Original article

Comparison of neutrophil respiratory oxidative burst activity between flow cytometry using dihydrorhodamine (DHR) 123 and conventional nitroblue tetrazolium test (NBT)

Nurasyikin Yusof[1], Norafiza Mohd Yasin[2], Rabeya Yousuf[3], Asrul Abdul Wahab[4], Suria Abdul Aziz[5]

Abstract:

Background*:** Neutrophil plays a defense role against bacteria and fungi specifically by the neutrophil phagoburst activity. The nitroblue tetrazolium (NBT) test is the gold standard method to measure this but the flow cytometry assays also have been developed. The aim of the study was to evaluate the performance of flow cytometry using Dihydrorhodamine (DHR) 123 and to compare with the conventional NBT test. ***Methods: This study was carried out to determine the phagoburst activity at Universiti Kebangsaan Malaysia Medical Centre on 26 normal healthy donors and 5 chronic myeloid leukemia (CML) patients using both methods. ***Results:*** In flow cytometry, higher fluorescence intensity was observed in Phorbol myristate acetate (PMA) stimulated neutrophils. Comparing the mean fluorescent intensity (MFI) between CML and healthy donor showed CML patients have significant lower MFI (312.15 vs 738.22; $p<0.05$) indicating poorer respiratory burst in CML patients. The PMA stimulated positive cells were higher in flow cytometry than the NBT (98.06% vs 87.5%). Although there was poor correlation between two methods, a good agreement between flow cytometry assay and NBT test results was observed in terms of positive and negative results in simulated and unstimulated cells. Moreover, in flow cytometry, >95% of stimulated cells suggest the higher sensitivity. All samples with negative NBT showed negative DHR, reflecting high degree of agreement between these two methods and eliminating possible false negative result in flow cytometry. ***Conclusion:*** This study showed that flow cytometry assay has superior technical advantages compared to NBT test as it is easy to perform, quick, more sensitive and requires only a small amount of blood sample.

Keywords: Dihydrorhodamine 123; ntrobluetetrazolium test; phagocytic burst activity; neutrophils function.

Introduction

Neutrophil plays a major role in defense against bacterial and fungal pathogens. It rapidly migrates to the inflamed tissues and activate the microbicidal mechanisms by releasing proteolytic enzymes and antimicrobial peptides as well as producing oxidative stress by increasing reactive oxygen species (ROS), a phenomenon called respiratory oxidative burst activity. [1,2,3] This respiratory burst activity is performed by multicomponent enzyme nicotinamide adenine dinucleotide phosphate reduced (NADPH) oxidase with an increase in oxygen consumption.[4,5]

1. Nurasyikin Yusof, Departments of Patholog & Diagnostic Laboratory Services, Kuala Lumpur, Malaysia. Email: drsyikinby@gmail.com.
2. Norafiza Mohd Yasin, Universiti Kebangsaan Malaysia Medical Centre, Kuala Lumpur, Malaysia. Institute for Medical Research, Email: norafizayasin@gmail.com
3. Rabeya Yousuf, Department of Diagnostic Laboratory Services, Kuala Lumpur, Malaysia. Email: rabeya@ppukm.ukm.edu.my
4. Asrul Abdul Wahab, Department of Microbiology. Kuala Lumpur, Malaysia. Email: saw@ppukm.ukm.edu.my
5. Suria Abdul Aziz, Departments of Pathology& Diagnostic Laboratory Services. Kuala Lumpur, Malaysia. Email: suria.abdulaziz@gmail.com

Correspondence: Dr. Nurasyikin Yusof. Assoc. Prof.Department of Pathology, Universiti Kebangsaan Malaysia Medical Centre, Jalan Yaacob Latif, Bandar Tun Razak, 56000 Cheras, Kuala Lumpur, Malaysia. Email: drsyikin@ppukm.ukm.edu.my

ROS is composed of superoxide anion (O2-) and hydrogen peroxide (H2O2) and is highly toxic to ingested microorganisms leading to bacterial killing, thus allowing the bactericidal action of neutrophil.[5,6]

The defect in neutrophil activity results in having a poor defense against infection which can be quantitative or qualitative/ functional defects. The functional defect with failure of reactive oxidative burst activity is well described in chronic granulomatous disorder (CGD) where the NADPH-oxidase function is impaired secondary to specific mutations in the genes that encode their components.[7,8] The functional defect may also be seen secondary to haematological malignancy for example chronic myeloid leukaemia (CML). [9, 10]

