicate the number of cells with one, two, three or four daughter nuclei, respectively, and N is the total number of cells analyzed.

$$\mathrm{NDI} = \frac{M_1 + 2M_2 + 3M_3 + 4M_4}{N}$$

$$\%\mathrm{BNC\ in\ all\ cells} = \frac{M_2}{N} \times 100\%$$

BNC identification and MN scoring were performed in accordance with the standardized criteria compiled by the Human MicroNucleus project (*15*). In conventional MN scoring for 0 and 2 Gy samples, a minimum of 250 BNCs was scored per spot per slide, for a total of 1,000 BNCs. In conventional MN scoring for 4 Gy samples, a minimum of 125 BNCs was scored per spot per slide, for a total of 500 BNCs. In triage MN scoring for 0 and 2 Gy samples, a minimum of 200 BNCs was scored per spot. In triage MN scoring for 4 Gy samples, a minimum of 100 BNCs was scored per spot. Detailed information on conventional and triage MN scores and time can be found in Supplementary Tables S1, S2 and S3 (https://doi.org/10.1667/RADE-00191.1.S1; https://doi.org/10.1667/RADE-00191.1.S2; https://doi.org/10.1667/RADE-00191.1.S3, respectively).

DRC Construction with Shortened 48 h and Conventional 72 h CBMN Assay

Due to a limited number of donors available for DRC construction, a modified approach was used instead of the recommended approach of multiple DRCs constructed for 3 age groups separately for males and females by ISO 17099 (*11*). In our DRC construction, radiation-induced MN/BNC (MN/BNC at irradiated doses – MN/BNC at 0 Gy) was used instead of observed MN/BNC to account for the donor-specific background MN frequency in males and females of different ages. To reduce the risk of upper and lower dose over-estimation in the 95% confidence limit, a pooled induced MN/BNC from the 3 donors was used for DRC construction (*36*).

For increased statistical reliability, a minimum of 10,000 BNCs for 0, 0.15 and 0.3 Gy, 5,000 BNCs for 0.5 and 0.75 Gy, 3,000 BNCs for 1, 1.5, 2 Gy, 1,500 BNCs for 3 Gy and 1,000 BNCs for 4 Gy were manually scored in Giemsa-stained slides per donor and condition at 400× magnification.

Poisson distribution was first verified in observed MN distributions with GOF Poisson R files kindly provided by M. Higueras. The R files are based on the same Shiny R application developed by Fernández-Fontelo et al. and Higueras et al. (*37, 38*). The dispersion index and results from over-dispersion (*u* test), zero-inflated Poisson (ZIP) (*Z*-test) and Bayesian (ZIP versus Poisson) tests were reported (*39–42*).

As the observed MN distributions showed mixed conclusions after Poisson validation (Supplementary Table S4; https://doi.org/10.1667/RADE-22-00191.1.S4), with lower doses often showing over-dispersion, a Quasipoisson model was used for generalized linear modelling. DRCs were constructed using BiodoseTools (*43*) with iteratively reweighted least squares. Linear-quadratic DRC coefficients (C, α, β), their standard errors and P values calculated with *F* test were reported.

Dose Estimation

Dose estimation was performed in WB and PBMC cultures of 48 h culture (24 h at Cyt-B) and 72 h culture (44 h at Cyt-B), using conventional radiation-induced MN frequency scored in 500/1,000 BNCs and triage radiation-induced MN frequency scored in 100/200 BNCs and their respective DRCs with Dose Estimate v 5.2 (*44*).

Other Statistical Analysis

Average values of NDI, percentage of BNC and MN frequency from duplicate cultures of the first part of the study were used in the figures. Raw data of duplicate cultures can be found in Supplementary Table S5 (https://doi.org/10.1667/RADE-22-00191.1.S5). Moreover, the coefficient of variance (CV) was within 20% (*11*) in 2 and 4 Gy MN frequency in 500/1,000 BNCs (Supplementary Table S1; https://doi.org/10.1667/RADE-22-00191.1.S6). Graphical representation and statistical analyses were carried out with R ver 4.2.2 (*45*), RStudio v 2022.07.2 Build 576 (*46*) and the ''tidyverse'' package (*47*). Parametric statistics of *t*-tests and one-way ANOVA were carried out in GraphPad Prism 9.5.0 after verification of both normality and equal variance assumptions. P values < 0.05 were significant.

RESULTS

Cell-Cycle Progression Parameters in 48 h (24 h at Cyt-B), 72 h (24 h at Cyt-B) and 72 h (44 h at Cyt-B)

NDI and %BNC were first compared in the three culture conditions (Fig. 1A). NDI and %BNC were significantly

