Original Article

J Babol Univ Med Sci
Vol 20, Issu 6; June 2018. P:20-26

Evaluating the Performance of Flow Cytometric Method in Identification of Platelet Resistance in Patients with Acute Myeloid Leukemia (AML)

M. Ghorbani (PhD)[1*], M.T. Hojjati (PhD)[2]

1.Department of Laboratory Sciences, Faculty of Paramedicine, AJA University of Medical Sciences, Tehran, I.R.Iran
2.Laboratory Science Research Center, Golestan University of Medical Sciences, Gorgan, I.R.Iran

J Babol Univ Med Sci; 20(6); June 2018; PP: 20-26

Received: Nov 4th 2017, Revised: Jan 18th 2018, Accepted: Feb 26th 2018.

ABSTRACT

BACKGROUND AND OBJECTIVE: Immune platelet resistance is a condition in which antibodies are produced against platelet antigens and cause damage to injected platelets by phagocytes and macrophages. The aim of this study is to evaluate the efficacy of flow cytometry in predicting the results of platelet injections and platelet resistance and selecting compatible platelet for patients.

METHODS: This case-control study was performed on 15 patients with acute myeloid leukemia (AML) with two or multiple injections of platelets and 15 healthy subjects without a history of platelet injection. After marking the platelets with 5-Chloromethylfluorescein diacetate (CMFDA) and their adjacency to the serum of patients, the amount of phagocytosis of platelets by monocytes was assessed in the two groups.

FINDINGS: The mean phagocytic power of platelets by monocyte was 18.27 ± 2.86% in the control group, 68.47 ± 10.40% in the group of patients with immune platelet resistance, and 36.73 ± 15.21 in the group of patients without immune platelet resistance (p = 0.001). In addition, there was a significant negative correlation between phagocytic power of platelets by monocyte and Corrected Count Increment (CCI) at 1 and 24 hours (p = - 0.001).

CONCLUSION: Based on the results of this study and considering the high sensitivity of the flow cytometric method, the use of CMFDA is highly appropriate for evaluating platelet studies, and it can also be used for platelet crossmatch by flow cytometry.

KEY WORD: *Platelet Resistance, Flow Cytometry, CCI, Phagocytosis, 5-Chloromethylfluorescein diacetate.*

Please cite this article as follows:

Ghorbani M, Hojjati MT. Evaluating the Performance of Flow Cytometric Method in Identification of Platelet Resistance in Patients with Acute Myeloid Leukemia (AML). J Babol Univ Med Sci. 2018;20(6):20-6.

•Corresponding Author: M.T. Hojjati (PhD)
Address: Department of Laboratory Sciences, Golestan University of Medical Sciences, Gorgan, I.R.Iran
Tel: +98 17 32436108
E-mail: mthhematology@gmail.com

[DOR: 20.1001.1.15614107.1397.20.6.3.1]
[DOI: 10.18869/acadpub.jbums.20.6.20]

Introduction

One of the complications that occur in patients with frequent platelet transfusion is platelet resistance, which produces antibodies against platelet antigens and cause damage to transfused platelets by phagocytes and macrophages. Platelet resistance is defined as the lack of increase in platelet count up to optimal therapeutic levels after two or more platelet transfusion, which can be estimated by calculating Corrected Count Increment (CCI) at 1 and 24 hours after platelet transfusion (1 – 3). Patients with acute leukemia are among the people who receive platelet due to thrombocytopenia (4, 5), which may lead to platelet resistance and lack of increase in platelet count in these patients, and may lead to severe bleeding and even death from bleeding (6). The prevalence of platelet resistance in hematology and oncology patients ranges from 7 to 34% (7). The causes of platelet resistance are divided into two categories: immune and non-immune. Non-immune causes may include fever, infection, splenomegaly, and intravascular coagulation, while immune causes may include alloantibodies against human leukocyte antigen (HLA) or alloantibodies against human platelet antigens (HPAs) (8, 9). Some of the important human platelet antigens that production of antibodies against them causes moderate to severe thrombocytopenia includes HPA-1, HPA-2, HPA-15, and HLA-I (10, 11). One of the most effective cells in destroying transfused platelets in susceptible individuals is monocyte. This cell has different types of receptors for the Fc portions of the IgG molecules. After the production of IgG alloantibodies against the HPA or HLA antigens, the Fab portions of the antibody adhere to the antigen of the surface of platelet and produce sensitized platelets. The exposure of sensitized platelets to monocytes and macrophages leads to phagocytosis and ultimately to destruction (12). In this condition, the platelet transfusion is inefficient and the patient's platelet will not increase as expected. Multiple assays using flow cytometry beads coated with soluble HLA antigens are a new type of assay that has been introduced in recent years. Since laboratory identification of anti-HLA antibodies is very difficult, in most patients, there are anti-HLA antibodies along with these antibodies (13), a flow cytometry method based on the phagocytic potency of monocyte was used in this study to evaluate the results of platelet transfusion. The aim of this study is to evaluate the efficacy of flow cytometry in predicting the results of platelet transfusion and platelet resistance and the selection of compatible platelets for patients.

