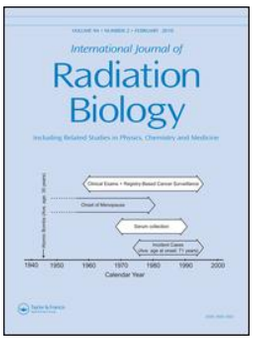

International Journal of Radiation Biology

ISSN: 0955-3002 (Print) 1362-3095 (Online) Journal homepage: http://www.tandfonline.com/loi/irab20

# Influence of anticoagulants and storage temperatures on blood counts and mitotic index of blood samples collected for cytogenetic biodosimetry

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**To cite this article:** Yohei Fujishima, Syuki Kanahama, Shigeki Hagino, Shiori Natsubori, Hitoshi Saito, Ayaka Azumaya, Kentaro Ariyoshi, Akifumi Nakata, Kosuke Kasai, Kyogo Yamada, Yasushi Mariya, Mitsuaki A. Yoshida & Tomisato Miura (2018): Influence of anticoagulants and storage temperatures on blood counts and mitotic index of blood samples collected for cytogenetic biodosimetry, International Journal of Radiation Biology, DOI: 10.1080/09553002.2019.1539882

**To link to this article:** https://doi.org/10.1080/09553002.2019.1539882

Accepted author version posted online: 29 Oct 2018.
Published online: 16 Nov 2018.

INTERNATIONAL JOURNAL OF RADIATION BIOLOGY
https://doi.org/10.1080/09553002.2019.1539882

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ORIGINAL ARTICLE



# Influence of anticoagulants and storage temperatures on blood counts and mitotic index of blood samples collected for cytogenetic biodosimetry

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**ABSTRACT**

**Purpose:** In order to establish suitable protocols of blood culture to obtain sufficient numbers of metaphases for dicentric chromosome assay (DCA), we have examined the effect of storage temperature, storage time, and anticoagulant type.

**Materials and methods:** Peripheral blood was collected from five healthy donors with lithium heparin and ethylenediaminetetraacetic acid dipotassium salt (EDTA-2K). These samples were irradiated with X-rays at 3 Gy or sham; the samples were further divided into groups that were either stored at room temperature (RT) or 5.2 ± 1.0 °C. After 6, 24, 48, 72, and 168 h of storage, both blood counts and the mitotic index (MI) were analyzed.

**Results:** Heparinized blood samples stored under cold conditions exhibited low white blood cell, lymphocyte, and platelet counts. EDTA-treated blood samples did not show such obvious changes in cell counts. After 6 h of storage, heparinized blood samples stored at RT had MI of 21.5–29.3%. Similar MI was obtained in the EDTA-washed group stored for 6, 24, 48, and 72 h.

**Conclusions:** Our study confirms that heparinized blood samples should be stored at RT to get sufficient metaphases for DCA, and that EDTA blood samples also can be used for blood culture after washing and storage under 5.2 ± 1.0 °C.

**ARTICLE HISTORY**
Received 13 June 2018
Revised 23 September 2018
Accepted 5 October 2018

**KEYWORDS**
Cytogenetic biodosimetry; mitotic index; blood storage; anticoagulants

## Introduction

In the event of a radiation accident, there are several methods by which a person's exposure level to radiation can be assessed. Amongst these, dicentric chromosome assay (DCA) known as one of the cytogenetic biodosimetry methods, is considered to be the gold standard in assessing radiation dose for cases of acute ionizing radiation exposure (IAEA 2011). This method is based on analyzing the frequencies of chromosomal aberrations, such as dicentric chromosomes (Dic) in lymphocytes cultured from blood samples; the radiation exposure dose can then be deduced using ex vivo irradiation calibration curves (Lloyd and Edwards 1983). As of now, it is suggested that DCA may also be used to assess radiation dose in case of low-dose rate chronic radiation exposure events, and as a validation method for medical exposure conditions (Jiang et al. 2000; Zakeri et al. 2011; Tanaka et al. 2013; Abe et al. 2015).