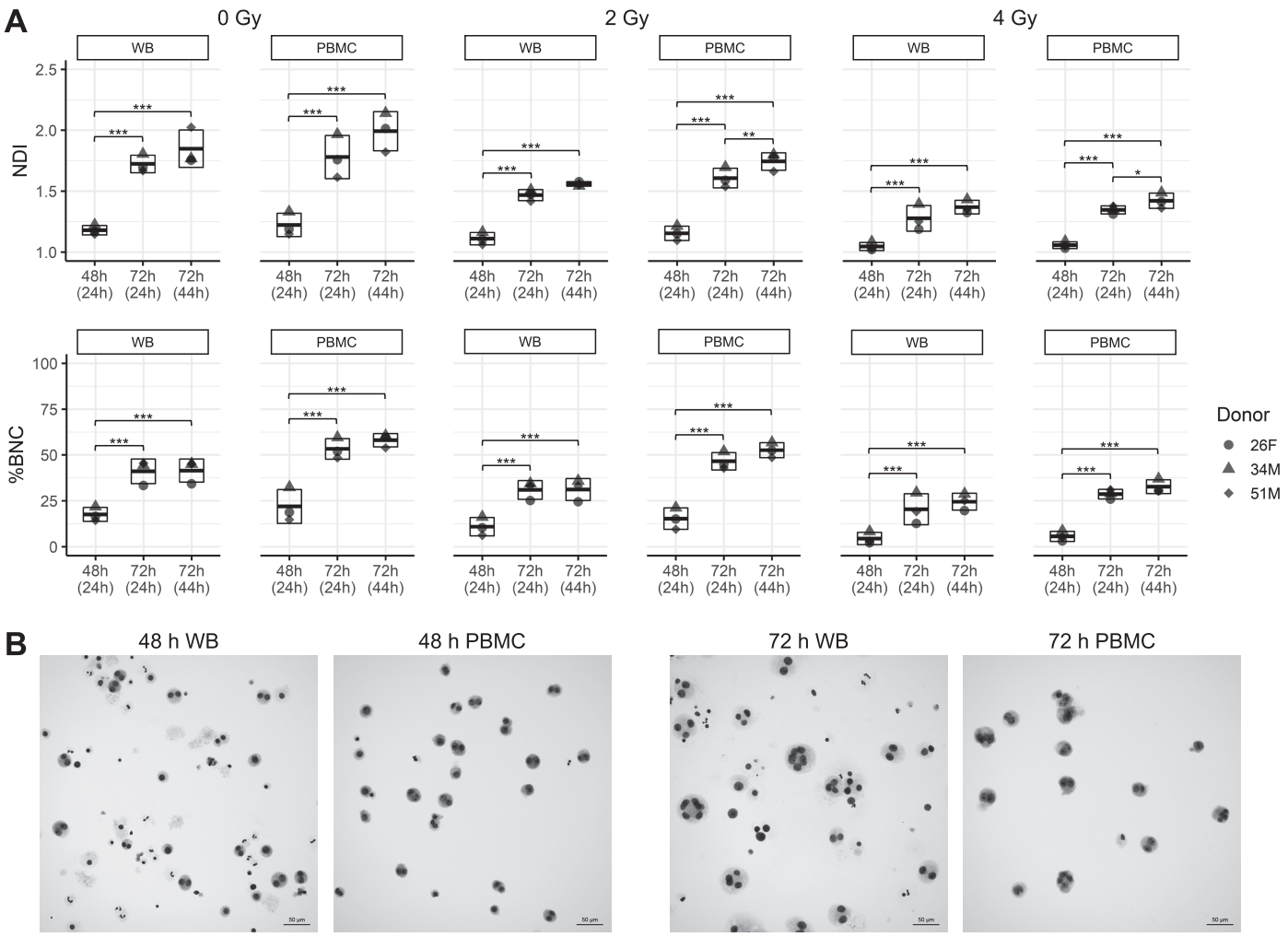

FIG. 1. Panel A: NDI and percentage of BNC (%BNC) in all culture conditions for 0, 2 and 4 Gy WB and PBMCs. The 500 cells with intact cytoplasmic and nuclear membrane were scored. Average values from duplicate cultures are shown. Cross bars represent Mean ± SD. One-way ANOVA with Tukey's post hoc test was performed. *$P < 0.05$, **$P < 0.01$, ***$P < 0.0001$. Panel B: Representative images of 0 Gy 48 and 72 h WB and PBMC cultures under a light microscope. Scale bars represent 50 μm.

lower in 48 h than 72 h cultures for 0, 2 and 4 Gy cells. In 72 h cultures, length of Cyt-B addition did not greatly affect cell-cycle progression as mostly similar NDI and %BNC were seen for 24 h at Cyt-B and 44 h at Cyt-B. Likewise, as seen in representative images at 0 Gy (Fig. 1B), 48 h cultures showed many mono- and binucleated cells while 72 h cultures showed many multi-nucleated cells.

Conventional MN Frequency and Observed Triage MN Distribution in 48 h (24 h at Cyt-B), 72 h (24 h at Cyt-B) and 72 h (44 h at Cyt-B)

In Fig. 2, conventional MN frequencies in both WB and PBMCs were compared in the three culture conditions. Similar, but not significantly different, MN frequencies between WB and PBMCs were seen in 0, 2 and 4 Gy cells for all conditions except for 4 Gy 48 h (24 h at Cyt-B). Variations of MN frequencies in PBMC cultures were wider than WB cultures.

Next, Fig. 3 shows the observed triage MN distributions in 2 and 4 Gy WB and PBMC cultures. Triage MN distributions were adjusted to 200 BNCs for 2 Gy and 100 BNCs for 4 Gy samples. As seen from the line graphs, MN distribution patterns were distinct between 2 and 4 Gy cultures regardless of culture type and condition.

DRCs of Shortened 48 h (24 h at Cyt-B) and Conventional 72 h (44 h at Cyt-B) CBMN Assay

As culture period and length of Cyt-B treatment had some effect on cell-cycle progression and MN frequency, we focused on subsequent comparisons between the shortened 48 h culture (24 h at Cyt-B) and the more frequently used conventional 72 h culture (44 h at Cyt-B). For both WB and PBMCs, DRCs were constructed with pooled radiation-induced MN/BNC to account for the different age and gender of the three donors (Fig. 4). DRC coefficients and SE, and their F test P values were shown in Table 1.