Methods

After approval by the Ethics Committee of Iranian Blood Transfusion Organization (Ir.Tmi.rec.1393.10), this case-control study was performed among 15 patients with AML admitted to Shariati Hospital with history of platelet transfusion twice or more, while 15 healthy subjects without a history of transfusion of blood and blood products were considered as control group. Sample size was determined based on previous studies (14). After completing the questionnaire by a colleague in Shariati Hospital, 5 ml of whole blood sample was collected from AML patients and control subjects in an anticoagulant EDTA tube and centrifuged for 10 minutes at 1200 g. The plasma of patients and controls were isolated in 0.5 ml microtube and stored in freezer at -70 °C until the time of use. After platelet transfusion, a whole blood sample was collected from the patient 1 hour and 24 hours after the transfusion, and platelet counting was performed by the Cell Counter (KX-21, Sysmex, Japan) and CCI at 1 and 24 hours was calculated according to the Formula (15).

To obtain a platelet source, six whole blood samples from healthy people with blood type O were poured into a Falcon 15 ml tube and were mixed with each other. After 15 minutes of centrifugation at 200 g, the supernatant containing PRP (Platelet Rich Plasma) was isolated and was poured into a separate tube. Then, the amount of platelet present in PRP was counted with a cell counter and the number was adjusted to 5 × 108/ml platelets. To evaluate the purity of the platelets in PRP, flow cytometry analysis was done with CD61 antibody (bdbiosciences, USA). In addition, 5-Chloromethylfluorescein diacetate (CMFDA) (Lifetechnology, USA), which is a non-toxic substance for cells, was analyzed at a final concentration of 5 μM for evaluation of live cells by flow cytometry. Furthermore, platelets cross – match against HLA antigens was performed for all patients according to the method described in the articles (16). Isolation of monocyte from whole blood was performed using the technique of monocyte adhesion to culture medium flask (17, 18) and the percentage of viable cells was determined using Trypan Blue 0.4% Solution. In addition, CD14 antibody (bdbiosciences, USA) was used to evaluate the purity of monocytes cultured through flow cytometry. After adjacency of the labelled platelets with CMFDA and serum, the CMFDA-labelled and antibody-sensitized platelets were adjacent to monocyte-rich mononuclear cells. To investigate the phagocytic potency, anti-CD14 monoclonal antibodies

[DOR: 20.1001.1.15614107.1397.20.6.3.1]
[DOI: 10.18869/acadpub.jbums.20.6.20]

were added to cell suspension. Since CMFDA-labelled platelet produces color in the color spectrum of fluorescein isothiocyanate (FITC), its phagocytic potency was evaluated by examining CMFDA-positive monocytes (19). To interpret the results after gating the platelet populations and monocyte-rich mononuclear cells, the percentage of platelets that were conjugated with Anti-Human IgG antibody to FITC and monocyte-rich mononuclear cells that were positive in terms of both CD14 and CMFDA markers (double positive) were calculated, respectively. To assess the difference between the patients and controls in terms of platelet cross – match assay and FMPA,U Mann-Whitney tests were used, and to assess the difference between the FMPA tests in the three groups of platelet resistance (5 people), normal (10 people), and control (15 people), Kruskal-wallis assay was used and $p < 0.05$ was considered significant.

Results

CCI assessment in patients: The patients' CCI levels were calculated based on the number of platelets before and after transfusion, and the number and type of transfused platelet units. The CCI assessment showed that 5 out of 15 patients had immune platelet resistance (table 1). There was a statistically significant difference between the mean CCI at 1 hour (6300 ± 1202 vs. 10530 ± 2857) and 24 hours (5540±993 vs. 8470±1666) in the two groups of patients with immune platelet resistance and patients with non-immune platelet resistance, respectively (p=0.001).