As mentioned above, the DCA using human peripheral blood lymphocytes (PBLs) is used routinely to detect and evaluate radiation-induced chromosomal aberrations in victims of radiation accidents and in the monitoring of populations facing occupational exposure to radiation hazards. In usual, blood samples that cannot be processed at the site of collection (such as a hospital) immediately are stored and transported to biodosimetry laboratories. According to the International Atomic Energy Agency (IAEA) recommendations, collected blood samples must be heparinized and stored at 18–25 °C before being cultured for DCA. However, it is often difficult to maintain samples within this temperature range without special equipment. Furthermore, blood samples exposed to changes in storage temperatures during shipping have been reported to exhibit loss of lymphocyte viability, due to which metaphase spreads for DCA cannot be obtained from such samples (Granath et al. 1996). An inter-laboratory comparison exercise simulating an accidental radiation emergency situation has shown that transport times of blood samples from the site of an emergency to the laboratories where the DCA was to be performed, ranged from 24 to 75 h (Wilkins et al. 2008; Oestreicher et al. 2017). A recent inter-laboratory comparison study has also shown that the transportation time of blood samples from the European

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Union to a Japanese laboratory was 63 h (Oestreicher et al. 2017). These studies have also shown that storage temperatures of the blood samples during transport could range from 11 to 30 °C; however, despite such large variations in temperature, blood cultures were successful and DCA was still possible on these transported samples.

In consideration of these inter-comparison exercises, however, it is necessary to confirm protocols and methodologies where blood culture is still possible in case samples for DCA must be stored and transported before being processed and analyzed. Since dose assessment using DCA must be performed in more than 1000 metaphase spreads for reliable results, it is important to clarify how conditions of blood sample storage influence the blood culturing process. In this study, we examine the effects of different anticoagulants, storage temperatures, time periods of storage, and ionizing radiation on the stability and usability of blood samples for DCA.

## Materials and methods

### Volunteers and blood collection

Peripheral blood samples were collected from five healthy male donors without any history of smoking and ionizing radiation exposure beyond those used for routine diagnostics. After obtaining informed consents from the volunteers, peripheral blood was collected by venipuncture into vacutainers containing either lithium heparin (BD Medical, Franklin Lakes, NJ) or ethylenediaminetetraacetic acid dipotassium salt (EDTA-2K) (BD Medical, Franklin Lakes, NJ) as an anticoagulant. The informed-consent forms were approved by the Committee of Medical Ethics of Hirosaki University Graduate School of Health Sciences, Hirosaki, Japan (approval number: 2012-278).

### Ex vivo irradiation and blood storage

Each blood sample was divided into 60 groups as per the experimental conditions it was exposed to; 0 or 3 Gy of irradiation, storage temperature of 5.2 ± 1.0 °C (cold condition) or 20.3 ± 0.1 °C (room temperature [RT]), storage for 6, 24, 48, 72, or 168 h, and treatment with two different types of anticoagulants: heparin and EDTA-2K. A subgroup of the EDTA-treated blood samples were grouped into an 'EDTA-wash' group, where these samples were suspended in RPMI 1640 medium (Thermo Fisher Scientific, Waltham, MA) supplemented with 2% heat-inactivated fetal bovine serum (FBS, Sigma-Aldrich, St. Louis, MO) and 60 μg/ml Kanamycin (Thermo Fisher Scientific, Waltham, MA), and centrifuged at 300 × *g* for 8 min; the cell pellets obtained through this procedure were resuspended in the same medium, and the procedure was repeated three times to remove EDTA from the cells before they were cultured in RPMI 1640. For irradiation treatments, blood samples were exposed to X-rays (150 kVp, 20 mA, with 0.5 mm Al, and 0.3 mm Cu filters, 1.0 Gy/min) using an X-ray generator (MBR-1520R-3, Hitachi Medical Corporation, Tokyo, Japan) at RT. The cumulative radiation dose was monitored with a thimble ionization chamber (TN31013, PTW, Freiburg, Germany) connected to the dosimeter (MZ-BD-3 (Type 153), Hitachi Medical Corporation, Tokyo, Japan), which was embedded to the X-ray generator in real time and the X-ray irradiation was automatically stopped at the set dose. The dosimeter and detector were calibrated annually by the Japan Quality Assurance Organization which satisfies national standard traceability and the ISO/IEC 17025 requirements. Following this, the irradiated blood samples were incubated at 37 °C for 2 h for DNA repair. The blood samples were stored in temperature-controlled shipping packages (TACPack 1525FS, Tamai Kasei Co. Ltd., Otaru, Japan) for the RT-condition group, or in refrigerators for the cold-condition group. During blood storage, temperature and humidity levels were monitored using a data logger (Thermo recorder TR-77Ui, T&D corp., Nagano, Japan).