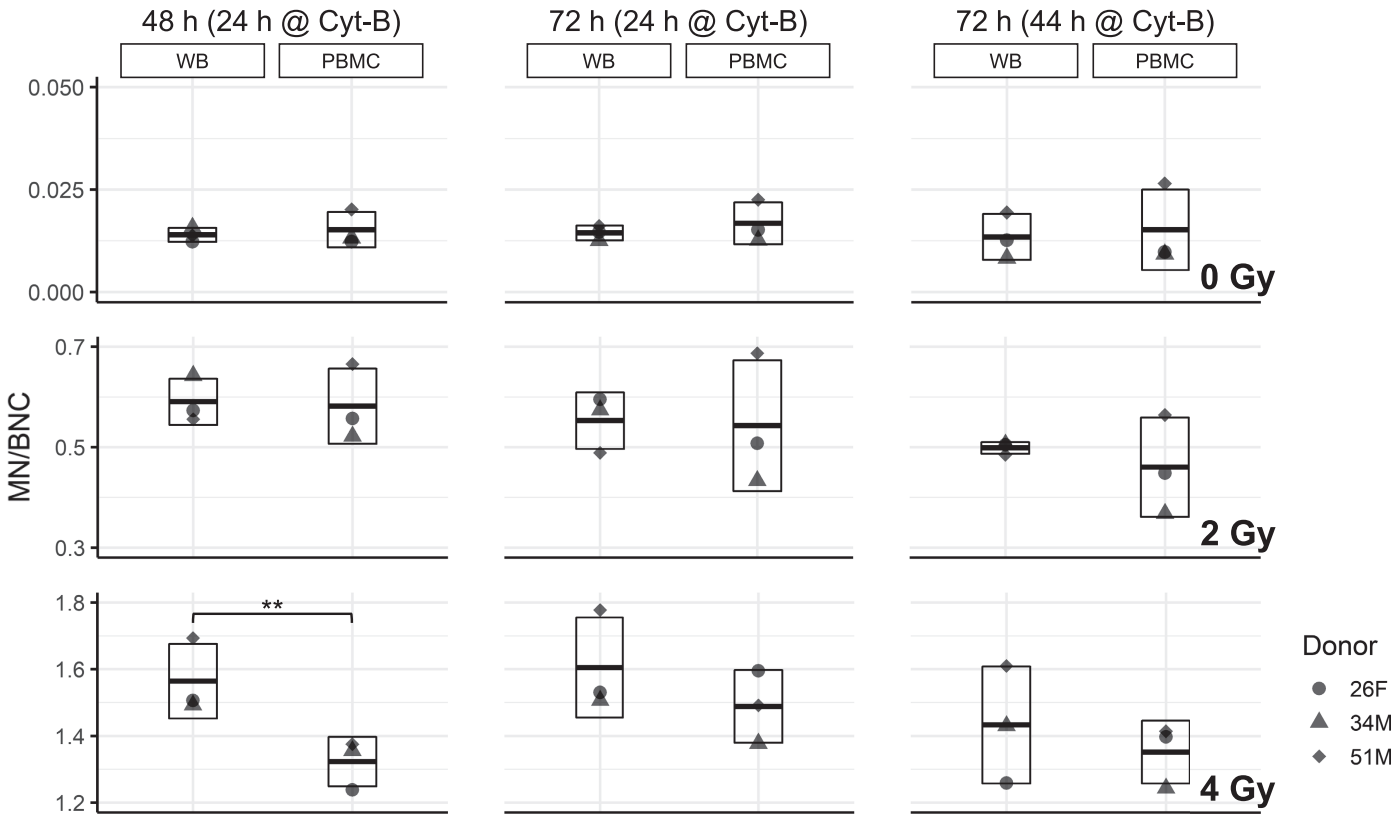

FIG. 2. Conventional MN frequencies of WB and PBMCs cultured in 48/72 h and Cyt-B added at 24/44 h. Average values from duplicate cultures are shown. Cross bars represent Mean ± SD. Unpaired two-tailed *t* test was performed. **P < 0.01.

Despite differences in DRCs seen in each donor (Supplementary Fig. S1, Table S6; https://doi.org/10.1667/RADE-22-00191.1.S6), pooled DRCs were very similar for WB and PBMC cultures of 48 h, while DRCs differed between WB and PBMC cultures of 72 h.

Time Taken for Triage MN Scoring

In Table 2, the average time taken for triage MN scoring for 0, 2 and 4 Gy was shown. For 48 h cultures of both WB and PBMCs, scoring time was within 8 min for 0 Gy, and 20 min for 2 and 4 Gy. For 72 h cultures of both WB and