Flow cytometric studies: The average purity of monocyte-rich mononuclear cells that were isolated from peripheral blood by the technique of monocyte adhesion to culture medium was 26.56 ± 2.51%. Considering that the percentage of isolated monocytes was low in mononuclear cells, Nycoprep isolation method was used (purity higher than 50% is acceptable). Mean percentage of monocyte-rich mononuclear cells isolated from peripheral blood by Nycoprep method was 74.69 ± 0.85. Considering that the percentage of monocytes in mononuclear cells by Nycoprep method was more than cell culture method, the Nycoprep method was used for the isolation of monocyte-rich mononuclear cells in this study (Fig. 1). Evaluation of the purity of isolated platelets and CMFDA-labelled platelet showed that the average percentage of platelet purity in the three samples was 97.25±0.25 and the mean percentage of CMFDA-labelled platelet cells in three samples was determined to be 99.48±0.18 (Fig. 2).

The mean phagocytic potency of platelet by monocyte in the control group was 18.27±2.86%, in group of patients with immune platelet resistance was 68.47±10.40%, and in the group of patients with non-immune platelet resistance was 36.73±15.21% (p=0.001). This difference was also significant in the two groups of patients with platelet resistance and normal subjects (p=0.001). The results also showed that there is a significant and inverse association in the phagocytic potency of platelet by monocyte in 1-hour CCI (r=-0.885, p=0.001), and in 24-hour CCI (r=-0.884, and p=0.001) of patients (Fig. 3).

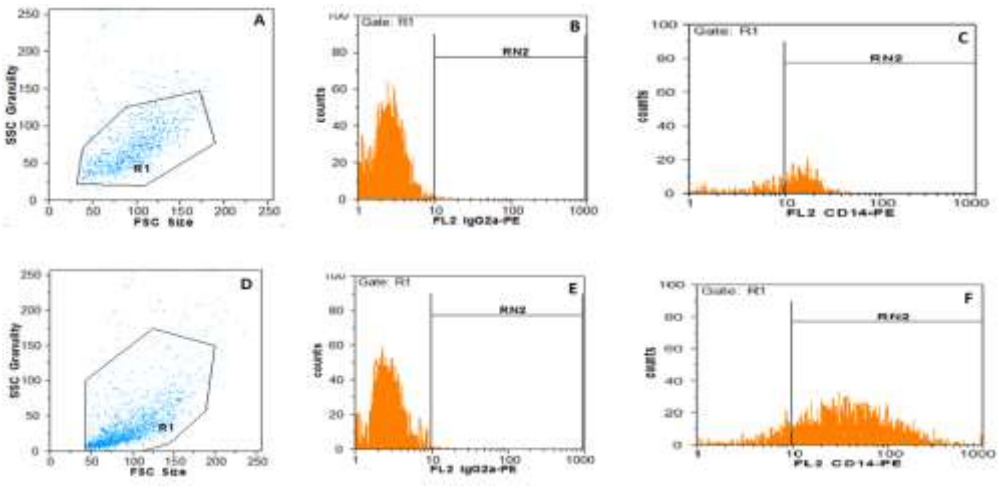


Figure 1. The flow cytometric chart of surface expression of CD14 in monocytes isolated from peripheral blood. Adhesion to the culture flask method: (A) Gate of monocyte-rich mononuclear cells (B) PE isotype control (C) surface expression of CD14 in monocyte-rich mononuclear cells isolated from peripheral blood. Nycoprep method: (D) Gate of monocyte-rich mononuclear cells (E) PE isotype control (F) surface expression of CD14 in monocyte-rich mononuclear cells isolated from peripheral blood

[DOR: 20.1001.1.15614107.1397.20.6.3.1]
[DOI: 10.18869/acadpub.jbums.20.6.20]

