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Flow Cytometric Measurement of CD41/CD61, CD42b Platelet Receptors and Platelet Factor 3 Activity in Lyophilized Infusible Platelet Membrane Preparation

Keimer Teke, Saleh Nasiri*, Kamran Mousavi Hosseini

Blood Transfusion Research Center, High Institute for Research and Education in Transfusion Medicine, Tehran, Iran

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*Corresponding author:
Saleh Nasiri
Blood Transfusion Research Center, High Institute for Research and Education in Transfusion Medicine, Tehran, Iran
Email: salehnasiri2012@gmail.com

ABSTRACT

Background: The short life time of human platelet units has led to a chronic shortage of fresh platelets in blood transfusion centers. Many approaches have been investigated experimentally to produce new hemostatically active platelet products that are capable of long term storage. Infusible platelet membrane (IPM) prepared from fresh or outdated human platelets have been developed as an alternative to standard platelet concentrates with the additional advantage of long shelf life. We aimed to measure the presence of CD41/CD61, CD42b platelet receptors and platelet factor 3 (PF3) activity during IPM preparation.
Methods: After pooling of fresh platelet concentrates, freeze-thawing, washing, formulation and lyophilization steps were performed.
Results: Flow cytometric analysis of CD41, CD61 and CD42b after lyophilization of IPM found to be 45.9%, 51.3% and 44.4% with PF3 activity of 39% in contrast to 81.5%, 87.5% and 81.1%, respectively on fresh pooled platelet concentrates with 100% PF3 activity.
Conclusion: The results showed that lyophilized IPM product can preserve major adhesion (CD42b) and aggregation (CD41/CD61) platelet receptors and may retain some PF3 activity at acceptable level which demonstrates hemostatic property of the lyophilized IPM.

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Introduction

Platelet transfusion is an effective therapy to control bleeding in thrombocytopenic patients. Unfortunately, platelet units are generally stored in blood banks for 3-5 days, thereafter they are discarded.[1, 2] For preserving platelets for a longer period, a number of attempts have been taken to develop substitutes for platelets as possible alternatives to currently available platelet concentrates.[3] A number of studies have shown that infusible platelet membrane (IPM) is the best candidate as a platelet substitute with a certain degree of hemostatic property both *in vitro* and *in vivo* experiments.[4-11] GPIb-IX-V complex is a membrane receptor complex on surface of the platelets that mediates the first critical step in platelet adhesion, facilitating binding to vWF on damaged subendothelium under high fluid shear stress conditions.[12-14] Aggregation begins after activation of the complex of GPIIb/IIIa (CD41/CD61) receptor which allows these receptors to bind with vWF or fibrinogen.[15] Platelet factor 3 as a phospholipid lipoprotein blood coagulation factor derived from platelets acts with certain plasma thromboplastin factors to convert prothrombin to thrombin. The PF3 assay relies on the principle that incubation of platelet-rich plasma (PRP) with kaolin activates the procoagulant activity of platelets, resulting in a progressive shortening of both recalcification time and Russell viper venom time. In this research, kaolin clotting time method was used to measure PF3 activity.[16,

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[17] The purpose of the present in vitro study was to measure CD41/CD61, CD42b and PF3 adhesion, aggregation and procoagulant activity during preparation of lyophilized IPM from fresh platelet concentrates.

Materials and Methods

Preparation of IPM

Five random units of conventional platelet concentrate was prepared in Tehran Blood Transfusion Center following a routine procedure. They were immediately pooled and centrifuged for 15 min at 1000 RPM to remove contaminating red cells and white cells. The supernatant was centrifuged for 30 min at 2500 RPM to remove plasma. The precipitate was resuspended in 15 ml physiological saline solution. For lysis and disruption of platelets, freeze-thaw procedure was repeated three times at -80°C and room temperature for 6 and 2 h, respectively. The solution was washed twice with physiological saline solution for removing any intracellular component by centrifugation (30 min at 2500 RPM). The precipitate was resuspended in 15 ml of the same solution.

