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Isolation of Nucleated Red Blood Cell from Peripheral Blood of β-Thalassemia Major Patients using CD71 Magnetic Beads and Future Application

Abstract— Introduction: Isolation of specific cell types is important in providing a better understanding of hematological disorders. The knowledge of molecular biology aspect in β-thalassemia is still limited. This is because hemoglobin disorder involves various erythropoietic processes in which the genetic information is lack due to enucleation of red blood cells occurs in bone marrow. It is invasive to collect samples from bone marrow and cord blood although nucleated red blood cells (NRBCs) are abundant in these sites. NRBCs are precursors of red blood cells and typically found in peripheral blood (PB) of β-thalassemia major patients and abundant post-splenectomy. The utilization of PB NRBCs will provide a further understanding of the molecular aspects of ineffective erythropoiesis in β-thalassemia major patients. Objective: The objective of this study was to isolate the NRBCs using CD71 magnetic beads from PB of β-thalassemia major; non-splenectomy and post-splenectomy patients. Methods: NRBCs were isolated from 6 mL PB of β-thalassemia major patients based on density gradient and magnetic activated cell sorting (MACS) for NRBCs enrichment using a CD71 marker. Cell count was determined by using hemocytometer (Weber Scientific, NJ, USA) and BD FACSCanto^{TM} II flow cytometry (Becton-Dickson, NJ, USA) was performed for method validation. Results: NRBCs were successfully isolated from the PB of both non-splenectomy and post-splenectomy β-thalassemia major patients with >90% specificity by flow cytometric analysis. The median number of enriched NRBCs (x10^4) was 58.5 (283) and 340 (338) respectively using hemocytometer. Conclusion: The MACS method was found to be convenient and efficient in the isolation of the targeted cells for downstream applications.

***Keywords*—β-thalassemia major, CD71, MACS, NRBCs.**

1 INTRODUCTION

β-thalassemia major is a genetic disease that is associated with severe anemia requiring life-long transfusion of red blood cells (RBCs). Molecular understanding of β-thalassemia major particularly on the ineffective erythropoiesis will provide some evidence in the pathophysiology of this condition and yet is still limited. It involves various erythropoietic processes where the genetic information is lack due to reducing or absence of β-globin chain synthesis. Absence or unpaired of other globin chains will precipitate in developing erythroid cells and causes oxidative damage to erythroid membrane and cytoskeleton [1]. This results in hemolysis of erythroid progenitors and leads to ineffective erythropoiesis, representing as an anemic phenotype in thalassemia [2].

Nucleated red blood cells (NRBCs) are immature erythrocytes, commonly known as erythroblasts at various stages that can be seen during pregnancy and in neonatal blood under physiologic conditions. The presence of NRBCs in peripheral blood (PB) is also associated with the increased erythroid activity or seen in various bone marrow diseases and in thalassemia major [3]. Enucleation process occurs at the final stage of the maturation of RBCs in bone marrow. Hence, no NRBCs are found in the PB of normal individuals. NRBCs are important as it contains the genetic information involving erythropoietic activity. They have become target cell type for a molecular understanding of diseases in the early and prenatal diagnosis of bone marrow disorder [4, 5]. NRBCs are highly present in cord blood and bone marrow samples. However, bone marrow examination is not routinely done for diagnosis of thalassemia patients due to its invasive procedure. In addition, PB specimen is adequate, easy to obtain and reliable for

diagnosis of thalassemia cases. NRBCs are present in the PB of thalassemia major patients due to ineffective erythropoiesis and they are abundant in the blood of post-splenectomy patients [6]. There are significant differences in the number of PB NRBCs of splenectomy and non-splenectomy β-thalassemia major patients. Therefore, the utilization of NRBCs from β-thalassemia major patients especially in post-splenectomy patients is expected to provide the information on the genetic aspects of this genetic disease.

