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Overnight Storage of Blood in ACD Tubes at 4°C Increases NK Cell Fraction in Peripheral Blood Mononuclear Cells

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Abstract. A considerable variabilility in the effects of sample handling on NK cytotoxicity has been observed. Using flow cytometry, NK cytotoxicity assays and lymphocyte subset analysis of Ficoll-Hypaque-separated peripheral blood mononuclear cells (PBMCs) isolated from whole blood stored under various conditions were performed. The NK cytotoxicity of samples in heparin tubes stored overnight at 4 and 22°C, as well as at 22°C in acid citrate dextrose (ACD) tubes, was lower than that of a fresh sample. However, the NK cytotoxicity of samples in an ACD tube stored at 4°C was similar to that of a fresh sample. Based on lymphocyte subset analysis, samples in an ACD tube stored at 4°C showed a lower percentage of CD3+ T cells and a higher percentage of CD16/56+ NK cells compared to samples stored under other conditions. The NK cytotoxicity of fresh samples and samples in ACD tubes stored in a Styrofoam cooler box did not differ significantly; however, the differences were inconsistent. Overnight storage of peripheral blood in ACD tubes at 4°C is optimum for retention of NK cytotoxicity, the level of which is similar to that of fresh blood. This may be associated with an increased NK-cell fraction in Ficoll–Hypaque-separated PBMCs after overnight storage.

Key words: Anticoagulant, temperature, acid citrate dextrose tube, natural killer cells, cytotoxicity

Introduction

Estimation of NK cytotoxicity is a useful cellular bioassay for assessment of various clinical conditions, including AIDS, systemic lupus erythematosus, hematologic malignancy, solid tumor, and hemophagocytic lymphohistiocytosis (HLH) [1–3]. Assessment of NK cytotoxicity is not automated and requires a labor-intensive process including PBMC isolation by density gradient centrifugation, manual cell counting and culture procedures, as well as 3–4 hours of incubation with a detection step based on percent lysis of target cells (i.e., K562 cell line) [4]. Therefore, establishing an NK cytotoxicity assay at hospitals with small-scale laboratories is problematic. Furthermore, standardization of the cellular bioassay is difficult due to variation in the skill sets of technicians. NK cytotoxicity assays for the diagnosis of diseases (such as HLH) and for data collection used in multi-center studies are thus often performed in the central laboratory.

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Typically, NK cytotoxicity assays cannot be performed in a single day, and the storage of whole blood becomes inevitable. For this reason, it is important to understand the factors, including the type of anticoagulant and storage temperature, that affect the quality of whole blood during transportation and storage. Identification of these factors will facilitate determination of the optimal storage conditions for human peripheral blood samples used for NK cytotoxicity assays.

Previous studies have evaluated the effects of sample handling on NK cytotoxicity, and reported considerable variability [5–7]. One important aspect of NK cytotoxicity specimen handling is differential change in specific subpopulations of stored whole blood samples after density gradient centrifugation. Any alteration in the fraction of NK cells, which subsequently function as effector cells, can affect NK cytotoxicity assays. However, there have been no reports of an association between NK cytotoxicity results and changes in the fraction of NK cells among PBMCs isolated from stored samples by density gradient centrifugation.

In this study, we investigated the effects of storage temperature and various anticoagulants on human peripheral blood samples for the assessment of NK cytotoxicity. We also examined the association between NK cytotoxicity and changes in cell fractions in peripheral blood mononuclear cells (PBMC) isolated by density gradient centrifugation after overnight storage. Furthermore, we determined whether transportation in a Styrofoam cooler box with ice packs preserved NK cell activity.

Materials and Methods

Sample preparation. All subjects signed a consent form approved by the Institutional Review Board of Chonnam National University Hwasun Hospital. Peripheral whole blood was collected from four normal healthy donors. Blood (60 mL) from each donor was collected in Becton–Dickinson Vacutainer tubes; 10 mL was collected in each of three tubes containing sodium heparin (heparin tube) and in each of another three tubes containing acid citrate dextrose (ACD tube) solution A. Two samples – one in an ACD tube and one in a heparin tube – were subjected to NK cytotoxicity assay immediately after collection. The remaining samples were stored at 4°C (refrigerator) or 22°C (room temperature) overnight (18–24 h) and NK cytotoxicity assayed the following day.