### Blood count analyses and blood smear preparation

Blood counts were obtained using a fully automated hematology analyzer XE-5000 (Sysmex Corporation, Kobe, Japan) outfitted with a specially designed software. Following this, May-Grünwald and Giemsa (MGG)-stained blood smears were prepared by the slide maker Sp-1000i (Sysmex Corporation, Kobe, Japan) for microscopic observation using an Axio Imager.Z2 (Carl Zeiss Microscopy GmbH, Jena, Germany) of blood cells.

### Whole blood culture and harvest

Lymphocytes from all blood samples were cultured using a standard protocol for whole blood cell culture (IAEA 2011). Briefly, aliquots of whole blood (0.5 ml) were placed in 15 ml tubes containing 4.5 ml of RPMI 1640 medium supplemented with 20% heat-inactivated FBS, 60 μg/ml kanamycin, 2% phytohemagglutinin (PHA) (Remel Europe, Dartford, UK), and KaryoMAX colcemid (Thermo Fisher Scientific, Waltham, MA) at a final concentration of 0.05 μg/ml. The cultures were maintained in a humidified incubator at 37 °C with 5% $CO_2$ for 48 h. Cultured lymphocytes were harvested with hypotonic treatment using 75 mM potassium chloride and fixed in cold fixative (3:1 methanol/glacial acetic acid). The fixed cells were stored at −30 °C before slide preparation.

### Metaphase spreading and analysis of mitotic index

The fixed cells were centrifuged at 300 × *g* for 8 min to obtain cell pellets, which were then resuspended in cold fixative. Air-dried slides were prepared by the automated metaphase spreader HANABI-PIV (ADStec Co., Chiba, Japan) on pre-cleaned glass slides (Matsunami Glass Industry Ltd., Osaka, Japan). Each slide was immersed in 4% Giemsa solution (Merck KGaA, Darmstadt, Germany) with Gurr buffer (pH 6.8, Thermo Fisher Scientific, Waltham, MA) for 10 min, then washed with tap water and air-dried. The Giemsa-stained metaphase spreads and blast cells were counted up to at least 500 blasts under a microscope (Axio Imager.Z2, Carl