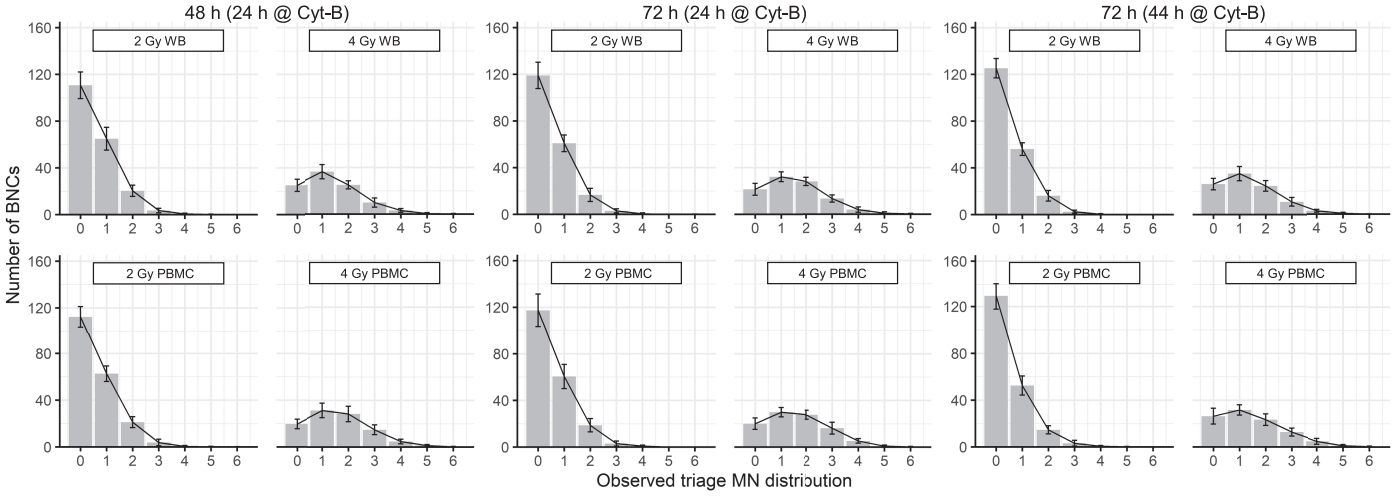

FIG. 3. Observed triage MN distributions for 2 and 4 Gy WB and PBMCs in three culture conditions. As shown by the line graph, MN distribution patterns were distinct between 2 and 4 Gy samples regardless of culture type and conditions. MN distributions were adjusted to 200 BNCs for 2 Gy and 100 BNCs for 4 Gy. Bar chart represents the mean while error bars show SD of three donors.

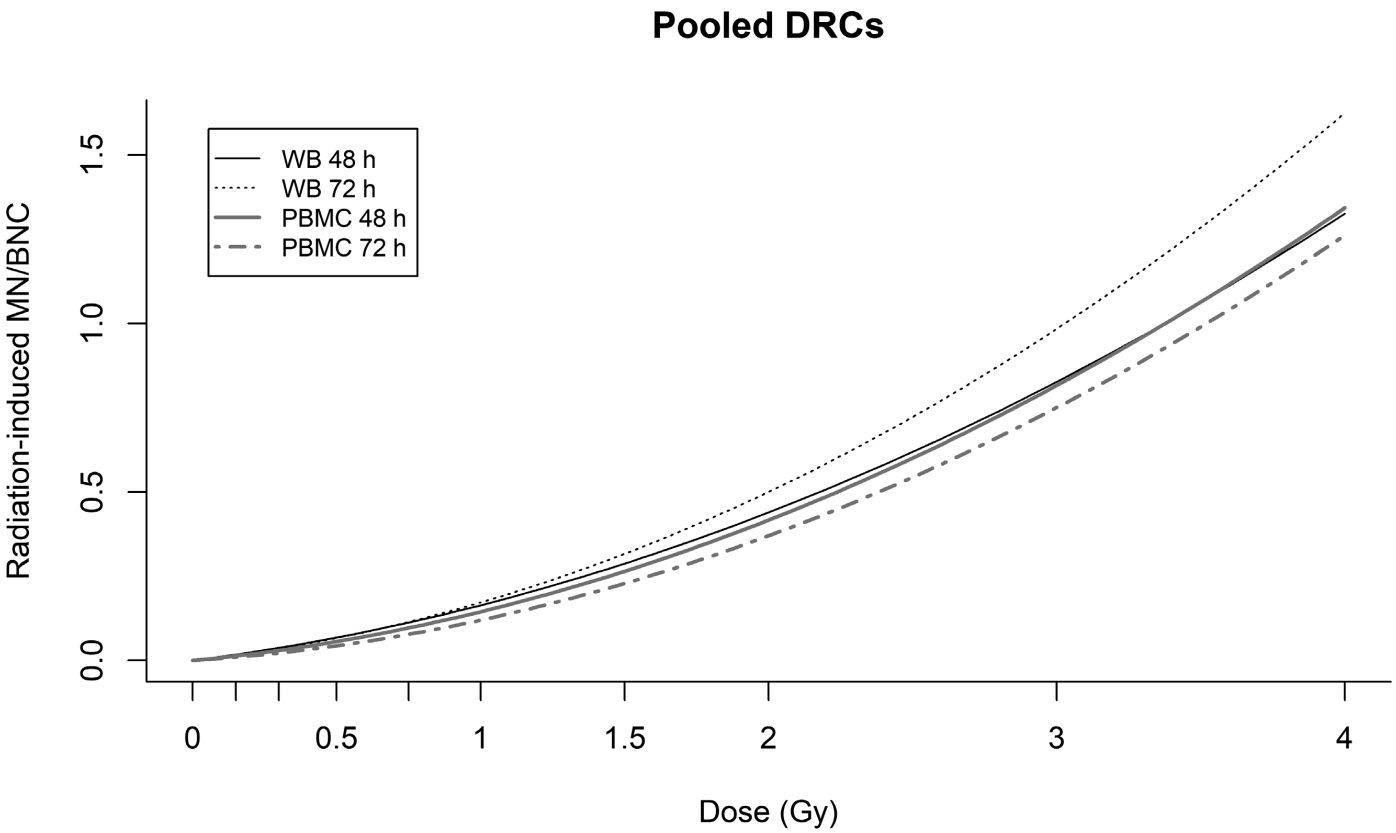

FIG. 4. Pooled DRCs of shortened 48 h (24 h at Cyt-B) and conventional 72 h (44 h at Cyt-B) WB and PBMC cultures with radiation-induced MN/BNC.