Fresh or overnight samples were layered onto the Ficoll–Hypaque (d=1.077 g/mL, Lymphoprep^{TM}, Axis-Shield, Oslo, Norway). PBMCs were isolated by density-gradient centrifugation and counted using Turk solution (a mixture of acetic acid and gentian violet), which hemolyzes RBCs and stains the nuclei of leukocytes blue. These PBMCs were used as effector cells in NK cytotoxicity assays against the K562 cell line using the flow cytometry-based assay, and as materials for granulocyte counting and lymphocyte subset analysis by flow cytometry.

Flow cytometric analysis of granulocyte counting and lymphocyte subset. Blood (69 mL) from seven normal healthy donors was collected; 10 mL was collected in each of three heparin tubes and three ACD tubes, and 3 mL was collected in each of three tubes containing ethylenediaminetetraacetic acid (EDTA). PBMCs were prepared from samples stored overnight at 4 and 22°C using Ficoll–Hypaque density gradient centrifugation. To analyze the baseline percentages of granulocytes, T lymphocytes, B lymphocytes and NK cells, whole blood lysis of fresh samples in the EDTA tubes was performed. During the flow cytometric analysis, granulocytes were set on a cytogram of $CD45^{dim}/SS^{high}$, and lymphocytes were gated based on low side-scatter (SS) and bright CD45-PC5 (IOtest, Beckman Coulter) staining. Three-color flow cytometric lymphocyte subset analysis was performed using a FC500 (Beckman Coulter, Fullerton, CA) with CXP software for a panel consisting of anti-CD3, CD19, CD16/56 monoclonal antibodies (IOtest, Beckman Coulter).

NK cell percentage in effector cells used in NK cytotoxicity assay. NK cell percentages in Ficoll–Hypaque-separated PBMC (effector cells) were calculated using the formula:

$$\frac{\text{Gated lymphocytes}}{\text{Lymph-, mono-, granulo-}} \times \frac{\text{NK cells}}{\text{T cells, B cell, NK cells}} \times 100$$

During flow cytometric analysis, lymphocyte ($SS^{low}CD45^{bright}$), monocyte ($SS^{intermediate}CD45^{dim}$), and granulocyte ($SS^{high}CD45^{low}$) populations were identified based on side-scatter and CD45-PC5 staining.

Flow cytometric NK cell cytotoxicity against the K562 cell line. Flow cytometry-based NK cytotoxicity assays using Calcein AM (CAM, 0.05 μM) stained effector cells were performed [4]. The NK-sensitive cell line K562, erythromyeloid blast cells, were used as target cells and maintained in long-term culture in RPMI medium with 10% FBS. CAM-stained PBMCs were used as effector cells. The target and effector cells were plated in V bottom 96-well microtiter plates with E:T ratios ranging from 50:1 to 12.5:1. The cells in each well were mixed gently by pipetting, and then centrifuged at 1,500 rpm for 1 minute and incubated from 1–4 hours at 37°C in a humidified incubator containing 5% CO2. After incubation, the mixed cells in 200 μL of complete medium were transferred to tubes. Next, 10-μL propidium iodide (PI) (Sigma; 1 μg/mL) was added to each tube 5–10 min before acquisition. Flow cytometric analysis was performed using a FC500. The instrument was set for analysis using the CXP software (Beckman Coulter, Fullerton, CA) with a threshold of 100 on FSC to exclude debris. At least 2,000 target cells per sample were acquired. To determine the rate of K562 cell lysis, the proportion of PI-positive cells was calculated. To calculate net percent lysis of target cells, the percentage of PI-positive target cells in media alone was subtracted from that in each sample.

Simulated transport of samples using a Styrofoam cooler box with ice pack. Blood (20 mL) from seven healthy donors was collected in two 10-mL ACD tubes. Ficoll–Hypaque separated-PBMCs obtained from fresh samples in ACD tubes were subjected immediately to NK-cytotoxicity assay. The other samples were used for transportation simulation in a Styrofoam cooler box with ice packs. One or two frozen ice packs, as well as blood samples in ACD tubes enfolded with bubble wrap and a thermometer, were placed in the Styrofoam cooler box. The box was sent to another laboratory by car and then returned to the original laboratory the following day for assessment of NK cytotoxicity. When the Styrofoam box was opened, the temperature in the box was measured and the NK cytotoxicity of Ficoll–Hypaque–separated PBMCs in the transported box was assessed.