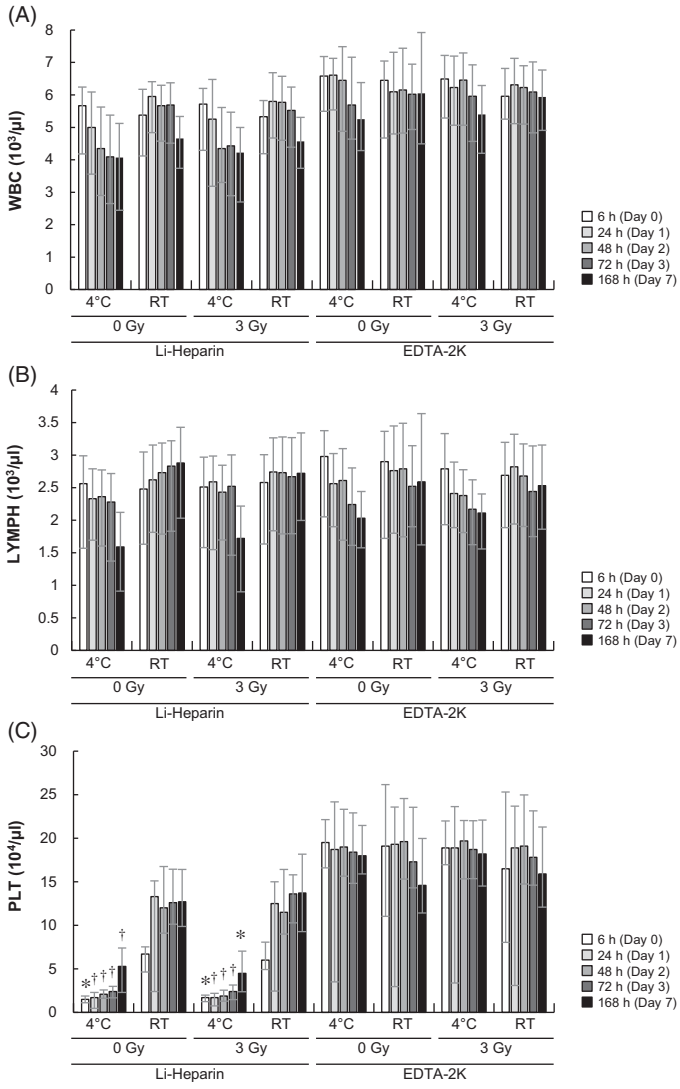


Figure 1. Effect of temperature, storage time, anticoagulant type, and irradiation on the cell counts. (A) White Blood Cells (WBCs), (B) lymphocytes, and (C) platelets. In panel (C), significant differences between RT-stored heparinized blood samples and other storage conditions at each time point as detected using the Wilcoxon rank sum test are marked with *indicates $p < .05$ and †indicates $p < .01$.

Table 1. The correlation between white blood cell (WBC), lymphocyte (LYMPH), platelet (PLT), and absolute immature platelet counts (A-IPC) versus storage time.

| | Storage temperature | Anti coagulant | 0 Gy | | 3 Gy | |
|---|---|---|---|---|---|---|
| | | | $\rho$ Value | $p$ Value | $\rho$ Value | $p$ Value |
| WBC | Cold | Li-Heparin | −0.486 | .01 | −0.502 | .01 |
| | | EDTA-2K | −0.396 | .05 | −0.469 | .02 |
| | RT | Li-Heparin | −0.224 | .28 | −0.188 | .37 |
| | | EDTA-2K | −0.006 | .98 | −0.112 | .59 |
| LYMPH | Cold | Li-Heparin | −0.463 | .02 | −0.298 | .15 |
| | | EDTA-2K | −0.486 | .01 | −0.479 | .02 |
| | RT | Li-Heparin | 0.259 | .21 | 0.218 | .30 |
| | | EDTA-2K | −0.018 | .93 | −0.128 | .54 |
| PLT | Cold | Li-Heparin | 0.790 | <.01 | 0.707 | <.01 |
| | | EDTA-2K | −0.232 | .27 | −0.228 | .27 |
| | RT | Li-Heparin | 0.471 | .02 | 0.571 | <.01 |
| | | EDTA-2K | −0.257 | .21 | −0.133 | .52 |
| A-IPC | Cold | Li-Heparin | 0.628 | <.01 | 0.729 | <.01 |
| | | EDTA-2K | 0.093 | .70 | 0.628 | <.01 |
| | RT | Li-Heparin | 0.531 | .02 | 0.620 | <.01 |
| | | EDTA-2K | 0.535 | .02 | 0.714 | <.01 |

Statistically, significances were assessed using Spearman's rank correlation coefficient.