The reduce function in neutrophils is usually identified by Nitro Blue Tetrazolium (NBT) dye reduction test that uses the histochemical demonstration of intracytoplasmic NBT reduction (blue dye formazan). The reaction depends upon the production of neutrophil NADPH oxidase.[5,11] and is based on a microscopic evaluation of stained neutrophils for the presence of intracellular blue dye formazan. This makes it highly dependent on observer experience.[12] It also involves complicated steps, requiring a large amount of blood and time-consuming neutrophils isolation procedures from whole blood.[13] PolymorPrep is a density media that is ready-made, sterile and endotoxin tested solution used for the isolation of neutrophils from whole blood. After cells separation procedure, the neutrophils layer needs to be isolated manually by skillful person. The utility of NBT test are in the detection and diagnosis of the primary immune disorder.[6, 10.14]

The use of flow cytometry has been introduced to reliably identify the Primary immunodeficiency disorders by granulocyte activation[15] This technique uses Dihydrorhodamine (DHR)123 as fluorogenic substrate where it is oxidized to rhodamine R123 by cellular hydrogen peroxide as a result of respiratory burst activity after addition of stimulant Phorbol 12-myristate 13-acetate (PMA) in the sample.[11] The formation of intracellular R123 gives a fluorescent signal that is detected and analyzed by flow. [5,6,11] This is the most sensitive technique available for detection of phagoburst activities in response to a variety of physiological and pathological conditions.[16]. As opposed to the NBT method, the flowcytometry requires small amount of blood and does not need the neutrophil isolation. Thus, this simple and highly sensitive assay can measure the phagoburst activity

in thousands of neutrophils very rapidly.[17,18]

Currently, at Universiti Kebangsaan Malaysia Medical Centre (UKMMC), we are using the NBT test to measure the neutrophil respiratory burst activity. However, we are planning to introduce the DHR 123 flow cytometry assay. It is important to evaluate the DHR 123 flow cytometry assay in our current laboratory setting. The objective of this study was to evaluate DHR 123 flow cytometry assay in determining neutrophils respiratory burst activity and to compare it with the NBT test.

Materials and Method

The study was carried out at the Immunology and flow cytometry lab, Department of Laboratory and Diagnostic Services, UKMMC.

A total of 31 samples were included in this study where 26 were from healthy individuals and five were from CML patients. Healthy individuals were selected randomly from adults having no medical illness, not on any medication and having normal absolute neutrophil count. The CML cases were selected based on the positive BCR-ABL status. All the participants were explained about the test and written informed consent was obtained before sample taking.

Ten ml of fresh venous blood sample was collected from each participant in two heparin anticoagulant tubes with 5mls in each tube where one tube was run by DHR 123 flow cytometry assay and another for traditional NBT test. Both methods were run on the same day to reduce the variability of the results.

DHR 123 flow cytometry assay

For DHR 123 flow cytometry assay, the sample was prcoessed within 12 hours of collection. The reagents were prepared and the procedure was done as recommended by the manufacturer. Two 100μl samples were taken from the 5ml sample taken earlier and placed in two separate falcon tubes and used as unstimulaed and stimulated tubes respectively. The blood samples were incubated in an ice bath for 10 minutes to cool them down to 0°C. Twenty microliters of PMA was then added to stimulant tube while 20 μl of PBS was added to the unstimulated tube and incubated at 37°C for 15 minutes followed by addition of 20 μl of working DHR 123 solutions to each tube and re-incubate at 37⁰C for another 10 minutes. FACS lysing solutions (Becton Dickinson, San Jose, CA) were added to the tubes and left at room temperature for 20 minutes and

centrifuged. The supernatant was discarded and the cells were resuspended in washing solution and re-centrifuged. Again the supernatant was discarded and replaced with DNA staining solution for cytometric discrimination of bacteria during leukocytes analysis. Upon stimulation, fluorescent signal was detected and analyzed using a flow cytometer FACSCalibur E97501067 and FACSComp 6.0 software using the blue-green excitation light (488 nm argon-ion laser).