Statistical analysis. Statistical analysis was performed using the Wilcoxon Signed–Rank Test. Groups being compared were considered to be significantly different if the *P* values were <0.05. Cytotoxicity results are expressed as the mean percent lysis±standard deviation (SD).

Results

NK cytotoxicity of samples stored in ACD tubes at 4°C is similar to that of fresh samples. To determine the optimal storage conditions for human peripheral blood samples used for the assessment of NK cytotoxicity, the NK cytotoxicity of Ficoll-Hypaque-separated PBMCs obtained from whole blood stored under different conditions was compared to fresh PBMCs. Samples that were stored in heparin tubes at 4 and 22°C showed lower NK cytotoxicity than fresh samples (84.5% for fresh, 59.0% for 4°C and 49% for 22°C, E:T ratio=50:1). When samples were stored in ACD tubes, the NK cytotoxicity at 22°C was lower than in fresh samples (78.2% for fresh and 53.3% for 22°C, E:T ratio=50:1, $P<0.05$), while the NK cytotoxicity of samples stored at 4°C was not significantly different from that of fresh samples (78.2% for fresh and 77.6 for 4°C, E:T ratio=50:1). The NK cytotoxicity of samples stored overnight in ACD tubes at 4°C was similar to that of the fresh samples. Furthermore, the NK cytotoxicity of fresh samples in heparin tubes was higher than samples in ACD tubes **(Figure 1)**.

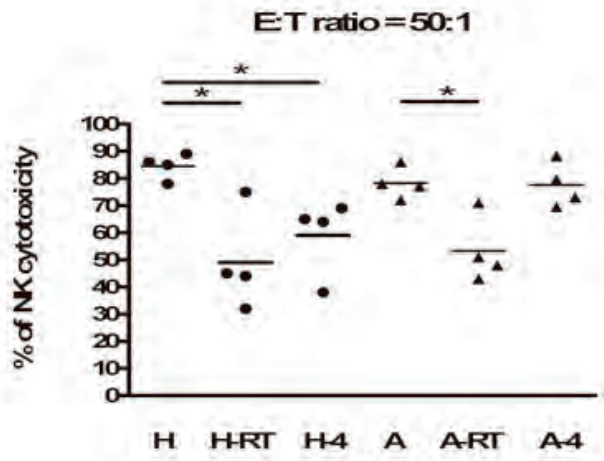
Changes in the percentage of granulocyte and lymphocyte subsets of PBMCs isolated by density gradient centrifugation after overnight storage. To explore the association between NK cytotoxicity and changes in cell fractions of PBMCs isolated by density gradient centrifugation after overnight storage, the cell fractions in PBMCs were used as effector cells and examined using flow cytometric analysis. Despite the use of various anticoagulants (heparin or ACD), PBMCs separated from the samples stored at 4°C had significant granulocyte contamination (27% for ACD, 32.4% for heparin), while PBMCs from samples stored at 22°C contained less than 1% granulocytes. Based on lymphocyte subset analysis, samples stored overnight in ACD tubes at 4°C showed significantly lower percentages of CD3+T cells than did samples stored in other conditions (75% for baseline, 47% for ACD at 4°C, 66% for heparin at 4°C, 71% for ACD at 22°C, and 67% for heparin at 22°C). The percentage of CD16/56+ NK cells in samples stored in ACD tubes at 4°C was higher than in samples stored under other conditions (10% for baseline, 27% for ACD at 4°C, 13% for heparin at 4°C, 11% for ACD at 22°C, and 11% for heparin at 22°C). There was no statistical difference among storage conditions based on the percentage of CD19+B cells. Among effector cells used for NK cytotoxicity assays, the percentage of NK cells was significantly higher in ACD tubes at 4°C than under other conditions (17% for ACD at 4°C, 5% for ACD at 22°C, 9% for heparin at 4°C, and 8% for heparin at 22°C) **(Figure 2)**.