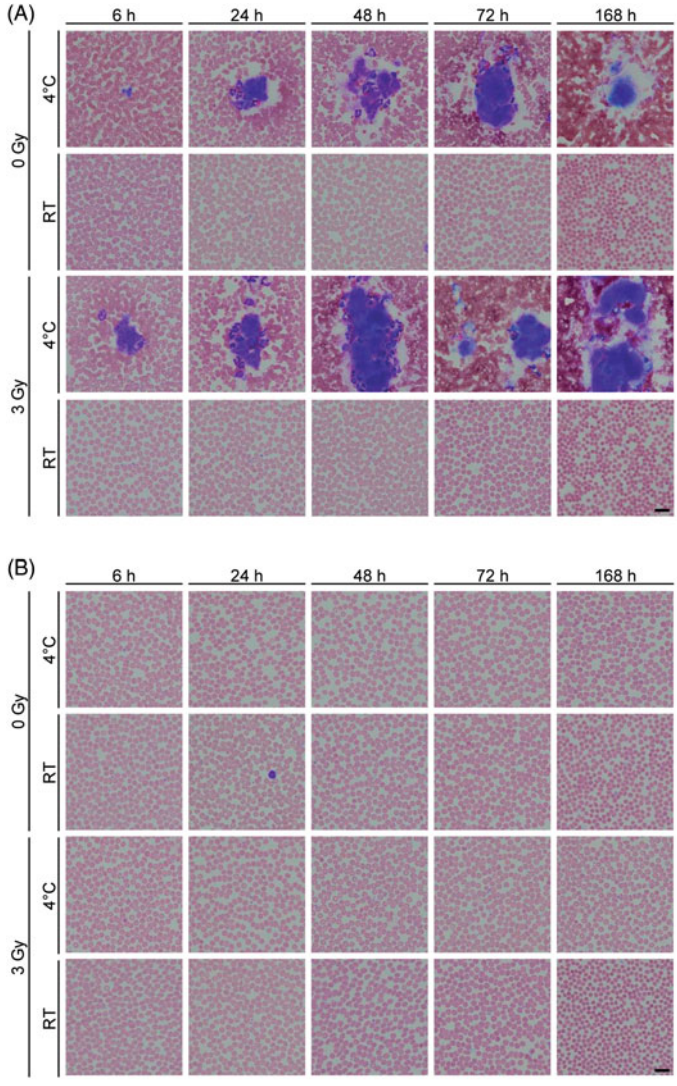


Figure 2. Microscopic observations of MGG-stained blood smears. (A) Heparinized blood. (B) EDTA-treated blood. Scale bars represent 20 µm.

Zeiss Microscopy GmbH, Jena, Germany) to obtain lymphoblast counts and record the number of cells in metaphase; following this the mitotic index (MI) was calculated as follows (IAEA 2011):

$$\text{MI}\ (\%) = \frac{\#\text{metaphases}}{\#\text{metaphases} + \text{blasts}} \times 100$$

## Statistics

All results are presented as median ±95% CI of five samples unless otherwise specified. Results were considered to be statistically significant if $P$ values <.05 were obtained. The correlation between white blood cell (WBC), lymphocyte, platelet, and absolute immature platelet counts versus storage time was assessed using Spearman's rank correlation coefficient. A two-way ANOVA was applied to the platelet count data to identify storage temperature and radiation effects for each storage time point. All statistical analyses were performed using R version 3.3.2 (R Core Team 2016).