Before acquiring data, a "live" gate was set in the red fluorescence histogram on those events which had at least the same DNA content as human diploid cells (i.e. exclusion of bacteria aggregates having same scatter light properties as leukocytes (Figure 1A). The percentage of cells having produced reactive oxygen metabolites were analyzed as well as their mean fluorescence intensity (MFI) (amount of cleaved substrate activity). For that purpose, the relevant neutrophil cluster was gated in the software program in the scatter gram of forward scatter (FSC) vs. side scatter (SC) (Figure 1B) and its green fluorescence-1 histogram (FL1) was analyzed (Figure 2). The neutrophil population was identified by its typical location at moderate SC and selected by gating (Figure 1B). A histogram of R123 fluorescence FL1 (Figure 2 A-D) was obtained for the gated region and percentage of R123 positive cell that reflects the percentage of cells having produced reactive oxygen metabolites were determined as well as the geometric mean recorded from the display statistics were noted. The MFI was automatically calculated by the analyzer and represents fluorescence intensity of stimulated neutrophils representing the degree of the neutrophil activity.
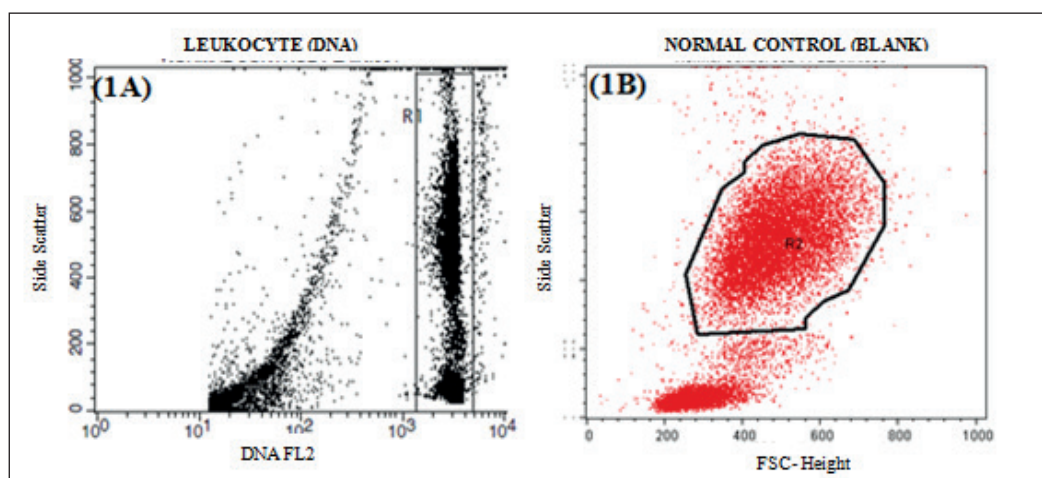

Fig. 1 Live gate on leukocyte DNA (R1) (1A). Flow cytometry dot plot of FSC vs SC of a lysed whole blood specimen (1B). The population of neutrophils is differentiated by their characteristic size (FSC, x-axis) and granularity (SC, y-axis). In this representative unstimulated sample, the neutrophils population can be clearly identified, and has been gated as the population of interest.

Nitroblue Tetrazolium Slide Test (NBT)

The NBT test performed according to the method of Gentle and Thompson. [19] The neutrophil was isolates from 5 ml of blood sample using PolymorphPrep solution. The stimulated and unstimulated tubes were prepared where in stimulated tube 100 µl of neutrophils suspension was mixed with 100µl of NBT and 100µl of PMA (stimulant) while in unstimulated tube, 100µl of neutrophils suspension was mixed with 100µl of NBT and 100µl of PBS. The tubes were incubated at 37°C water bath for 10 minutes and further incubated at RT for 10 minutes. Smears of fixed cells were prepared and a total of 200 cells were counted microscopically. Neutrophils showing formazan deposits were recorded as positive. The results were considered within normal range which is between 63-90% for PMA stimulated NBT positive cells and 0-38% for unstimulated NBT positive cells.

Statistical analysis

Data were collected immediately after performing the test and analyzed by Statistical Package for the Social Sciences (SPSS) version-25. For the DHR flow cytometric assay, fluorescent signal was detected for both PMA stimulated tube and the unstimulated tube. Comparison of fluorescence signal by PMA stimulation was done between the CML group and healthy donor group by Mann-Whitney U test. For

the NBT test, NBT positive cells in PMA stimulated samples and in unstimulated samples were obtained. Data was obtained as percent values. Pearson Correlation study was done in order to determine the relationship between the two methods.

Ethical clearance: This study commenced once approval from the research ethics committee of UKMMC has been obtained.