Changes in NK cell activity in ACD tubes stored in a Styrofoam cooler box with ice packs during simulated transportation. The NK activity of samples stored overnight in ACD tubes at 4°C was similar to that of fresh samples. Thus, a simulated package system using a Styrofoam cooler box with ice packs for shipping blood samples was evaluated to determine if this method could preserve NK cell activity. The temperature inside the cooler box was checked immediately upon opening and ranged from 4 to 10°C. **Figure 3** indicates variable changes in the NK-cell activity of PBMCs isolated from each sample in ACD tubes stored in Styrofoam cooler boxes with ice packs. Although the NK-cell activities of fresh samples and stored samples in the cooler box did not differ significantly ($P>0.05$ at E:T ratio =50:1.25:1), the differences in each sample were inconsistent.

Discussion

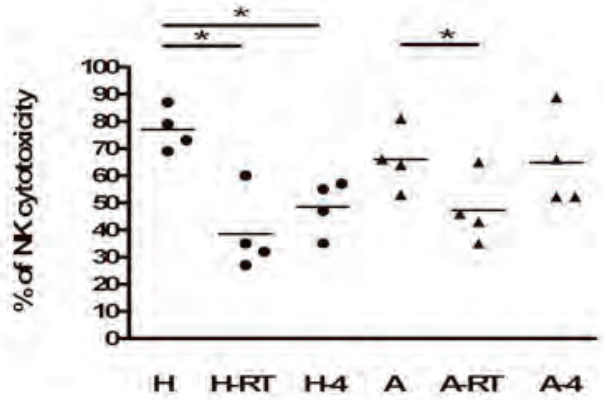
The NK cytotoxicity test of clinical samples performed at the central research laboratory uses predominantly PBMCs from whole blood samples transported overnight. The first consideration regarding specimen handling was the choice of anticoagulant. Anticoagulants commonly used for immunological assays, such as NK cytotoxicity assays, include heparin, EDTA, and ACD [8]. However, EDTA is not a suitable anticoagulant for NK cytotoxicity assays. Overnight storage of whole blood samples reduces their cytotoxic activity, particularly if the blood is anticoagulated with EDTA rather than heparin [7,9]. Furthermore, EDTA is known to inhibit NK activity, most likely by chelating the divalent cations necessary for NK lysis. In contrast, for the shipment of whole blood, ACD is the preferred anticoagulant since dextrose increases cell

A



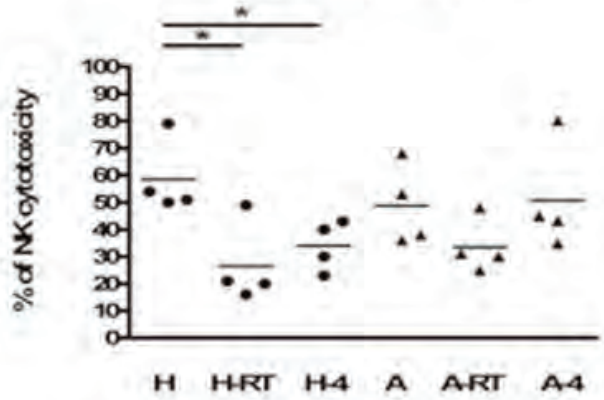
	Fresh	Overnight storage as whole blood	
		RT (°C)	4 (°C)
Heparin	84.5 ± 4.7 (H)	49.0 ± 18.3 (H-RT)	59.0 ± 14.2 (H-4)
ACD	78.2 ± 5.8 (A)	53.3 ± 6.1 (A-RT)	77.6 ± 8.2 (A-4)

B



	Fresh	Overnight storage as whole blood	
		RT (°C)	4 (°C)
Heparin	77.0 ± 7.8 (H)	38.5 ± 14.7 (H-RT)	48.5 ± 10.0 (H-4)
ACD	66.0 ±11.5 (A)	50.8 ± 20.0 (A-RT)	64.8 ± 17.3 (A-4)