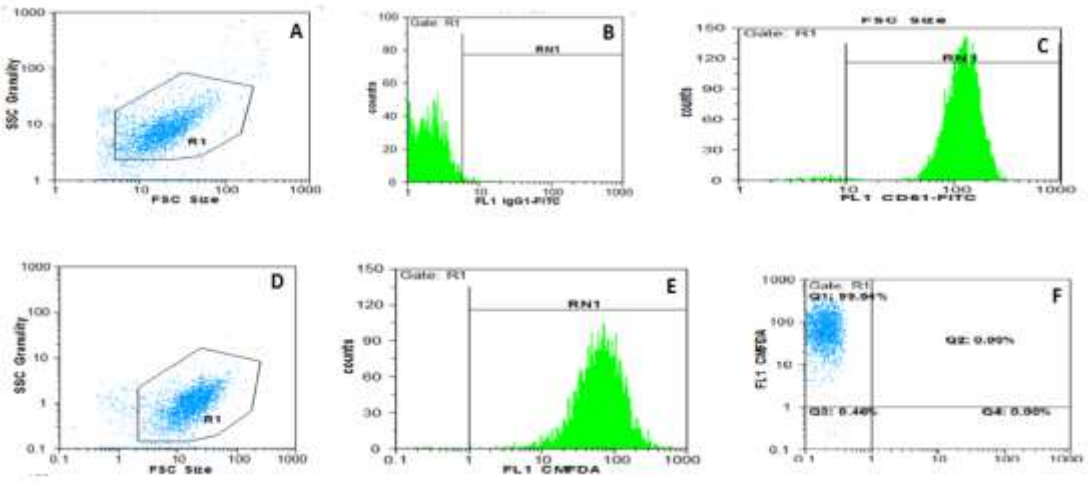


Figure 2. Flow cytometric results. Surface expression of CD61 in platelets isolated from peripheral blood: (A) Gate of platelet population (B) FITC Isotype Control (C) Surface Expression of CD61 in Platelet Cells Isolated from Peripheral Blood. Flow cytometric results of the expression of CMFDA in labelled platelets: (D) Gate of platelet population (E) The expression of CMFDA in platelets isolated from peripheral blood (F) Histogram showing the expression of CMFDA in platelets.

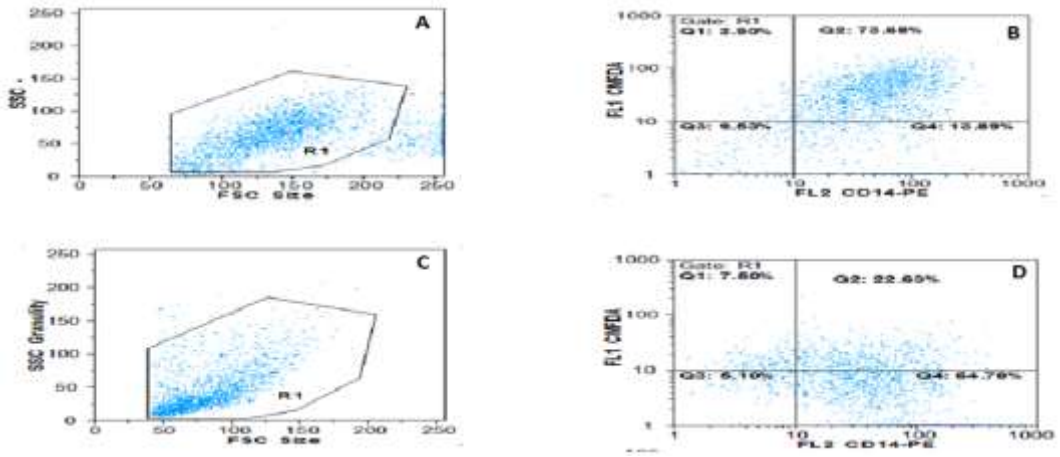


Figure 3. Flow cytometric diagram of phagocytic potency of platelets by monocytes: (A and C) Gate of monocyte population. (B) Surface expression of CD14 and CMFDA in monocyte cells in positive control sample. (D) Surface expression of CD14 and CMFDA in monocyte cells in study patients. The dual positive cells (region Q2) in Figures B and D represent the level of phagocytosis of platelets by monocytes.

