

CD4 COUNT FROM CRYOPRESERVATION OF BUFFY COAT AND PBMC

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ABSTRACT

This study aimed to determine CD4 count from cryopreservation of Buffy coat (BC) and Peripheral Blood Mononuclear Cell (PBMC) with and without ficoll. Fifteen EDTA Blood sample (2 ml for each tube) were drawn from one adult healthy subject. The samples were categorized into five group before measuring the CD4 level (which were fresh whole blood [Group(G)-I], BC without ficoll [fresh <G-II> and frozen <G-III>], and PBMC resulted from BC and ficoll isolation [fresh <G-IV> and frozen <G-V>]. Each group was replicated three times. Blood storage before preparation was less than four hours. Two months cryopreservation using liquid nitrogen (in 40% FBS, 10% DMSO, and RPMI) was conducted. The mean value of CD4 count (cell / μ l) were 522 (G-I), 1410 (G-II), 906 (G-III), 807 (G-IV), and 733 (G-V). CD4 count, after 2 month preservation in liquid nitrogen, of the BC sample (G-III) was higher (906 cell / μ l) than PBMC (G-IV) sample (733 cell / μ l).

Keywords: CD4, Buffy coat, PBMC, Cryopreservation

INTRODUCTION

Cryopreservation is the only method currently available for long term storage cell, that it can be used for various assays and purposes in medical area (Nazarpour, 20012). Mononuclear cells were commonly used as thawed samples for flow cytometry (Dogan, 2004; Nazarpour, 2012; wuchter, 2000). However there were minimum cells amount required for assays (such as in p-glycoprotein, CD4, CD3, CD8, and B cells) with flow cytometry (Subira, 2002 wuchter, 2000). On some cases in Sanglah Hospital, only small volume of peripheral blood could be taken for cryopreservation. Therefore on this study, we analyzed CD4 count between thawed Buffy coat (BC) and Pheripheral Blood Mononuclear Cells (PBMCs) collected from 2 ml blood for each sample.

MATERIALS AND METHODS

This research was approved by Sanglah Hospital – Faculty of Medicine, Udayana University ethics committee. This observational study was conducted in Balai Besar Veteriner (BBVet) and Laboratory of Clinical Pathology Sanglah Hospital Denpasar Bali on period of May 1st – July 31st, 2015.

Samples Preparation Procedure

Peripheral blood samples were drawn with aseptic technique from one healthy adult, who had written informed consent. Fifteen samples in Ethylene Diamine Tetra-Acetic Acid (EDTA) tubes (2 ml peripheral blood for each tube) that were collected, were divided into five different procedures

(Table1) before measuring CD4 count (which were: fresh whole blood [without BC or PBMCs isolation, and cryopreservation] group G-I, BC isolation [fresh <group G-II> and frozen <group G-III>], and BF isolation continued by PBMCs isolation [*fresh* <group G-IV> and frozen <group G-V>]). Each procedure replicated three times. Blood storage time until processing less than 4 hours. The samples in group III and V were cryopreserved for two months before conducting CD4 count.

Buffy Coats (BCs) Isolation

BCs were isolated from peripheral blood samples by using a refrigerated centrifuge (3000 rpm, r: 15.5 cm, swing rotor, break off) for 5 minutes.

PBMCs Isolation

Same volume of phosphate buffer saline (PBS) were added into BCs. The diluted BCs were gently layered and isolated by Ficoll Paque plus GE (3000 rpm, r: 15.5 cm, 20 minutes, refrigerated centrifuge, swing rotor, break off). PBMCs layers were collected. PBMCs were washed with 5 ml PBS for two times. Then, the PBMCs were prepared differently for group IV and group V.

For group IV, the PBMCs were diluted with 1ml PBS. Meanwhile for group V, PBMCs were suspended in media for cryopreservation.

Cryopreservation

In the tubes of PBMCs or BCs suspension in RPMI, 40% fetal bovine serum (FBS) and 10% dimethyl sulphoxide (DMSO) were added. The total volume were 1 ml. Then, those were stored gradually to lower temperature, which were in 4°C (30 minutes), -80 °C (overnight), and liquid nitrogen (two months).

Thawing

After two months cryopreserved in liquid nitrogen, BCs or PBMCs were thawed in a water bath (37°C) and then diluted in pre-warmed PBS. The diluted cells were washed with 10 ml pre-warmed PBS. Pellet were collected and suspended in 1 ml PBS.

CD4 count test

CD4 were measured by FACs count with CD4 - CD3 BD reagent.

RESULTS AND DISCUSSION

Fresh whole blood were commonly used for CD4 count. Mean value of CD4 count on fresh whole blood samples on this study was 522 cells/ μ l (Table 1.) Compared with mononuclear cells, the assay using whole blood samples were more convenient and rapid (Hanekom, 2004; Mallone 2011). The storage time before isolation and cryopreservation was a critical parameter affecting cell recovery and function. Viability PBMCs in blood samples stored within 8 hours (94% cells) was better than 24 hours blood storage (86-92% PBMCs) (Bull, 2007; Mallone 2011). On this study the time from venipuncture until processing was less than 4 hours.