Results

DHR 123 flow cytometry assay

The DHR flow cytometry assay was performed within less than one hour. In figure-2, the flowcytometry analysis results for both control and CML groups showed unstimulated sample have low R123 fluorescence intensity of $<10^1$ whereas the PMA stimulated neutrophils revealed a high intensity of $>10^2$ indicating presence of phagocytic burst activity.
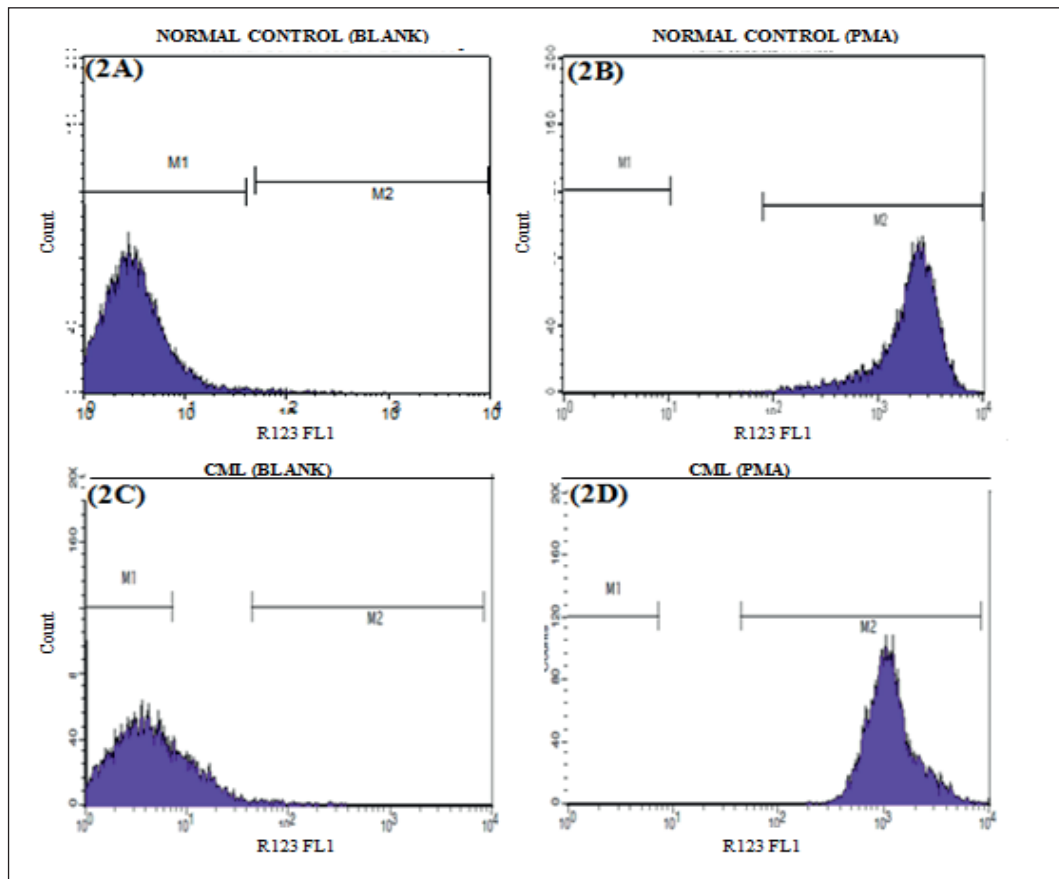

Fig. 2 Flow cytometry histogram plot showed R123 fluorescence results. 2(A) and 2(B) is unstimulated and PMA stimulated for normal healthy subjects while 2(C) and 2(D) is unstimulated and PMA stimulated for CML cases.

The DHR 123 flow cytometry in both CML group and healthy donor showed higher rhodamine fluorescence with stimulation by PMA. On comparing the MFI values between CML patients and healthy donors, it revealed CML patients have statistically significant lower MFI compared to healthy donor (312.15 vs 738.22; $p<0.05$) which reflects poorer respiratory burst activity. (Table -1).

Table 1 Comparison of MFI of DHR 123 flow cytometry in CML group and healthy donor.

	Diagnosis	Median	Mann-Whitney U	P value
DHR(%)	Healthy	98.00	55.00	0.581
	CML	98.00		
Geomean DHR(+)	Healthy	2115.81	0.00	0.001*
	CML	1002.00		
Geomean BLANK	Healthy	2.80	4.50	0.001*
	CML	3.73		
MFI	Healthy	738.22	0.00	0.001*
	CML	312.15		

*Significant P<0.05

In figure-3, Box and Whisker Plots showed the median MFI values for PMA stimulated neutrophils in CML subjects were statistically significantly lower than control healthy subjects(p<0.05).

Fig. 3 Box and Whisker Plots showed MFI for PMA stimulated CML group and healthy donor. Horizontal line indicates median MFI value. Median MFI for CML case was significantly lower than healthy person.