PBMCs, scoring time was within 4 min for 0 Gy, 9 min for 2 Gy and 12 min for 4 Gy.

Dose Estimations Compared with the Number of BNCs Scored and Culture Period

In Fig. 5, both triage and conventional dose estimations were compared based on the number of BNCs scored (i.e., triage MN scoring in 100/200 BNCs vs. conventional MN scoring in 500/1,000 BNCs) and culture period (i.e., shortened 48 h vs. conventional 72 h culture). For triage dose estimates, error bars depicted the minimum and maximum estimated doses if dose estimations were reported based on the first 100/200 BNCs for triage in a realistic emergency triage situation, while the mean was the dose estimated from the averaged MN scores. The number of BNCs scored for MN did not affect dose estimation as doses were within ± 0.2 Gy. Estimated doses from averaged MN scores were mostly within ± 0.5 Gy of the actual doses and were generally higher in 48 than 72 h cultures.

DISCUSSION

Triage scoring and dose estimation with cytogenetic markers during a radiation mass-casualty should be efficient and reliable, such that individuals exposed to whole-body equivalent ≥ 2 Gy can be quickly distinguished from the worried well for immediate medical treatment. The current ''gold standard'' of DCA requires only 48 h for cell culture, but is limited by the long scoring time and prior knowledge

TABLE 1
Coefficients and Statistical Results of Pooled CBMN DRCs

Culture condition	Culture type	C ($\pm$ SE$_C$)	P value (C)	α ($\pm$ SE$_\alpha$)	P value (α)	β ($\pm$ SE$_\beta$)	P value (β)
48 h (24 h at Cyt-B)	WB	8.14E-08 $\pm$ 4.92E-03	-	1.08E-01 $\pm$ 1.66E-02	6.77E-04	5.59E-02 $\pm$ 6.72E-03	1.42E-04
	PBMC	8.26E-08 $\pm$ 4.57E-03	-	8.08E-02 $\pm$ 1.63E-02	3.24E-03	6.38E-02 $\pm$ 7.01E-03	7.93E-05
72 h (44 h at Cyt-B)	WB	8.12E-08 $\pm$ 5.29E-03	-	9.41E-02 $\pm$ 1.90E-02	3.34E-03	7.80E-02 $\pm$ 8.04E-03	5.20E-05
	PBMC	7.86E-08 $\pm$ 2.81E-03	-	5.45E-02 $\pm$ 1.05E-02	2.59E-03	6.53E-02 $\pm$ 4.63E-03	4.28E-06

Notes. Linear-quadratic DRC equation is represented as $Y = C + \alpha D + \beta D^2$, where C, α, β are coefficients, Y is the radiation-induced MN/BNC and D is the dose. Curve fitting was performed with Biodose Tools. P values of C were excluded as radiation-induced MN frequencies at 0 Gy were adjusted to 0.

TABLE 2
Time Taken for Triage MN Scoring in Three Culture Conditions

Culture period	Dose	Culture type	Scoring time (min:s)		
			26-year-old female	34-year-old male	51-year-old male
48h (24h at Cyt-B)	0 Gy	WB	6:32 ± 0:37	3:48 ± 0:17	7:45 ± 0:28
		PBMC	5:39 ± 0:40	4:37 ± 0:28	8:04 ± 1:24
	2 Gy	WB	12:59 ± 0:48	7:43 ± 0:38	18:34 ± 2:01
		PBMC	9:49 ± 0:46	10:19 ± 0:28	~25 min*
	4 Gy	WB	14:18 ± 1:49	10:42 ± 0:59	~20 min*
		PBMC	14:27 ± 1:41*	9:23 ± 1:15	~20 min*
72h (44h at Cyt-B)	0 Gy	WB	3:34 ± 0:14	2:16 ± 0:09	2:51 ± 0:12
		PBMC	2:40 ± 0:21	2:50 ± 0:16	3:33 ± 0:22
	2 Gy	WB	8:53 ± 0:34	5:33 ± 0:30	5:50 ± 0:30
		PBMC	4:51 ± 0:34	6:21 ± 0:53	7:34 ± 0:48
	4 Gy	WB	11:34 ± 0:55	3:53 ± 0:21	4:41 ± 0:41
		PBMC	7:39 ± 0:24	5:20 ± 0:28	7:43 ± 0:52

Notes. Time taken to score 200 BNCs were recorded for 0 and 2 Gy. Time taken to score 100 BNCs were recorded for 4 Gy. Time taken was expressed as Mean ± SD.

* As insufficient cells were scored, estimated time taken to scan the whole drop was recorded.