Discussion

Our results indicate that there was a significant difference between the mean phagocytic potency of platelets by monocytes in both patient and control groups, and there was also a significant difference between the mean phagocytic potency of platelets by monocytes in the group of patients with platelet resistance and patients without platelet resistance. In addition, there was a significant negative correlation between the phagocytic potency of platelets by monocytes and the CCI at 1-hour and 24 hours. In the present study, it tried to simulate body condition at the time of platelet transfusion; we adjoined the patient's serum, containing the possible antibodies, to a series of pooled platelet antigens to sensitize the platelet and finally, placed it in the vicinity of monocyte to start the phagocytosis process. Lim et al. (19) in their study used a flow cytometric method based on phagocytosis of platelets by monocytes to predict the results of platelet transfusion. They measured CCI after platelet transfusion, performed a platelet cross – match assay, and performed flow cytometric method based on phagocytosis of platelets by monocytes, and used ELISA method to assess anti-HPA or anti-HLA antibodies. They found that there was a significant negative correlation between phagocytosis of platelets by monocytes and CCI of patients. They stated the results of the comparison between the cross – match

[DOR: 20.1001.1.15614107.1397.20.6.3.1]
[DOI: 10.18869/acadpub.jbums.20.6.20]

method and phagocytosis of platelets by monocytes as follows; if phagocytosis of platelets by monocytes is high, platelet cross – match assay may be considered as an effective screening method, but if phagocytosis of platelets by monocytes is low even if the platelet cross – match assay is high, it is not reliable. Therefore, the effective role of flow cytometric method in identifying this disorder is emphasized in studies, especially in alloimmune individuals, for whom HLA-compatible platelets are not available (20). In the present study, all 5 patients with high FMPA had a positive platelet cross – match, but of 10 patients with low FMPA (and normal CCI), 6 had positive cross – match, while their CCI was normal, and cross – match was negative only in four of these patients. A set of factors are effective, including the patient-related factors and the characteristics of the transfused platelets such as the number of transfused platelets, the platelet source (random or apheresis), the compatibility of ABO between donor and recipient, and the duration of platelet maintenance on CCI after clinical transfusion and hemorrhage in patients with thrombocytopenia (21). However, there is still no complete study of how these factors can be considered in predicting the results of platelet transfusion, and it seems that the level of hemorrhage cannot be estimated by examining these factors (22). Because discovering the presence of antiplatelet antibodies in the platelet cross – match does not necessarily mean platelet destruction. Meanwhile, only anti-HLA and anti-GP-IIb / IIIa antibodies have been reported to play a predictive role in platelet resistance (23).

It is also necessary to consider that we would observe platelet resistance in cases of the presence of splenomegaly and storage of platelets for more than three days due to non-immunological reasons (24). Considering the high sensitivity of the flow cytometric method, the use of CMFDA is very suitable for platelet examination, and since this fluorescent substance does not alter platelet function or, in other words, has no effect on platelet activity, it can be used for platelet cross – match by flow cytometry and can also be used to achieve better clinical results in identifying platelet resistance.

Acknowledgement

Hereby, we express our deepest sense of gratitude and indebtedness to Deputy of Research and Technology of Tehran Medical University for the Islamic Republic of Iran's Army for their support.

[DOR: 20.1001.1.15614107.1397.20.6.3.1]
[DOI: 10.18869/acadpub.jbums.20.6.20]

References

1.Bub CB, Martinelli BM, Avelino TM, Gonçalez AC, Barjas-Castro Mde L, Castro V. platelet antibody detection by flow cytometry,An effective method to evaluate and give transfusional support in platelet refractoriness. Rev Bras Hematol Hemoter. 2013;35(4):252-5

2.Zhai J, Ding M, Yang T, Zuo B, Weng Z, Zhao Y, et al. Flow cytometric immunobead assay for quantitative detection of platelet autoantibodies in immune thrombocytopenia patients. J Transl Med. 2017;15(1):214.

3.Zhao Y, Zhu M, Jiang M, Zuo B, Wu Q, Ruan C1, et al. An improved flow cytometric immunobead array to detect autoantibodies in plasma from patientswith immune thrombocytopenic purpura. Clin Chim Acta. 2015 Jan 1;438:396-400.

4.Heal JM, Blumberg N, Masel D. An evaluation of crossmatching, HLA, and ABO matching for platelet transfusions to refractory patients. Blood. 1987;70(1):23-30.

5.Freedman J, Gafni A, Garvey MB, Blanchette V. A cost-effectiveness evaluation of platelet crossmatching and HLA matching in the management of alloimmunized thrombocytopenic patients.Transfusion.1989; 29: 201-7

6.Petz LD, Garratty G, Calhoun L, Clark BD, Terasaki PI, Gresens C, et al. Selecting donors of platelets for refractory patients on the basis of HLA antibody specificity. Transfusion. 2000;40(12):1446-56.