C



	Fresh	Overnight storage as whole blood	
		RT (°C)	4 (°C)
Heparin	58.5 ± 3.8 (H)	26.5 ± 15.2 (H-RT)	34.0 ± 9.2 (H-4)
ACD	53.3 ±12.3 (A)	33.5 ± 10.0 (A-RT)	47.3 ± 12.7 (A-4)

Figure 1. Effect of storage temperature and anticoagulant type on NK cytotoxicity in fresh and stored samples. *Blood from donors (n=4) was collected in three tubes containing sodium heparin and in another three tubes containing acid citrate dextrose (ACD). NK cytotoxicity using fresh samples assayed immediately after collection, whereas NK cytotoxicity using stored samples assayed the following day. PBMCs isolated by density-gradient centrifugation were coincubated with K562 for 4hr in three different E:T ratios of 50:1 (A), 25:1 (B), and 12.5:1 (C). H, fresh sample in heparin tube; H-RT, stored sample in heparin tube at room temperature (RT); H-4, stored sample at 4°C; A, fresh sample in ACD tube; A-RT, stored sample in ACD tube at RT; A-4, stored sample in ACD tube at 4°C. * **P** < 0.05 compared with fresh samples. All samples were tested in duplicate (flow-cytometry based assay). Data represent the average of two determinations.*

survival and maintains cell functionality. Therefore, sodium heparin and ACD have been used as preferred anticoagulants for the NK cytotoxicity assay. In addition to the type of anticoagulant, storage temperature was also an important concern regarding the handling of blood samples prior to NK cytotoxicity assay.

In this study, the effects of different types of anticoagulant (heparin and ACD) and storage temperature (4 and 22°C) on peripheral blood samples for the assessment of NK cytotoxicity were evaluated **(Figure 1)**. Samples stored in heparin tubes at 4 and 22°C, as well as in ACD tubes at 22°C, showed significantly lower NK cytotoxicity than did fresh samples. These results agree with previous reports demonstrating a 10-20% decrease in NK cell activity in samples stored for 18–24 hours after collection, as compared with freshly drawn blood [10]. However, the NK cytotoxicity decreased further at 22°C than at 4°C regardless of the type of anticoagulant used. This contrasted with the report of Son *et al.* [7], who showed a change from 42.9% at baseline to 24.8% at 4°C and to 36.4% at 22°C in heparin tubes.

Therefore, any association between changes in the percentage of NK cells among effector cells after storage and NK cytotoxicity assays was investigated. The percentage of NK cells among effector cells used for NK cytotoxicity was significantly higher in ACD tubes at 4°C (17%) than under other conditions (5–9%). Samples stored overnight in ACD tubes at 4°C showed a significantly higher percentage of CD16/CD56+ NK cells and a lower percentage of CD3+T cells than did samples stored under other conditions. It is possible that overnight storage changes the density of lymphocyte subpopulations. Specimen storage at 4°C resulted in a 70% decrease in the proportion of T cells, even after only 24 hours of storage [11]. Furthermore, Santos *et al.* [12] reported a significant change in T lymphocyte subsets when blood samples were processed by Histopaque gradient sedimentation at 48 hours post-collection (from 83.5% to 72.8% at 24 hours and 7.7% at 48 hours). However, using the lysis method, there was no interval change in lymphocyte subset percentages (from 80.9% to 75.8% at 24 hours and 77.6% at 48 hours).

Regardless of the type of anticoagulant (heparin or ACD), PBMCs isolated from samples stored overnight at 4°C had a high level of granulocyte contamination. It was reported that specimen storage at 4°C increases the yield of granulocytes [12]. The granulocyte contamination in PBMCs isolated by density gradient centrifugation can be explained by overnight storage at 4°C, which decreases the granulocyte density compared to Ficoll–Hypaque (d=1.077 gm/mL). After density gradient centrifugation, granulocytes (at a lower density), as well as lymphocytes and monocytes, were retained at the plasma-Ficoll-Hypaque solution interface. The weak association between PBMCs and other cells (granulocytes, non-nucleated red cells, and debris) in the gradient sedimentation could result in a technical error in PBMC separation from red cells and granulocytes. These results agree with those of previous studies [9,13,14]; namely, that storage of whole blood for 18 hours increases erythrocyte and granulocyte contamination, which decreases the effector/target cell ratio.