Other important parameters in affecting cells recovery were the processes on isolation, cryopreservation, and thawing. Those processes may reduce cell recovery, such as in CD19 (8%), and HLA-DR+ CD38 (3-6%) (Reimann, 2000; Maloone 2011). It was showed on Table 1 that thawed samples (G-III and G-V) had a lower CD4 count compared to the fresh samples (G-II and G-IV).

Tabel 1. CD4 Count From Five Different Preparation Procedures

Group	Samples Preparation Steps Before CD4 test					Mean Value Of CD4 count (cells / μ l)
	<i>Fresh whole blood</i>	BC Isolation	PBMC isolation	Cryo-preservation	Thawing	
G-I	V	-	-	-	-	522
G-II	V	V	-	-	-	1410
G-III	V	V	-	V	V	906
G-IV	V	V	V	-	-	807
G-V	V	V	V	V	V	733

V= yes ; - = no

Nine percent decrease of CD4 count related to cryopreservation and thawing on PBMCs groups, which were G-IV (807 cells / μ l) and G-V (733 cell / μ l). Some studies reported that viability PBMCs for CD4 test after cryopreservation in liquid nitrogen was on the range of 81-95%. One of the factors associated with the loss were increase of T cell CD4 apoptosis (Owen, 2007; Malone 2011). On this research, thawed BCs, G-III, showed bigger percentage of loss on CD4 count, which were 35% reduce from the mean value of fresh BCs group, G-II. However, after two months cryopreservation, mean value of CD4 count in BCs samples G-III (906 cells/ μ l) were higher than PBMC samples G-V (733 cells / μ l). Comparing to PBMCs, use of BCs were cheaper and more rapid in sample preparation. A study should be conducted to explore further about viability cells in longer cryopreservation in both BCs and PBMCs sample. In this study, after two months cryopreservation in liquid nitrogen and thawing, CD4 count mean value of BCs samples (906 cells / μ l) were higher than those on PBMCs sample (733 cells / μ l).

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REFERENCES

- Bull M., Lee D, Stucky J. Chiu Y.L., Rubin A. Horton H. McElrath M.J. 2007. Defining blood processing parameters for optimal detection of cryopreserved antigen-specific responses for HIV vaccine trials. *J Immunol Methods* 322:57–6
- Dogan A.L., Kars A., Canpinar H., Güç D., Hayran M., Criss W.E., Kansu E. 2004. Prediction of Clinical Response to Chemotherapy by *In Vitro* Chemosensitivity Assay In Acute Leukemia. *Turkish Journal of Cancer* .Volume 34, No.2, 2004. P : 75-80
- Hanekom WA, Hughes J, Mavinkurve M Mendillo M, Watkins M, Gamiendien H, Gelderbloem Sj., Sidibana M, Mansoor N, Davids V, Murray RA, Hawkrige A, Haslett PA, Ress S. Hussey GD, Kaplan G. 2004. Novel application of a whole blood intracellular cytokine detection assay to quantitate specific T-cell frequency

- in field studies. *J Immunol Methods*. 291:185–95.
- Mallone R., Mannering S.I., Brooks-Worrell B.M., Durinovic-Belló I., Cilio C.M., Wong F.S. and Schloot N. C. 2010. Isolation and preservation of peripheral blood mononuclear cells for analysis of islet antigen-reactive T cell responses: position statement of the T-Cell Workshop Committee of the Immunology of Diabetes Society. *Clinical and Experimental Immunology*. 163: 33–49
- Nazarpour R., Zabihi E, Alijanpour E, Abedian Z, Mehdizadeh H, Rahimi F. Optimization of human Peripheral Blood Mononuclear Cells (PBMCs) cryopreservation. 2012. *Int J Mol Cell Med Spring*. Vol 1 No2. P. 88-92
- Owen RE, Sinclair E, Emu B . Heitman JW, Hirschhorn, D.F, Epling , C.L., Tan Q.x, Custer, B, Harris J.M., Jacobson, MA, McCune J.M., Martin J.N., Hecht, F.M., Deeks, S.G., Norris P.J. 2007.
- Loss of T cell responses following long-term cryopreservation. *J Immunol Methods*. 326:93– 115.
- Reimann KA, Chernoff M, Wilkening CL, Nickerson CE, Landay AL. 2000.
- Preservation of lymphocyte immunophenotype and proliferative responses in cryopreserved peripheral blood mononuclear cells from human immunodeficiency virus type 1-infected donors: implications for multicenter clinical trials. *The ACTG Immunology Advanced Technology Laboratories. Clin Diagn Lab Immunol*. 7:352–9.
- Subira, D, Castanon S., Aceituno E., Hernandez J, Jimenez- Garofano C., Jimenez A, Jimenez A.M., Roman A., Orfao A. 2002. Flow Cytometric Analysis of Cerebrospinal Fluid Samples and Its Usefulness in Routine Clinical Practice . *Am. J. Clin. Pathol*. 117: 952-958
- Wuchter C, Karawajew L., Ruppert V., Schrappe M., Büchner T., Schoch C., Haferlach T, Arbott J.H., Ratei R., Dörken B., Ludwig W.D. 2000. Clinical significance of P-glycoprotein expression and function for response to induction chemotherapy, relapse rate and overall survival in acute leukemia. *Haematologica* 85:711-72.