7.Moncharmont P, Rigal D. Prevalence of platelet-specific antibodies in the recipients of platelet units with transfusion adverse event. J Soc Franc Trans Sang. 2012;19(6):333-7.

8.Apelseth TO1, Hervig T, Bruserud O. Current practice and future directions for optimization of platelet transfusions in patients with severe therapy-induced cytopenia. Blood. 2011:25;113-22.

9.Eldad Hod and Joseph Schwartz. Platelet transfusion refractoriness. Brit J Haematol. 2008:142;348-60.

10.Mohanty D, KullkarniB, Ghosh K, Nair S,Khare A. Human platelet specific Antigens and their Importance. Ind pediat. 2004;41(8):797-805

11.Ertel K, Al-Tawil M, Santoso S, Kroll H. Relevance of the HPA-15 (Gov) polymorphism on CD109 in alloimmune thrombocytopenic syndromes. Transfusion. 2005;45(3):366-73.

12.Armour KL, Smith CS, Turner CP, Kirton CM, Wilkes AM, Hadley AG, Ghevaert C, Williamson LM, Clark MR. Low-affinity FcγR interactions can decide the fate of novel human IgG-sensitised red blood cells and platelets. Eur J Immunol. 2014;44(3):905-14.

13.Blajchman MA, Slichter SJ, Heddle NM, Murphy MF. New strategies for the optimal use of platelet transfusions. ASH Education Program Book. 2008;21(2)198-203.

14.Lim J, Kim Y, Han K, Kim M, Lee KY, Kim WI, et al. Flow cytometric monocyte phagocytic assay for predicting platelet transfusion outcome. Transfusion. 2002;42(3):309-16.

15.Shivdasani AR, Haluska FG, Dock LN. GraftVersus-Host disease associated with transfusion of blood from unrelated hla-homozygous donor. The New Engl. J.Med. 1993;328(11):766-70.

16.Costa A.N, Scolari MP, Iannelli S. ELISA AntiHLA Antibody screeing Identifies Non-Compeletmentfixing Antibodies Responsible for Acute Graft Rejection. A case Report. Eury J Immunogenetics. 1996;383-7.

17.deAlmeida MC, Silva A, Barral A, Netto MB. A simple method for human peripheral blood monocyte isolation. MEMORIAS-INSTITUTO OSWALDO CRUZ. 2000;95(2):221-4.

18.Bøyum A, Brincker Fjerdingstad H, Martinsen I, Lea T, Løvhaug D. Separation of human lymphocytesfrom citrated blood by density gradient (NycoPrep) centrifugation: monocyte depletion depending upon activation of membrane potassium channels. Scandin J Immuno. 2002;56(1):76-84.

19.Gates K1, MacPherson BR. Retrospective evaluation of flow cytometry as a platelet crossmatching procedure. Cytometry. 1994;18(3):123-8.

[DOR: 20.1001.1.15614107.1397.20.6.3.1]
[DOI: 10.18869/acadpub.jbums.20.6.20]

20.Triulzi DJ, Assmann SF, Strauss RG, Ness P, Hess JR ,Kaufman RM, et al. The impact of platelet transfusion characteristics on posttransfusion platelet increments and clinical bleeding in patients with hypoproliferative thrombocytopenia. Blood. 2012;119(23):5553-62.

21.Slichter SJ, Davis K, EnrightH, Braine H, Gernsheimer T, Kao K-J, et al. Factors affecting posttransfusion platelet increments, platelet refractoriness, and platelet transfusion intervals in thrombocytopenic patients. Blood. 2005;105(10):4106-14.

22.Levin MD, van der Holt B, de Veld JC, Gratama JW, de Vries W, van't Veer MB. The value of crossmatch tests and panel tests as a screening tool to predict the outcome of platelet transfusion in a non-selected haematological population of patients. Vox Sang. 2004;87:291-8.

23.Levin MD, de Veld JC, van der Holt B, van 't Veer MB. Immune and nonimmune causes of low recovery from leukodepleted platelet transfusions: a prospective study. Ann Hematol. Ann Hematol. 2003;82(6):357-62.

[DOR: 20.1001.1.15614107.1397.20.6.3.1]

[DOI: 10.18869/acadpub.jbums.20.6.20]