Advanced technology had introduced various methods of isolating NRBCs from the cord and maternal blood based on a combination of density gradient with antibody-binding techniques; fluorescence-activated cell sorting (FACS) [7] or magnetic-activated cell sorting (MACS) [8]. FACS separation uses conjugation of fluorescent labels to the antibodies in which cell separation is achieved by laser excitation signaling the target cell. MACS is based on conjugation to iron oxide containing microbeads that require the cells to be placed in a magnetic field; unlabeled cells are eluted and labeled cells are retained in the magnetic field until they are removed from the magnet for separation [9]. Despite higher yield and purity in FACS, it is less specific and high cell loss was reported as compared to MACS [10].

To date, various automated cell sorter such as FACS Aria and MoFlow are available commercially. However, the use of this cell sorter technique may be limited due to high cost, availability of equipment and requirement for specially-trained laboratory personnel. MACS method, however, is a bench-top technique that can be performed in most laboratories. Previous studies supported that MACS method is comparatively simple, fast and efficient to enrich fetal NRBCs from mononuclear cells (MNCs) in the maternal blood using erythroid cell surface markers ie CD71 [5, 11].

The objectives of this study were to isolate the NRBCs using CD71 magnetic beads from PB of β-thalassemia major patients; non-splenectomy and post-splenectomy and to compare the quantity of NRBCs enriched between both groups of patients. Hence, NRBCs isolated from thalassemia major patients can be used for further downstream molecular application in various research including epigenetic studies which need specific cells as the heterotopic interaction between various cells could impact the

2 METHODOLOGY

2.1 Blood Samples Collection

Six mL of PB was collected in vacutainer tubes containing the anticoagulant EDTA from 15 non-splenectomy and 7 post-splenectomy β-thalassemia major patients at Medical Specialist Clinic and Pediatric Day Care, Hospital USM. This study was approved by Human Ethics Research Committee, Universiti Sains Malaysia (USM/JEPeM/00004494). Informed consent was obtained from all patients prior to participation in this study. The collected blood was subjected to hematologic analysis (Hb, MCH, MCV, and NRBCs) using automated hematology Sysmex XN analyzer (Sysmex, Illinois, USA) and blood smear, stained with Wright's stain.

2.2 Isolation of Mononuclear Cell (MNCs)

All PB samples were subjected to isolation of MNCs using lymphoprep (Alere Technologies AS, Oslo, Norway) based on density gradient following the manufacturer's protocol. Briefly, each blood sample collected was diluted with phosphate buffer saline (PBS) (Gibco, CA, USA) in 1:2. The blood dilution was layered on 3 mL lymphoprep (Alere Technologies AS, Oslo, Norway) and subjected to centrifugation for 20 mins at 600 x g at 20°C. The MNCs were suspended at the middle layer between plasma and lymphoprep. The supernatant (plasma) was removed and the MNCs were pipetted into new centrifuge tube followed by two steps washing procedure with PBS. In the first washing step, cells were centrifuged at 300 x g at 20°C for 10 mins and the supernatant was discarded. The cells were then centrifuged at 200 x g at 20°C for another 10 mins in the second step to remove the platelets. The numbers of MNCs obtained in the form of pellets were determined by hemocytometer (Weber Scientific, NJ, USA) with 1:1 ratio of trypan blue (Sigma-Aldrich, MO, USA).

2.3 Isolation of nucleated red blood cells (NRBCs)

Figure 1 showed the summary of the NRBCs isolation process. Briefly, the cells suspension of MNCs was centrifuged at 300 x g for 10 mins and the cells pellets were resuspended in 80 µL buffer containing PBS, pH 7.2 and 2mM EDTA. The

cells were incubated with 20 µL anti-CD71 microbeads (Macs Miltenyl Biotec, CA, USA) (per 10^7 total cells) for 15 mins at 4-8°C. The cells were washed by adding 1 mL buffer and centrifuged at 300 x g for 10 mins. After complete removal of the supernatant, 500 µL of buffer was used to resuspend up to 10^8 cells and applied onto the MS column (Macs Miltenyl Biotec, CA, USA) that have been placed in the magnetic field of mini MACS separator (Macs Miltenyl Biotec, CA, USA) for magnetic separation. The pass-through unlabeled cells, $CD71^-$ were collected and the column was washed three times with 500 µL buffer. The MS column was then removed from the separator and placed in the collection tube. The NRBCs were collected by immediately flushing out 1 mL fraction of the magnetically labeled cells, $CD71^+$.