Although granulocyte contamination slightly decreased the NK cell ratio, the increase in the NK cell fraction in PBMCs separated from samples stored overnight in ACD tubes at 4°C resulted in a net increase in the percentage of NK cells among effector cells. This suggests that when samples are stored in ACD tubes at 4°C, the increased NK cell fraction in effector cells compensates for the impaired NK cytotoxicity during overnight storage; the results of NK cytotoxicity assays were similar to those on fresh PBMCs. Rehermann *et al.* [8] reported that ACD is preferred for the shipment of whole blood to central laboratories due to the dextrose in ACD anticoagulant, which increases cell survival and maintains cell functionality. Furthermore, Bergman *et al.* [15] found that incubation of PBMCs at 4°C for 24 h resulted in the lowest percentage of apoptotic cells compared to those incubated at 22 and 37°C.

A standard whole blood lysis method of fresh sample in EDTA is recommended when analyzing the lymphocyte subset to avoid differential losses among lymphocyte subpopulations during cell preparation [16]. The whole blood lysis method was applied to the baseline data of lymphocyte subset analysis in this study.

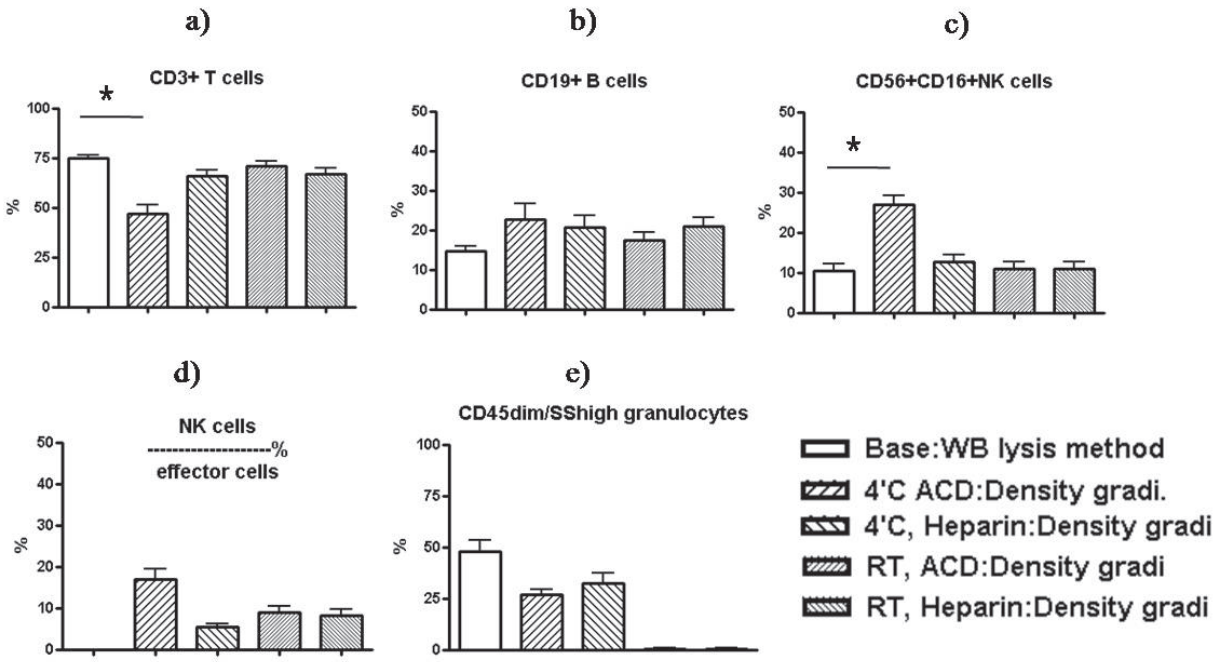


Figure. 2. Changes in the percentage of granulocyte and lymphocyte subsets of PBNCs isolated by density gradient centrifugation after overnight storage depending on anticoagulants and temperature. *Percentages of CD3+ T cells* ***(a)****, CD19+ B cells* ***(b)****, and CD56+CD16+NK cells* ***(c)*** *within the lymphocyte gate obtained by flow cytometry. The percentage of NK cells among effector cells in the NK cytotoxicity assay* ***(d)****, and that of granulocyte contamination within the cell fraction isolated by density-gradient centrifugation obtained from flow cytometry based on CD45dim/Side Scatter (SS_high)* ***(e)****. All blood samples were obtained from a normal individual (n=7) and the percentage of baseline (a, b, and c) was measured by flow cytometry using the whole blood lysis method in an EDTA tube. Blood samples were stored overnight (18–24 h) at 4 and room temperature as whole blood, and then separated by density-gradient centrifugation. Procedures are described in detail in the Materials and Methods. WB, whole blood; gradi, gradient; RT, room temperature. * P < 0.05 compared with base: WB lysis method.*