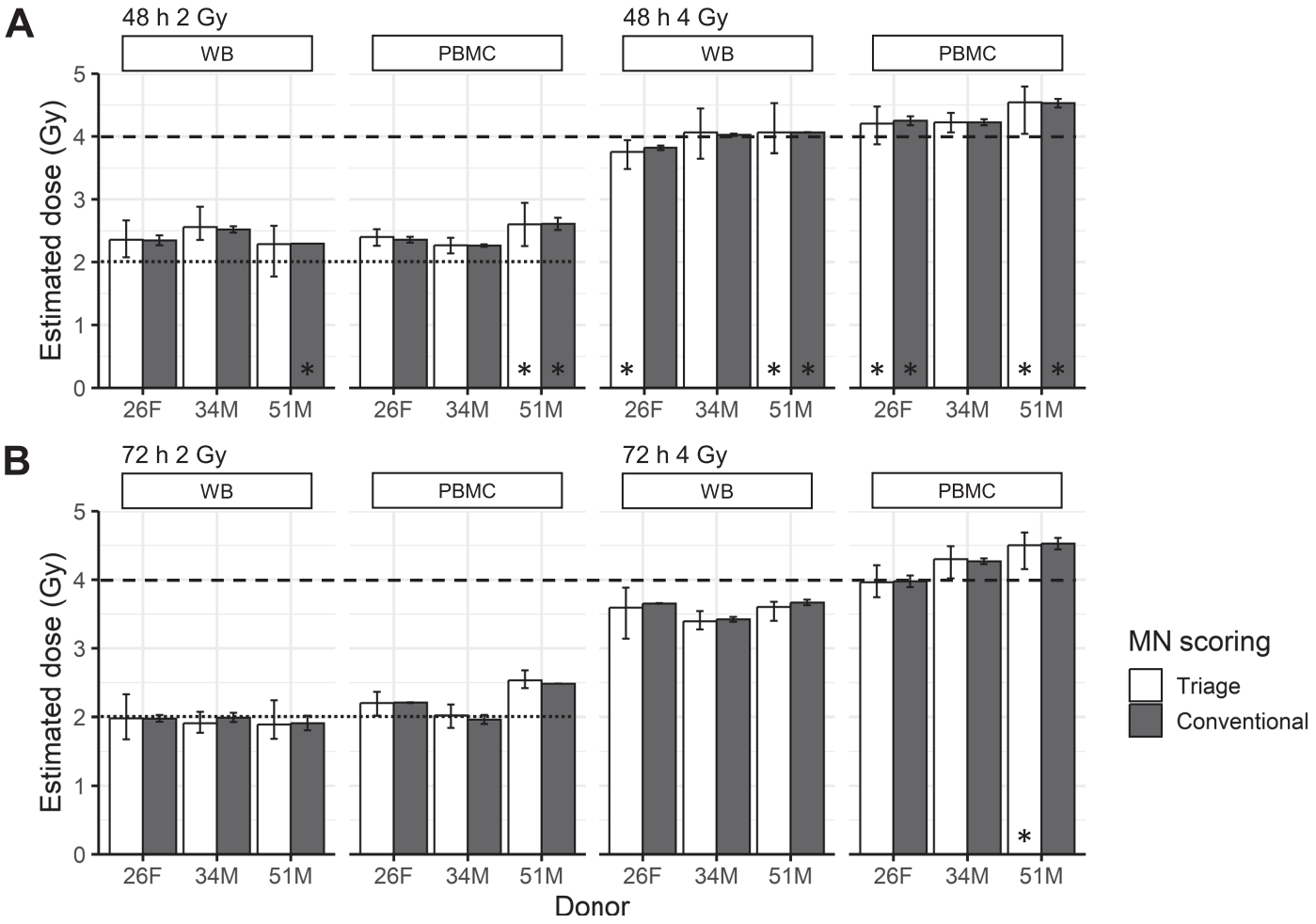

FIG. 5. Estimated doses from triage and conventional MN scoring of actual doses of 2 and 4 Gy. Bar chart height shows the dose averaged from eight 15 μl spots for triage MN score and dose averaged from duplicate cultures for conventional MN score. Error bar shows the minimum and maximum estimated doses. *Dose estimates with extrapolated MN frequency due to insufficient cells scored. Panel A: 48 h (24 h at Cyt-B) WB and PBMC cultures. Panel B: 72 h (44 h at Cyt-B) WB and PBMC cultures.

of chromosome karyotypes for scoring. On the other hand, CBMN assay is conventionally cultured for 72 h, but scoring is much faster as it involves visually identifying MN with a ''mean diameter within 1/16th to 1/3rd of the main nuclei'' (*15, 34*).

Recent attempts in reducing reporting time for estimated doses involve automated scoring and robotics for high-throughput analysis, but they can be very costly. In our study, we showed that a shortened 48 h CBMN culture is feasible for triage assessment for both WB and PBMC cultures up to 4 Gy. For individuals with low mitogen response to PHA stimulation, more slides can be made for scoring. Acute radiation syndrome symptoms such as skin erythema, nausea, diarrhea and low blood cell counts can also be used simultaneously to identify individuals exposed to higher doses up to 4 Gy (*48*). Furthermore, dose estimates were within ± 0.5 Gy of actual doses and triage MN scoring in 100/200 BNCs was sufficient for accurate dose estimations. Using manual triage scoring with light microscopy, 0 Gy samples can be identified within 8 min while 2 and 4 Gy samples can be scored within 20 min, in slides with appropriate cell concentrations.

The 72 h (44 h at Cyt-B) CBMN culture is the most commonly used (*49*) as it was shown to obtain an optimal BNC frequency for both WB and PBMC cultures (*13, 34*), although 68–70 h cultures were also used by multiple laboratories (*50*). Our results showed that the length of Cyt-B treatment did not significantly affect NDI and percentage of BNC for 72 h cultures. In addition, while our results showed PBMCs with higher NDI and percentage of BNC than WB cultures, the opposite was instead seen in Ellard and Parry's study (*32*). It is thus important to evaluate the optimal CBMN culture conditions in WB and PBMCs for multiple individuals in each population as differences in individual susceptibility to Cyt-B had been shown even within the same age group (*51*).