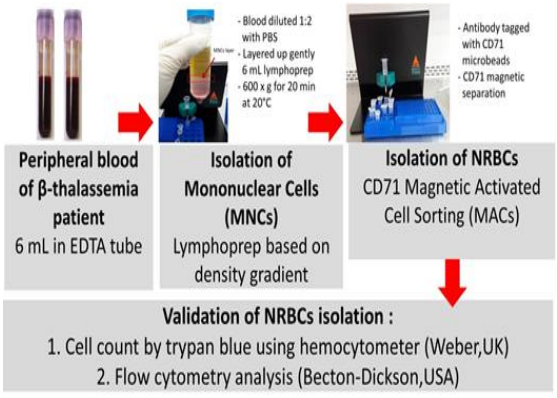

Figure 1: Cell sorting procedure using magnetic-activated cell sorting (MACS) with anti-CD71 for NRBCs isolation.

2.4 Flow Cytometry Analysis

Ten µL anti-CD71 monoclonal antibody conjugated to FITC (Macs Miltenyl Biotec, CA, USA) was added to 100 µL each of the fractions; negative, the unlabeled cells ($CD71^-$) and positive, the targeted cells ($CD71^+$). The cells were incubated for 15 mins at 4-8°C. The cells were washed twice with 1 mL of washing buffer. After removing the supernatant, the cells were resuspended in 500 µL buffer and ready for analysis by using BD FACSCanto™ II flow cytometry (Becton-Dickson, NJ, USA).

2.5 Statistical Analysis

Mann Whitney test analysis was used to compare the number of isolated NRBCs after CD71 MACS between non-splenectomy and post-splenectomy β- thalassemia major patients. Statistical analysis was performed using the Statistical Package for Social Sciences for Windows (SPSS Inc. version 22, Chicago, Illinois, USA). The results were considered to be significant when the *p*-value was <0.05.

3 RESULTS

PB film of both β-thalassemia major non-splenectomy (n=15) and post-splenectomy (n=7) samples showed circulating NRBCs with some dyserythropoietic features (nucleus and cytoplasmic bridging) (Figure 2). Hypochromic and microcytic RBC features were seen in the PB film of these patients. Numerous NRBCs were also seen in post-splenectomy patients as compared to non-splenectomy patients, parallel to the full blood count (FBC) results conducted using automated hematology system, Sysmex XN Analyzer (Table I). The number of NRBCs is approximately 31.59 x 10^3 ± 30.88 and 0.33 x 10^3 ± 0.32 respectively.

Based on flow cytometry results, it was shown that NRBCs have been successfully isolated from the PB of non-splenectomy and post-splenectomy β-thalassemia major patients, with >90% specificity by using combination method of a density gradient and CD71 MACS (Figure 3).

According to cell count results, the median number of enriched NRBCs (x10^4) were 58.5 (283) in non-splenectomy and 340 (338) in post-splenectomy β-thalassemia major patients (Table II). Isolated NRBCs from post-splenectomy patients were significantly higher compared to non-splenectomy patients (*p*<0.05).

Table I: Study population characteristics on hematological parameters and splenectomy status.