NBT test results

NBT assay took an average of four hours to complete. NBT positive cells in PMA stimulated samples and NBT negative cells in unstimulated samples were s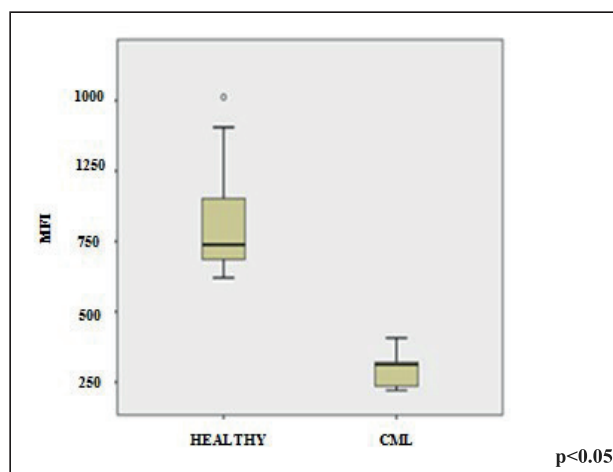hown in Figure 4A and 4B respectively. In figure 4C, NBT positive cells in CML patients. Even with isolation of neutrophils using PolymorPrep medium, the red cells contamination can interfere with the interpretation of either positive or negative cells (Figure 4D).
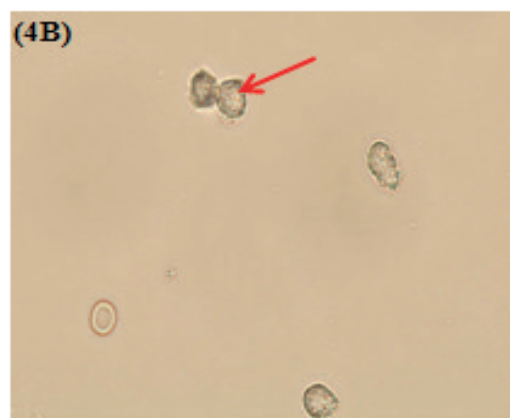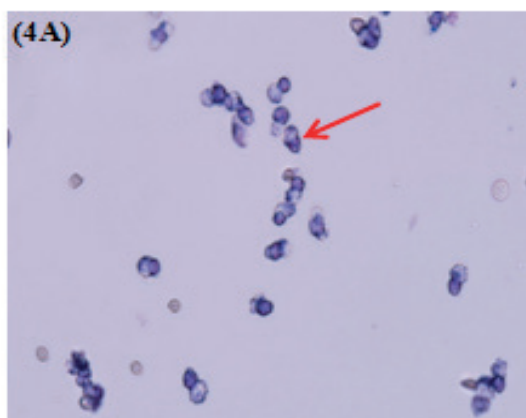

Fig. 4A & 4B (A) NBT blue formazan positive staining of neutrophil in PMA stimulated tube in healthy sample (arrow) (B) NBT negative cell (unstimulated tube) in normal healthy sample (arrow).

In this study, the DHR flowcytometry assay and NBT test results showed good agreement in terms of positive and negative results in simulated and unstimulated cells respectively but with poor correlation in the percentage values (r = 0.066, p=0.723). Although there was poor correlation between the percentage of stimulated cells in the two methods, the study revealed that the percentage value of stimulated cells in flow cytometry were higher than the NBT (98.06% vs 87.5%) (Table 2). This study also showed that all unstimulated samples of normal healthy participants and CML patients showed negative results for the NBT which were in accordance with the negative result in flowcytometry. Similarly, the stimulated cells showed positive results which agrees between the two tests.
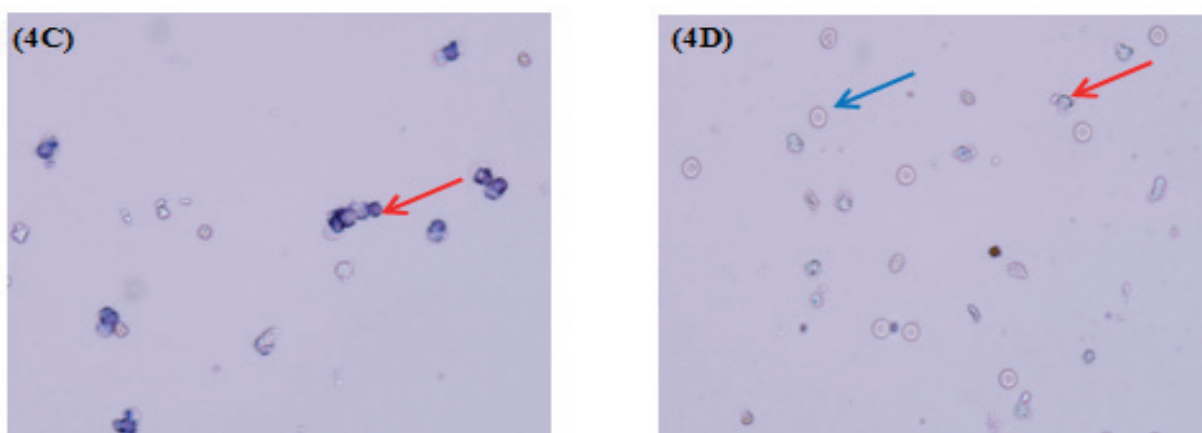
Fig. 4C & 4D (C) NBT positive cells in CML case (arrow). (D) NBT negative cells (red arrow) with red cells contamination (blue arrow).