Moreover, in Köksal et al.'s study, reducing CBMN culture time from 72 h to 48 h led to a conclusion that 48 h was too short to obtain desirable BNC frequencies for MN scoring (*52*). While this is undeniably true as a much lower percentage of BNC was seen in our 48 h cells (0 Gy: 15–32%, 2 Gy: 6–20%, 4 Gy: 2–9%), a sufficient number of BNCs was still able to be obtained for MN scoring of $>$ 10,000 BNCs in 0 Gy and $>$1,500 BNCs in 4 Gy for a 5 ml culture (500 μl WB) in the three donors used in DRC construction. Likewise, as shown by Rodrigues et al. (*22*) for 48 h cultures of 2 ml (200 μl WB), an approximate average of 1,400 and 800 BNCs scored in 0 and 4 Gy with imaging flow cytometry, respectively. Even though more time is needed to score MN in cultures showing low percentage of BNC, a shorter CBMN culture is highly applicable for triage as a reduced number of BNCs is scored to account for the lower percentage of BNC. In addition, by personal observation, it was easier to locate BNCs for MN scoring in 48 h cultures as cells were mostly mono- and bi-nucleated.

Some differences in MN frequency between shortened 48 h culture and conventional 72 h cultures were seen in our experiments in Figs. 2 and 4, though no strong conclusions can be drawn due to a low number of donors analyzed. In Rodrigues et al.'s study, a much lower MN frequency in 48 h (24 h at Cyt-B) than 72 h (24 h at Cyt-B) WB cultures in automatically scored cells with imaging flow cytometry (*22*), which could be due to damaged cells dividing slower than healthy cells (*53*). In Köksal et al.'s study comparing 48 h (24 h at Cyt-B) and 72 h (44 h at Cyt-B) CBMN assay, a lower MN frequency was also seen in 48 than 72 h WB cultures in manually scored cells (*52*). This finding was also supported other studies by Lee et al. (*20*) and Almássy et al. (*54*), where an increasing MN frequency was seen in elongated culture periods. However, differences in donor population could have also contributed to MN frequency variability (*55*). Nevertheless, a 48 h CBMN culture period was able to easily distinguish irradiated from unirradiated samples, thus supporting the use of a shortened CBMN assay for radiation triage as results are able to be reported at a much quicker time. It also reinforces the recommendation that the same culture protocols should be used for CBMN DRC construction and dose estimation.

Likewise, Cyt-B addition at 24 h or 44 h for conventional 72 h cultures could affect MN frequency and estimated doses. Our observation of a slightly higher MN frequency in 72 h (24 h at Cyt-B) than 72 h (44 h at Cyt-B) culture was also seen by Lee et al. (*20*) and Köksal et al. (*52*). When Cyt-B was added at 24 h in 72 h cultures, MN were likely scored in BNCs arrested after the first cell division. When Cyt-B was added at 44 h instead, MN were likely scored in BNCs arrested after the first cell division, if mitosis was delayed, and after the second cell division. As MN contains unstable aberrations such as acentric fragments or whole chromosomes (*34*), MN frequency could decrease with multiple cell divisions. As stated in EPR-Biodosimetry, Cyt-B should be preferably added at 24 h for ''biological dosimetry to ensure only first division cells are captured'' (*13*). As supported in Duthoo et al.'s study (*53*) of PHA-stimulated non-irradiated and irradiated T cell cycles, the estimated total cell division time was 16.6 h in non-irradiated cells. First-cycle divisions were completed in 48–72 h and unaffected by irradiation of up to 2 Gy after PHA stimulation. Furthermore, prolonged G2/M arrest was observed with increasing dose up to 4 Gy. The length of Cyt-B treatment is thus an important factor affecting MN frequency and hence, consistency in protocols for CBMN DRC construction and dose estimation is important.

Furthermore, to the best of our knowledge, this is the first time CBMN DRCs were constructed for the same donors comparing WB and PBMC cultures of 48 h (24 h at Cyt-B) and 72 h (44 h at Cyt-B). The 48 h cultures showed similar DRCs for WB and PBMCs, while a higher DRC was seen in 72 h cultures of WB than PBMCs. Similarly seen in Rodrigues et al.'s study, a much higher DRC was seen in 72 h than 48 h WB cultures for Cyt-B added at 24 h (*22*).

Likewise, Sioen et al. also showed a higher DRC in 70 h (23 h at Cyt-B) WB than fresh PBMC cultures of up to 2 Gy (*53*). On the contrary, in Lue et al.'s study comparing 70 h (44 h at Cyt-B) 50 μl WB and 500 μl PBMC cultures, DRCs in both types of cultures were very similar up to 6 Gy (*56*). Further study is required to better understand the differences in DNA repair mechanics and MN induction after irradiation in WB and PBMCs. Once again, population differences could have influenced the dose-response relationships observed, and hence CBMN assays of multiple conditions should be separately evaluated in each laboratory. Despite the differences in DRCs seen, our dose estimations of 48 and 72 h WB and PBMC cultures were mostly within ±0.5 Gy of actual doses.