ID	Hb (g/dL)	MCV (fL)	MCH (pg)	NRBC (10^3/uL)	Splenectomy status
1	6.1	67.6	21.5	0.27	No
2	9.3	75.5	25.3	0.21	No
3	6.5	66.9	22.2	0.06	No
4	5.6	76.1	24.8	0.17	No
5	7.3	63.8	20.0	0.97	No
6	6.5	77.2	23.9	0.15	No

7	7.5	71.0	22.4	0.31	No
8	9.2	78.1	25.8	0.12	No
9	8.2	62.4	19.8	0.42	No
10	5.85	75.0	18.8	0.61	No
11	8.9	70.5	22.8	0.09	No
12	6.8	69.1	19.3	0.13	No
13	7.1	61.2	18.7	0.17	No
14	7.1	62.8	18.1	1.09	No
15	10.0	71.7	23.0	0.15	No
16	6.4	115.0	25.9	81.35	Yes
17	6.7	90.3	24.2	4.44	Yes
18	8.5	76.4	24.2	4.68	Yes
19	6.2	76.0	21.6	57.89	Yes
20	9.1	69.5	20.9	17.12	Yes
21	9.0	66.7	20.4	7.15	Yes
22	5.3	74.4	19.4	48.49	Yes

*MCV: Mean corpuscular volume, MCH: Mean corpuscular hemoglobin.

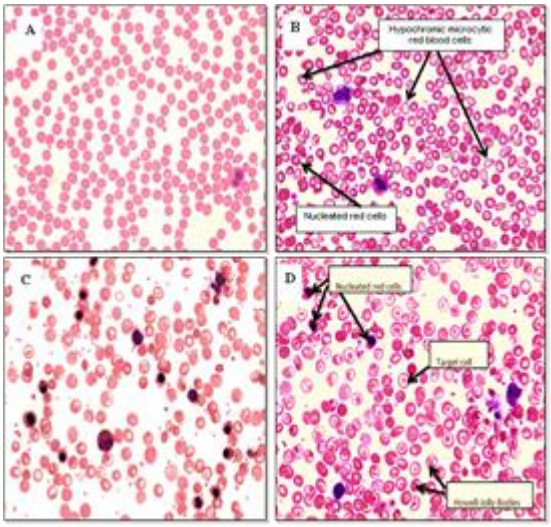

Figure 1: Peripheral blood films of thalassemia major patients A. Normal red blood cells (RBCs) B. β-thalassemia major patient showing hypochromic microcytic RBCs features C and D. Abundant nucleated red blood cells (NRBCs) in PB films of post-splenectomy compared to non-splenectomy β-thalassemia major patients in B.

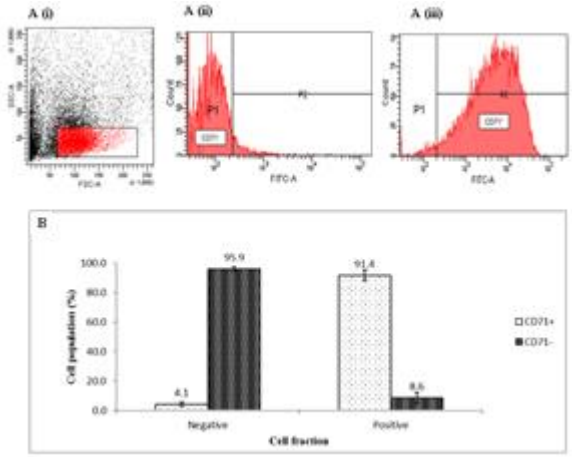

Figure 2: Flow cytometry analysis of RBCs positivity in β-thalassemia patients using Fluorescein isothiocyanate (FITC). A (i) Gating strategy for NRBCs. A (ii) Negative fraction. A (iii) Positive fraction. P1 represents CD71-population with 95.9%±1.42 in the positive fraction and 4.1%±1.42 in a negative fraction. B percentage of cells population in negative and positive fraction after the isolation of NRBCs using MACS CD71

Table II: Number of NRBCs recovered from PB of β-thalassemia major patients after CD71 MACS.