Table 2 Mean results for conventional NBT test and DHR 123 flow cytometry assays for normal healthy samples and CML patients.

	NBT test			DHR 123 flow cytometry assays			
	Unstimulated (Mean%)	cell Stimulated (Mean%)	cell Unstimulated cell (Mean%)	Stimulated cell (Mean%)	Geomean	MFI
Healthy	4.42	88.02	1.32	98	2245.11	802.67
CML patients	3.5	84.8	2.56	98.4	1112.16	299.18
Total	4.27	87.5	1.52	98.06	2062.37	721.46

* non-statistically significant poor correlation in the percentage of stimulated cells in NBT and DHR123 flow cytometry in normal healthy control (r = 0.066, p=0.723).

Discussion

This present study showed that the DHR 123 flow cytometry assay was simple, quick and easy method that takes only 1 hour compared to the NBT test that takes 4 hours. Previous studies also showed DHR 123 flow cytometry assay has several potential technical advantages. Blood sample required for analysis is as small as 0.1 ml making this assay ideal for studies especially in neonate and young children which is the most common age group investigated for CGD and haematological malignancy.[5,6,13]

On the other hand, the conventional NBT test required skillful personal to interpret the findings which include visual inspection of the peripheral blood neutrophils and therefore very much dependent on the observer experience.[12,20] The limitations of the NBT test has been shown in many studies regarding the artifactual changes that can occur following cells isolation procedures that potentially activate neutrophils leading to false negative results.[13,21] Therefore, it is important to maintain the physiological environment in order to preserve the normal functional state of cells during laboratory testing.[13] Other technical factors that affect the results in the NBT test are concentration of heparin and NBT, duration of blood stored before the assay and temperature of blood-NBT mixture incubation and many others.[12,20,22,23] These explain many discrepancies on the NBT test results between different institutions. There are also unstandardized ways of interpreting the NBT test specially to identify and counting the neutrophils with positive and negative result leading to different normal ranges used in different laboratories.[19,22]

Flow cytometry has the ability to measure multiple functions of neutrophils simultaneously, for example the measurement of the geomean and the MFI which provide added information on the degree of the phagoburst activity of the neutrophil as compared with the conventional NBT. This present study showed that the DHR flow cytometry assay can clearly distinguish between unstimulated and PMA stimulated neutrophils as described by previous studies.[16,24]

In comparison between the CML patients and normal healthy individuals, our study revealed that CML patients showed statistically significant lower MFI compared to the healthy adults (312.15 and 738.22 respectively, p<0.05), which implies that despite the normal neutrophils count of the CML patients, they have poorer activity compared to normal patients. Usually CML patient with positive BCR-ABL fusion protein have defective neutrophils function.[10] One recent study showed median MFI in CGD patients and normal controls were 432.5 and 902.5 respectively suggesting this method as a cost-effective tool for effective diagnosis of primary immune deficiency (PID) disorders.[25] Although genetic analysis is needed for definitive diagnosis of such diseases, this method adds an advantage by giving an alternative option for screening of suspected Primary Immunodeficiency diseases in haematological laboratories equipped with flow cytometer.[26]

Comparison between the two test methods showed there is poor statistical correlation between the percentage of PMA stimulated cells in DHR assay and the NBT test (r = 0.066, p=0.723). In a previous study on comparison between the DHR assays and NBT test showed a high degree of correlation between these two tests. They conclude that whole blood DHR assay is an accurate and sensitive measure of respiratory burst.[5] Our study supports this fact that although there was poor correlation between the percentages of NBT stimulated cells with percentage of DHR positive cells, the percentage of stimulated cells in flow cytometry method is higher than the NBT test. All of the DHR flow samples showed percentage stimulated cells of more than 95% suggesting the higher sensitivity. All samples with negative NBT showed negative DHR, reflecting high degree of agreement between these two tests and eliminating possible false negative result. These findings suggested that the DHR flow cytometry has more technical superiority for detection of phagoburst activity in the neutrophils.