The most common and convenient transportation system for maintaining the temperature at 4°C is a Styrofoam cooler box with ice packs. Unlike refrigerators, Styrofoam cooler boxes with ice packs can maintain a temperature of 4°C during transportation. In the transportation simulation study, the temperature inside the cooler box ranged from 4 to 10°C when the box was opened. Although the NK cytotoxicity of PBMCs stored overnight in ACD tubes at 4°C in a refrigerator was similar to that of fresh PBMCs, the NK cytotoxicity of PBMCs stored overnight in ACD tubes at 4 to 10°C in a Styrofoam cooler box with ice packs was inconsistent with that of fresh PBMCs. Olson *et al.* [17] reported that blood packages shipped overnight by commercial carriers may encounter extreme seasonal temperatures, and considerations in the design of shipping containers should include protection against extreme ambient temperature deviations. This transportation simulation study was performed during the spring season. If this study were performed during the summer or winter seasons, extreme temperature deviations within the Styrofoam cooler box may have occurred.

The NCCLS I/LA26-A document provides guidance for the performance of single cell immune response assays in clinical areas. However, this guideline described sample collection, transportation and preparation only in specific assays to evaluate CD4 and CD8 T-cell function. No guidelines for or standardization of sample collection, transportation, and preparation for NK-cell analysis have yet been established.

Our data suggest that the increased fraction of NK cells in Ficoll–Hypaque separated PBMCs used as effector cells may be associated with the observation that NK cytotoxicity of samples stored overnight in ACD tubes at 4°C was similar to that of fresh samples. Caution is required when using PBMCs isolated by density-gradient centrifugation from whole blood stored under various conditions

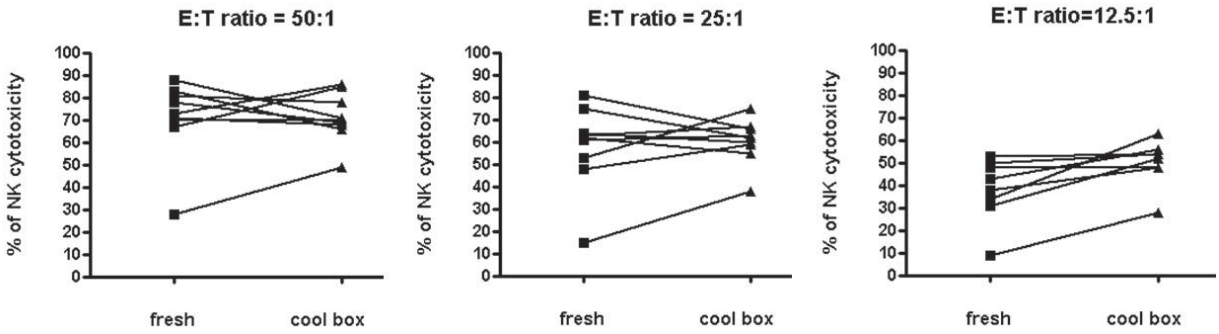


Figure. 3. Changes in NK cytotoxicity of PBMCs separated from fresh samples and samples in ACD tubes stored in a Styrofoam cooler box with cold packs during simulated 24 h-transportation. *Blood from healthy donors (n=7) was collected in tubes containing ACD. Variation in NK cell activities of PBMCs separated from each sample in ACD tubes stored in Styrofoam cooler boxes with ice packs. Although the NK cell activities of fresh samples and those stored in the cooler box was not significantly different (**P**>0.05 at E:T ratio =50:1.25:1), the differences were inconsistent. Data represent the average of two determinations. The cytotoxicity (% lysis) of different effector/target cell ratios (50:1, 25:1, and 12.5:1) against the K562 cell line was assessed using a flow cytometry-based assay.*