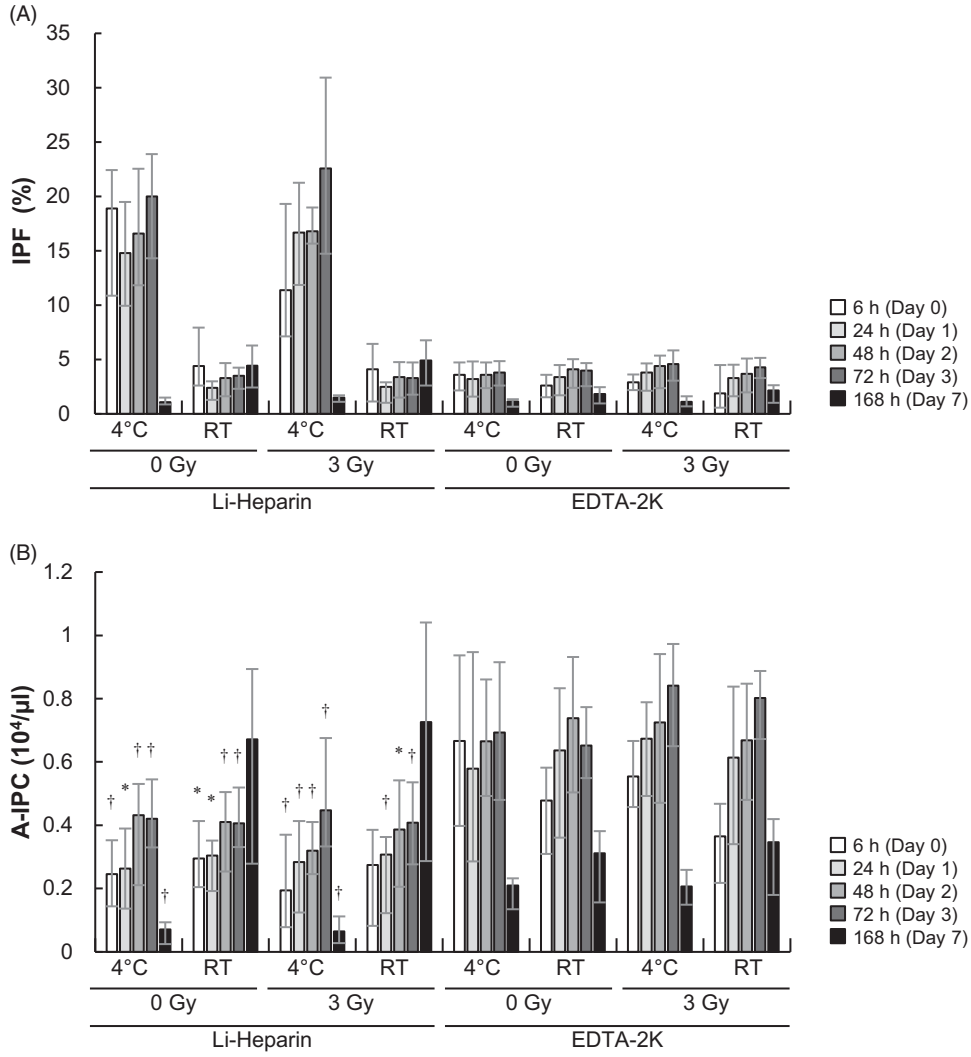


Figure 3. The transition of immature platelet fraction during blood storage. (A) IPF% and (B) A-IPC. In panel (B), significant differences between values of A-IPCs of heparinized blood samples under all storage conditions and those of the corresponding EDTA-treated blood samples using Wilcoxon rank sum test (one-sided) are marked with *indicates $p < .05$ and †indicates $p < .01$.

## Results

### *Effects of anticoagulant type, temperature, and storage time on blood counts of blood samples*

Changes in WBC counts over different time periods during storage were evaluated using the hematology analyzer XE-5000 (Figure 1(A)). WBC counts in blood samples stored under cold conditions were found to decrease significantly in all treatment groups (anticoagulant type and irradiation condition) as storage time increased (Table 1). In contrast, for blood samples stored at RT, changes in WBC counts were found to stabilize by 72 h and only a slight decrease is observed at 168 h. Lymphocyte counts (Figure 1(B) and Table 1) in all blood samples under different conditions were similar to those observed for WBC counts. In addition, we also found that irradiation treatment by 3 Gy does not significantly change WBC and lymphocyte counts during storage.

Platelet counts in cold-stored heparinized blood samples were found to decrease significantly as compared to that of RT-stored heparinized blood, irrespective of radiation exposure (Figure 1(C)). In particular, single platelets were absent in these samples under microscopic observations, but frequently exhibited the presence of clumped platelets detected by both the blood analyzer and microscopic observations (Figure 2(A)). This platelet-cluster formation was noted in samples at storage time points as early as 6 h, and the sizes and numbers of platelet clusters in such samples were found to increase with time. Moreover, radiation treatment appeared to enhance this cluster formation. It was also noted that heparinized blood samples showed increases in platelet counts (Table 1) as storage time increased. On the other hand, EDTA-treated blood samples did not show obvious changes in platelet counts except for samples stored at RT for 168 h, where clustering of cells was not identified (Figure 2(B)).

In our investigations, we have also observed that heparinized blood samples stored under cold conditions showed very high immature platelet fraction percentages (IPF%) (Figure 3(A)). The increase in IPF% in the irradiated group of these blood samples was found to be positively correlated with the storage time ($\rho = 0.605$, $p < .01$) until 72 h, though no such pattern was observed for the non-irradiated