Conclusion

DHR flow cytometry showed higher fluorescence intensity in PMA stimulated neutrophils. It showed statistically significant lower MFI in CML patients compared to healthy donor, reflecting poorer respiratory burst activity that coincides with the disease process of CML. The percentage of stimulated cells in flow cytometry were higher than the NBT (98.06% vs 87.5%), although it showed a poor correlation between two methods. Despite that, there is a good agreement between NBT test and the DHR 123 flow cytometry assay in terms of positive and negative results in simulated and unstimulated cells. In flow cytometry, presence of >95% of stimulated cells suggest the higher sensitivity. All samples with negative NBT showed negative DHR, reflecting high degree of agreement between these two tests and eliminating possible false negative result. Thus, DHR flow cytometry test is a quick, simple and easy method, has superior technical advantages and able to provide further information on the degree of phagoburst activity from the Geomean and MFI data. The flow cytometry methods thus represent a convenient alternative to the classical methods for measurement of neutrophils respiratory burst.

Informed consent: Informed consent has been obtained from the participants

Authors contribution: All authors contributed to the study conception and design and data analysis. Norafiza Yasin contributed in data collection and drafting of the manuscript. Nurasyikin Yusof contributed in drafting, critical review and finalizing the manuscript. Rabeya Yousuf contributed in drafting and finalizing the manuscript. Asrul Bin Abdul Wahab and Suria Abdul Aziz contributed in critical review of the manuscript. All authors read and approved the final manuscript.

References

1. Elbim C, Lizard G. Flow Cytometric Investigation of Neutrophil Oxidative Burst and Apoptosis in Physiological and Pathological Situations. *Cytometry A* 2009; **75** (6): 475-481. DOI: 10.1002/cyto.a.20726

2. Kuwabara WMT, Zhang L, Schuiki I, Curi R, Volchuk A, Alba-Loureiro TC. NADPH Oxidase-Dependent Production of Reactive Oxygen Species Induces Endoplasmatic Reticulum Stress in Neutrophil-Like HL60 Cells. *PLoS ONE* 2015; **10**(2): e0116410. DOI: 10.1371/journal.pone.0116410

3. Anita DC, Sulistyorini SL, Kadarsih S. The RAGE expression in glomerulus and the Malondialdehyde level in the kidney of Diabetes Mellitus rat model after exercise. *Bangladesh J. Med. Sci.* 2019; **18** (3): 484-490. https://doi.org/10.3329/bjms.v18i3.41615

4. Forman HJ, Torres M. Reactive Oxygen Species and Cell Signaling, Respiratory Burst in macrophage Signaling. *Am J Respir Crit Care Med* 2002; **166**: S4–S8. DOI: 10.1164/rccm.2206007

5. Richardson MP, Ayliffe MJ, Helbert M, Davies EG. A simple flow cytometry assay using dihydrorhodamine for the measurement of the neutrophil respiratory burst in whole blood: comparison with the quantitative nitrobluetetrazolium test. *J Immunol Methods* 1998; **219**: 187-193. DOI: 10.1016/s0022-1759(98)00136-7

6. Dimitrova G, Bunkall C, Lim D, Kendrick C. Comparison of two methods for the diagnosis of chronic granulomatous disease - neutrophil oxidative burst measured by the nitroblue tetrazolium slide test versus the dihydrorhodamine 123 flow cytometric assay. *N Z J Med Lab Sci* 2013; **67**: 45-51.

7. Kuijpers TW, Weening RS, Roos D. Clinical and laboratory work-up of patients with neutrophil shortage or dysfunction. *J Immunol Methods*. 1999;**232**(1-2):211-229. DOI: 10.1016/s0022-1759(99)00178-7

8. Avendaño A, Sales-Pardo I, Marin L, Marin P, Petriz J. Oxidative burst assessment and neutrophil–platelet complexes in unlysed whole blood. *J Immunol Methods* 2008; **339**: 124–131. DOI: 10.1016/j.jim.2008.09.003

9. Ajdary S, Hamidpoor L, Zandieh T. Function of neutrophils in different phases of chronic myelogenous Leukemia. *Iran. Biomed. J* 2002; **6**(2 & 3): 83-88.