for NK cell cytotoxicity analysis. It is possible that the increased NK cell fraction increased the actual NK cells/K562 target cell ratio more than expected, and compensated for the impaired NK cytotoxicity during overnight storage. In addition, the results of the transportation simulation study indicate considerable variability in the NK cytotoxicity of PBMCs in ACD tubes transported in Styrofoam cooler boxes with ice packs.

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References

1. Sutlu T, Alici E. Natural killer cell-based immunotherapy in cancer: current insights and future prospects. J Intern Med. 2009;266:154-181.
2. Whiteside TL, Bryant J, Day R, et al. Natural killer cytotoxicity in the diagnosis of immune dysfunction: criteria for a reproducible assay. J Clin Lab Anal. 1990;4:102-114.
3. Henter JI, Horne A, Arico M, et al. HLH-2004: Diagnostic and therapeutic guidelines for hemophagocytic lymphohistiocytosis. Pediatr Blood Cancer. 2007;48:124-131.
4. Jang YY, Cho D, Kim SK, et al. An improved flow cytometry-based natural killer cytotoxicity assay involving calcein AM staining of effector cells. Ann Clin Lab Sci. 2012;42:42-9.
5. Levin A, Massey R, Deinhardt F. Spontaneous human mononuclear cell cytotoxicity to cultured tumor cells: reproducibility of serial measurements with the use of a chromium-51-release microcytotoxicity assay. J Nat Cancer Ins. 1978;60:1283-1294.
6. Sulica A, Gherman M, Galatiuc C, et al. Inhibition of human natural killer cell activity by cytophilic immunoglobulin G. J Immunol. 1982;128:1031-1036.
7. Son BK, Roberts RL, Ank BJ, et al. Effects of anticoagulant, serum, and temperature on the natural killer activity of human peripheral blood mononuclear cells stored overnight. Clin Diagn Lab Immunol. 1996;3:260-264.
8. Rehermann B, Naoumov NV. Immunological techniques in viral hepatitis. J Hepatol. 2007;46:508-520.
9. Solovera JJ, Alvarez-Mon M, Casas J, et al. Inhibition of human natural killer (NK) activity by calcium channel modulators and a calmodulin antagonist. J Immunol. 1987;139:876-880.
10. Ojo-Amaize EA, Conley EJ, Peter JB. Decreased natural killer cell activity is associated with severity of chronic fatigue immune dysfunction syndrome. Cli Infect Dis. 1994;18:S157-S159.
11. Dzik WH, Neckers L. Mononuclear cell-surface antigens during storage of banked blood. Transplantation. 1984;38:67-71.
12. dos Santos AP, Bertho AL, Martins Rde M, et al. The sample processing time interval as an influential factor in flow cytometry analysis of lymphocyte subsets. Mem Inst Oswaldo Cruz. 2007;102:117-120.
13. Nicholson JK, Jones BM, Cross GD, et al. Comparison of T and B cell analyses on fresh and aged blood. J Immunol Methods. 1984;73:29-40.
14. Nicholson JK, Green TA. Selection of anticoagulants for lymphocyte immunophenotyping. Effect of specimen age on results. J Immunol Methods. 1993;165:31-35.
15. Bergman M, Bessler H, Salman H, et al. Relationship between temperature and apoptosis of human peripheral blood mononuclear cells. Int J Hematol. 2003;77:351-353.
16. Kotylo PK, Sample RB, Redmond NL, et al. Reference ranges for lymphocyte subsets. A comparison of standard vs rapid whole-blood lysis techniques. Arch Pathol Lab Med. 1991;115:181-184.
17. Olson WC, Smolkin ME, Farris EM, et al. Shipping blood to a central laboratory in multicenter clinical trials: effect of ambient temperature on specimen temperature, and effects of temperature on mononuclear cell yield, viability and immunologic function. J Transl Med. 2011;9:26.