MN scoring in 200 BNCs was shown by McNamee et al. to reliably identify individuals exposed to ≥1 Gy gamma irradiation with manual scoring on acridine orange-stained cells (*12*), and thus 200 BNCs is recommended by ISO 17099 (*11*) for initial triage assessment. For higher doses, 200 total MN can also be scored (*12*). We also showed that 100/200 BNCs were sufficient for triage dose estimates as doses were within ±0.2 Gy as compared to conventional MN scoring. Furthermore, for higher doses, 100 BNCs could be scored instead of 200 BNCs. As our paper's focus was on manual MN scoring with a multiple unit mechanical tally counter, the direct data output was the number of cells with 0 to 6 MN (i.e., MN distribution). However, additional calculations were required to obtain total cells scored and total MN. To quicken triage identification in different dose categories, we have shown that observed triage MN distribution can be directly used to distinguish 2 and 4 Gy WB and PBMC cultures for both 48 and 72 h cultures.

However, depending on the 15 μl spot analyzed by the same scorer, triage MN of 48 h cultures in 8 spots scored showed differences of up to 60 MN in 200 BNCs (CV: 7–17%, ±0.8 Gy) in 2 Gy and 50 MN in 100 BNCs (CV: 5–12%, ±0.8 Gy) in 4 Gy. In an international inter-laboratory MN scoring exercise (*57*), the median intra-scorer CV of 2 spots for 1 and 2 Gy for 1,000 BNCs was 14 and 11%, respectively. Our previous study also showed differences of up to 60 MN in 1,000 BNCs and inter-scorer CV of 1–10% in 2 Gy (*31*). As manual MN scoring is highly dependent on visual interpretation, doses estimated could result in the false-negative rejection for the triage threshold dose of 2 Gy. Some donors also showed low percentage of BNC as insufficient BNCs were scored for triage analysis on 2 and 4 Gy PBMC for a 15 μl spot after 48 h CBMN culture. As such, if irradiated individuals were identified with 48 h CBMN triage, we highly recommend multiple scorers to score the same 2-spot slide(s). If the CV is within 20%, the average MN frequency can be used for more reliable dose estimation.

Our study, however, has several limitations. First, only one scorer was used in this study as many slides needed to be analyzed for two experiments and six donors. Second, we did not compare the time taken from culture to dose estimate report production between manual and automated MN scoring. Third, duplicate cultures were not performed in DRC construction. As 30 ml peripheral blood from each donor was required for culture set-up of 4 conditions, duplicate cultures were not feasible due to lack of manpower and blood sample volume restrictions according to ethical guidelines in our study. Fourth, only three donors of similar age were used for DRCs. According to the ISO standard, at least three donors in different age groups and gender should be used for CBMN DRCs (*11*). Fifth, this study focused on the ideal radiation scenario of a homogenous whole-body irradiation. Accidental heterogeneous high dose exposures account for more than 95% of the cases (*58*). As this study serves as an initial evaluation on the feasibility of manual MN scoring in 48 h cultures for triage, the above limitations will be addressed in the next study. Lastly, individual donor variations were seen in both parts of the study and could affect dose estimation reliability. It was to be expected as MN is not as radiation specific as dicentrics and many factors such as age, gender and smoking history could have contributed to the variations. Increased sensitivity, especially in the lower doses, could be achieved by combining MN scoring with centromere staining to distinguish MN containing acentric fragments or whole chromosomes (*59*). However, at the higher doses of 0.75–2 Gy, similar dose estimates and their confidence limits were obtained in MN frequencies with or without centromere staining (*60*). From the triage point-of-view, conventional MN scoring is sufficient for high dose identification, though estimated dose differences caused by donor or scorer variation prove difficult to resolve.

In conclusion, our study showed that with a low-cost method of manual MN scoring on Giemsa-stained cells and shortened CBMN assay to 48 h (24 h at Cyt-B) for both WB and PBMCs, radiation triage identification of individuals exposed to a whole-body equivalent dose of ≥2 Gy can be easily performed. In our donor population, triage MN scoring was completed in 8 min for 0 Gy and in 20 min for 2 and 4 Gy samples. Discrimination between 2 or 4 Gy exposure was feasible based on observed MN distribution patterns. Triage dose estimates were also mostly within ±0.5 Gy of actual doses. We thus highly recommend the 48 h CBMN assay for initial triage in a radiation mass-casualty accident. If more reliable dose estimates are required, dose estimation with 1,000 BNCs in CBMN assay or 500 metaphases in DCA can be additionally performed on selected individuals.

SUPPLEMENTARY MATERIALS

Supplementary Table S1. Coefficient of variance (CV) for conventional MN frequency in duplicate cultures (Expt 1)

Supplementary Table S2. Scoring time (min:s) in (a) 48 h (24 h at Cyt-B) WB and (b) PBMC cultures; (c) 72 h (24 h at Cyt-B) WB and (d) PBMC cultures; (e) 72 h (44 h at Cyt-B) WB and (f) PBMC cultures.

Supplementary Table S3. Observed triage MN frequency in (a) 48 h (24 h at Cyt-B) WB and (b) PBMC cultures; (c) 72 h (24 h at Cyt-B) WB and (d) PBMC cultures; (e) 72 h (44 h at Cyt-B) WB and (f) PBMC cultures.

Supplementary Table S4. Raw data and Poisson test results for DRC construction for (a) 48 h (24 h at Cyt-B) WB and (b) PBMC cultures; (c) 72 h (44 h at Cyt-B) WB and (d) PBMC cultures.

Supplementary Table S5. Raw data of (a) NDI and (b) percentage of BNC in duplicate cultures (Expt 1)

Supplementary Fig. S1. CBMN DRCs of (a) each donor and (b) each culture condition.

Supplementary Table S6. Coefficients and statistical results of each donor's CBMN DRCs in 4 culture conditions.

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