10. Ahmed AA. Neutrophils phagocytic function in chronic myelogenous leukemia after imatinib mesylate therapy. *Iraqi. j. cancer med. genet.* 2013; **6**(1):65-71

11. Walrand S, Valeix S, Rodriguez C, Ligot P, Chassagne J, Vasson MP. Flow cytometry study of polymorphonuclear neutrophil oxidative burst: A comparison of three fluorescent probes. *Clin Chim Acta* 2003; **331**(1-2):103-110. DOI: 10.1016/s0009-8981(03)00086-x

12. Segal AW, Trustey SF, Levi AJ. Re-evaluation of nitroblue tetrazolium test. *Lancet* 1973; **302**(7834):879-883. DOI:https://doi.org/10.1016/S0140-6736(73)92007-2

13. van Eeden SF, Klut ME, Walker BAM, Hogg JC. The use of flow cytometry to measure neutrophil function. *J Immunol Methods* 1999; **232** (1-2):23-43. DOI: 10.1016/ S0022-1759(99)00148-9

14. Freeman R, King B. A modification to the N.B.T. test. *Lancet* 1971; **298** (7734):1154. https://doi.org/10.1016/ S0140-6736(71)91312-2

15. Fleisher TA, Madkaikar M, Rosenzweig SD. Application of Flow Cytometry in the Evaluation of Primary Immunodeficiencies. *Indian J Pediatr* 2016; **83**: 444–449. DOI: 10.1007/s12098-015-2011-0

16. Smith, JA, Weidemann MJ. Further characterization of the neutrophil oxidative burst by flowcytometry. *J Immunol Methods* 1993; **162**(2):261-268. DOI: 10.1016/0022-1759(93)90391-j

17. Albert-Vega C, Tawfik DM, Trouillet-Assant S, Vachot L, Mallet F, Textoris J. Immune Functional Assays, From Custom to Standardized Tests for Precision Medicine. *Front Immunol* 2018; **9**:2367. DOI: 10.3389/ fimmu.2018.02367

18. Bass DA, Parce JW, DeChatelet LR, Szejda P, Seeds MC, Thomas M. Flow cytometric studies of oxidative product formation by neutrophils: a graded response to membrane stimulation. *J Immunol* 1983; **130**(4):1910-1917.

19. Gentle TA, Thompson RA. Neutrophil function test in Clinical Immunology. In: Gooi HC, Champel H, eds. Clinical Immunology. A practical Approach. IRS Press, Oxford. 1990; 51-80.

20. Freeman R, King B. Technique for the performance of the nitro-blue tetrazolium (NBT) test. *J Clin Pathol.* 1972; **25**(10): 912–914. doi: 10.1136/jcp.25.10.912

21. Kuijpers TW, Tool AT, van der Schoot CE, Ginsel LA, Onderwater JJ, Roos D, Verhoeven AJ. Membrane surface antigen expression on neutrophils: a reappraisal of the use of surface markers for neutrophil activation. *Blood* 1991; **78**(4):1105-1111. https://doi.org/10.1182/ blood.V78.4.1105.1105

22. Feigin RD, Shackelford PG, Choi SC, Flake KK, Franklin FA Jr, Eisenberg CS. Nitroblue tetrazolium dye test as an aid in the differential diagnosis of febrile disorders. *J Pediatr* 1971; **78**(2):230-7. DOI: 10.1016/ s0022-3476(71)80005-7

23. Conklyn MJ, Neot, K, Showell HJ. Chemokine dependent up regulation of 11b on specific leukocyte subpopulations in human whole blood: effect on anticoagulant on rantes and MIP-1 beta stimulation. *Cytokine* 1996; **8**(10):762-766. DOI: 10.1006/cyto.1996.0101

24. Vowells SJ, Sekhsaria S, Malech HL, Shalit M, Fleisher TA. Flow cytometric analysis of the granulocyte respiratory burst: a comparison study of fluorescent probes. *J Immunol Methods* 1995; **178**(1):89-97. DOI: 10.1016/0022-1759(94)00247-t

25. Kuppuswamy G, Kathirvel K, Priyanka M, Devasia A, Kulkarni U, Abraham A, et al. Flow Cytometry Based Diagnosis of LAD, ALPS and CGD. *Indian J Hematol Blood Transfus* 2019; **35**: S1–S151. (Abstract)

26. Langer S, Radhakrishnan N, Pradhan S, Das J, Saraf A, Kotwal J. Clinical and Laboratory Profiles of 17 Cases of Chronic Granulomatous Disease in North India. *Indian J Hematol Blood Transfus* 2020. https://doi.org/10.1007/ s12288-020-01316-6.