	Median (IQR)		*p*-value*
	β-thalassemia		
	Non-splenectomy	Splenectomy	
No. of MNCs ($x10^7$)	2.14 (2.3)	4.1 (5.3)	-
Total no. of cells after MACS ($x10^4$)	58.5 (283)	340 (338)	0.012

MNCs, mononuclear cells; MACS, magnetic-activated cell sorting; NRBCs, nucleated red blood cells; IQR, interquartile range

*Mann Whitney test

4 DISCUSSION

Hematological disorders which derived from blood cells comprised of multiple components including RBCs, white blood cells (WBCs) and platelets. PB was commonly used in most of the

studies as it is much more convenient to obtain from human subjects [12]. DNA extraction of blood samples is derived from the nucleated cells which mostly comprised of the white blood cells.

However, in some cases such as β-thalassemia major patients, circulating immature RBCs are found in their blood due to ineffective erythropoietic process in bone marrow [13]. These immature RBCs are nucleated cells of various maturation stages, still possessing DNA that contains the specific genetic code information of the cells which have been confirmed in the present study that showed the existence of NRBCs in PB of both β-thalassemia groups; non-splenectomy and post-splenectomy patients. NRBCs also can be used as a marker of erythropoiesis stress which helps to provide optimum transfusion therapy in β-thalassemia major patients [14].

The NRBCs were successfully isolated by using CD71 Microbeads based on MACS strategy with higher NRBCs isolated from the post-splenectomy compared to non-splenectomy patients. This showed the effectiveness of the isolation technique as this is theoretically expected where an elevated level of circulating NRBCs was detected in splenectomy patients' with thalassemia intermedia [15].

Furthermore, the finding was consistent with the previous studies which used CD71 antibodies to isolate fetal NRBCs from cord blood [5, 16]. This finding supports the idea of isolating the NRBCs through specific antibodies, CD71 as the surface marker of NRBCs. The CD71 antigen is also known as the transferrin receptor that is crucial for iron transport into proliferating cells and its expression was reported downregulated when the cells differentiate into mature erythrocytes [17].

Moreover, the use of single CD71 antibody gave a better yield compared to the use of double antibodies, anti-CD14 for depletion of macrophage and monocytes before anti-CD71 for the targeted cells. Cells may be damaged by the magnetic field as additional manipulations such as washing and centrifugation were involved in order to isolate the targeted cells using two antibodies [5]. Therefore, CD71 is the marker of choice to isolate NRBCs from non-splenectomy and post-splenectomy β-thalassemia major patients for further downstream molecular studies.

This study suggested that NRBCs can be isolated from PB of non-splenectomy and post-splenectomy β-thalassemia patients with a single erythroid marker, CD71. The use of PB as the specimen is much more convenient for researchers to access and comfortable to the patients without going through the invasive procedures for bone marrow samples.

Moreover, the successful isolation of NRBCs in PB of thalassemia major patients would also enlighten the researchers to better understand the molecular aspect of ineffective erythropoiesis in β-thalassemia patients. For example, the technique can be part of the tremendously growing area of epigenetics studies of thalassemia; looking at how the genes involved are regulated through epigenetic mechanisms. The knowledge in this area is still limited due to technical restriction to isolate the specific cells as different cell types have different DNA methylation profile [18]. In addition, differential DNA methylation profile was seen during hematopoietic cell differentiation suggesting that the composition of multiple cell types during the process can give confound effect to the studies of whole tissue epigenetics [12].

Thus, the need for specific and proper NRBCs isolation from β-thalassemia major patients is crucial for eliminating these confounding effects in providing better understanding in the pathogenesis of the disease in term of epigenetics. Therefore, this is a good platform to obtain precursors of RBCs for genetic application.

5 CONCLUSION

MACS method is convenient, simple, and cost-effective that can be efficiently applied in isolating NRBCs for downstream molecular application not only from splenectomy patients but also from non-splenectomy β-thalassemia major patients. Furthermore, this procedure has also been optimized and validated. It is suggested for genetic studies related to RBCs disorders especially in conditions with high circulating NRBCs are found in PB for the source of erythroid precursors.

CONFLICTS OF INTEREST

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this paper.

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