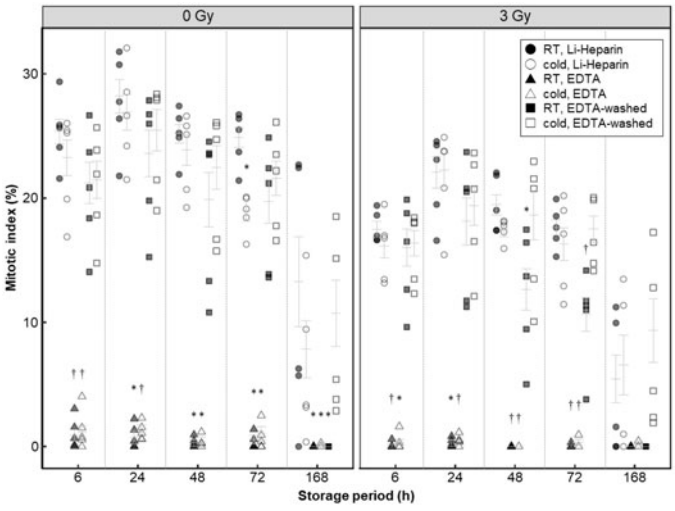


Figure 4. Effect of storage conditions on the mitotic index (MI) of blood samples: RT-stored (solid circle) and cold-stored (open circle) heparinized blood, RT-stored (solid triangle) and cold-stored (open triangle) EDTA-treated blood, and RT-stored (solid square) and cold-stored (open square) EDTA-washed samples. Each error bar represents median ±95% CI of five samples. Significant differences at each storage time point between RT-stored heparinized blood samples and other storage conditions as detected by the Wilcoxon rank sum test are marked with *indicates $p < .05$ and †indicates $p < .01$.

samples. In addition to the IPF% values, we also obtained measures of the absolute immature platelet count (A-IPC) by multiplying the IPF% with the optical platelet count (Figure 3(B)). Increases in the A-IPC values were observed in every storage group until 72 h (Table 1). Interestingly, values of A-IPCs of heparinized blood samples under all storage conditions were significantly lower than those of the corresponding EDTA-treated blood samples. Although there are large variations among five donors, we also observed that the A-IPC values of heparinized blood samples stored under cold conditions were highly reduced after 168 h of storage. In contrast, these values were extremely high for heparinized blood samples stored at RT.

### *Effects of anticoagulant type, temperature, and storage time on the MI of blood samples*

Figure 4 shows the effects of storage time, anticoagulant type, and irradiation on the MI values of blood samples. The non-irradiated heparinized blood samples stored at RT for 6 h demonstrate high MI values of 21.5–29.3%. MI values of these samples peaked at 24 h and continued to fall at later storage time points. All EDTA-treated blood samples had low MI values (only 0–4%); in addition, after 48 h of incubation for blood culture, it was observed that these blood samples became coagulated and very few cells were available for chromosome spreading. However, once the EDTA is removed by washing, MI values of 14.1–26.7% could be obtained (solid and open squares in Figure 4). Furthermore, it was observed that the MI values for all irradiated blood samples decreased by 5–10%.

Interestingly, it was possible to obtain fairly high MI values up to 72 h of storage from RT-stored heparinized, cold-stored heparinized, and cold-stored EDTA-washed blood samples. However, after 168 h of storage, blood samples from only 2 of the donors (donors A and D) still exhibited MI values of <15% under these three conditions. In contrast, MI values after 168 h of storage under the same conditions were found to be significantly lower (only 0–6.2%) in the other three donors. The kinetics of change in MI values for the blood samples from each donor, however, were similar for all three conditions (RT-stored heparinized, cold-stored heparinized, and cold-stored EDTA-washed samples).

## Discussion

According to IAEA recommendations, heparinized blood samples must be stored at 18–25 °C for use in cytogenetic biodosimetry (IAEA 2011); several inter-laboratory comparison studies have tested this recommendation and found it adequate for blood samples stored up to 75 h (Wilkins et al. 2008; Romm et al. 2012). Our results also suggest that heparinized blood stored at RT is the most suitable for cytogenetic dosimetry and tests that rely on measures of MI.

Heparin binds to antithrombin III and neutralizes the activity of thrombin and other coagulation factors (Rosenberg 1989). This neutralization activity is most effective at temperatures ranging between 37 and 25 °C, reduced at 15 °C, and incomplete at 0 °C (Machovich and Arányi 1978). Therefore, in heparinized blood samples stored under cold conditions, platelet clusters are formed, which create aggregations of WBCs, lymphocytes, and platelets; due to this, cell counts of these blood samples were tended to be lower than the EDTA-treated blood samples, becoming significantly reduced after longer storage periods. However, it is unclear why heparinized blood samples stored under cold conditions have increased IPF% values. Our results clearly indicate that it is inadvisable to store and transport heparinized blood samples for cytogenetic biodosimetry under cold conditions. Abe et al. reported that the analysis in 2000 metaphases is useful for detecting exposure to radiation doses less than 100 mSv as found in medical exposures like CT scan (Abe et al. 2015). Therefore, this transporting condition is especially important to obtain sufficient analyzable metaphases if an extremely low dose exposure is suspected.

It is well known that EDTA-treated blood samples are not suitable for blood culture to analyze chromosomes as cells from such samples show poor cell division and growth. Since EDTA chelates calcium ions, and calcium ions are necessary for PHA-induced lymphocyte transformation and cell-cycle progression (Elves et al. 1966; Whitney and Sutherland 1972), this anticoagulant can completely inhibit lymphoblast transformation at concentrations of 1.2–1.6 mM (Alford 1970). However, several studies showed that it is possible to obtain adequate cell cultures for metaphase spreads by supplementing EDTA-treated blood samples with metal ions (Chesters 1972; Inoue 1983). In this study, we have observed that if the EDTA in EDTA-treated blood samples stored under cold conditions is removed by washing, it is possible to culture cells successfully for metaphase spreads. Based on this result, we suggest that storing EDTA-treated blood samples under cold conditions could be a viable alternative when transporting blood samples meant for cytogenetic biodosimetry. As long as the EDTA is

washed out of the blood samples before culturing is done, this method of storing blood samples could be especially useful under emergency conditions where it may be difficult to obtain heparin as an anticoagulant. This method of storing blood samples is also likely to be useful if maintaining the samples at RT becomes difficult due to climatological factors during transport.

Further studies will be necessary to examine if the numbers of Dic are altered in blood samples due to storage conditions, such as temperature, storage time, and anticoagulant type. Some reports suggested that in blood samples irradiated with X-rays, storage time and temperature affect the frequency of chromosomal aberration occurrences in PBLs from such samples (Bajerska and Liniecki 1969a, 1969b; Ivanov et al. 1973; Gumrich et al. 1985; Virsik-Peuckert and Harder 1985). However, there are other studies which also demonstrated that blood storage conditions do not affect the frequencies of radiation-induced chromosomal aberrations in these cells (Vekemans and Leonard 1977; Tomkins and Scheid 1986). Besides these uncertainties, there are no reports on the frequencies of radiation-induced chromosomal aberrations in cells obtained and cultured from EDTA-treated blood samples. As part of our future studies, we plan to perform DCA using heparinized and EDTA-treated blood samples to investigate the effects of temperature, anticoagulant type, and storage times on the suitability of blood samples for usage in dose estimation assays.

In addition, although we have performed whole blood culture in this study, isolated lymphocyte culture is also an option for DCA. However, when whole blood is stored under cold conditions for extended periods of time, separation of peripheral blood mononuclear cells (PBMCs) for culture becomes difficult (Nicholson et al. 1984; Nicholson and Green 1993; Son et al. 1996). We have also experienced problems with obtaining PBMCs for culture from 24 h-old blood samples that had been stored and transported under cold conditions (personal observation).

In conclusion, our study has established that although heparinized blood samples stored at RT for no more than 72 h are most suitable for the cytogenetic biodosimetry, EDTA-treated blood samples stored under cold conditions for up to 72 h are also capable of yielding enough metaphase spreads for such assays, as long as the EDTA is removed via washing before cell culture. Although further work is necessary to confirm our observations, this study has laid the foundation for exploring alternative methods of blood sample storage during transportation, especially under conditions where the temperatures of the samples cannot be easily maintained.

## Acknowledgments

We thank the volunteers who provided peripheral blood samples for this study. We also thank Michio Nomura from the Sysmex Corporation for his kind advice on analyzing blood count data. A part of this study was performed partially at the Department of Laboratory Medicine in Mutsu General Hospital (Mutsu, Japan) and we express our deep appreciation of the laboratory members who supported us during this work. We thank Ikue Asari and Yuki Sato for their technical and secretarial support.

## Disclosure statement

The authors report no conflicts of interest. The authors alone are response for the content and writing of the article.

## Funding

This work was supported in part by the Japan Society for the Promotion of Science [Grant-in-Aid for Scientific Research (B), No. 26293270].

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## Funding

This work was supported in part by the Japan Society for the Promotion of Science [Grant-in-Aid for Scientific Research (B), No. 26293270].

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