Formulation

The sample of IPM was formulated with sucrose 1 M and human serum albumin 1% before lyophilization in a sterile condition.

Lyophilization

The samples of IPM were transferred in freezer -80C° with freezing rate -1C°/min for 90 minutes. After freezing, the frozen platelet solution was transferred to lyophilizator (VaCo 5-II, Zirbus Technology) under100 mili Torr vacuum pressure for 16 h. Freeze-dried platelet concentrates were rehydrated for approximately 2 hours in a closed box with moisture-saturated air at 37°C and then in 5 ml physiological saline solution.

Flow Cytometry Analysis

The pooled sample was tested using a CyFlow® ML flow cytometer (Partec GmbH Otto-Hahn-Straβe 32, D-48161 Munster, Germany) equipped with a 488-nm argon-ion laser and 635 nm red diode laser. For analysis of platelets in washed platelet suspensions, fluorescence parameters and light scattering were adjusted at logarithmic gain, and the threshold was set to eliminate events negative for platelet markers. Quality control procedures were followed; the flow cytometer setting was identical for all analyses and negative and positive controls were performed routinely.

PF3 Measurement

Platelet factor 3 assay was performed on pooled samples according to the kaolin clotting time of platelet rich plasma.[17] Platelet rich plasma or IPM, platelet poor plasma, kaolin and calcium chloride were used during the assay. Each test was repeated three times. The clotting time was converted to percent activity by use of a standard curve.

Results

Adhesion and aggregation of platelets play an important role during clotting of blood. The results of lyophilized IPM showed that adhesion and aggregation of CD42b and CD41/CD61 receptors can be preserved during freeze-thawing, washing and lyophilization which were found to be 44.4%, 45.9% and 51.3%, respectively (Table 1).

The main goal of this study was to demonstrate overall biological and procoagulant activity of IPM in the plasma without the presence of other stimulators such as agonists or injured endothelial cells for adhesion and aggregation pathways. The results of Table 2 demonstrated PF3 activity of 39% in the final lyophilized product. Our results showed that freeze-thawing step has more detrimental effects on IPM in comparison with lyophilization step.

Discussion

The principle of the experiment on IPM was introduced by Chao et al. in 1996. He concluded that glycoprotein Ib (CD42b), responsible for the binding of platelets to exposed subendothelial matrix at the site of vessel injury, is present in IPM; while the GPIIb/IIIa (CD41/CD61) complex, which is found on the surface of activated platelets, is absent in IPM. In fact, HLA antigens (class I and II) and GPIIb/IIIa epitopes seem to be lost from platelets during freeze-thawing, centrifugation

Table 1: Flow cytometry analysis of pooled fresh platelets*, freeze-thawed and rehydrated lyophilized samples of infusible platelet membrane (IPM).

Assay (%)	Pooled fresh platelet concentrate	Freeze-thawed IPM	Lyophilized IPM
CD42b	81.1	75.6	44.4
CD41	81.5	66.2	45.9
CD61	87.5	80.8	51.3

*Five fresh conventional platelet concentrates were pooled.

Table 2: Results of platelet factor 3 (PF3) assay during lyophilization of IPM

	μ±SD*	Range (s)	Activity** (%)
Fresh pooled platelet concentrate	10±1.0	9-11	100
Freeze-thawed IPM	36±1.7	35-38	41
Rehydrated lyophilized IPM	37±1.8	36-39	39
Negative control***	55±2.5	52-57	0

*The tests were repeated three times; **Percent activity was calculated by using standard curve; ***Negative control: Physiological saline was used instead of platelet rich plasma during the assay

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and especially after heat treatment. A high level of phospholipid-associated activity of PF3, which mediates the activation of prothrombin by complex of Va-Xa, is found in IPM.[4] Our results showed better preservation of GPIIb/IIIa due to the absence of heat-treated viral inactivation method such as pasteurization in our protocol. Our results was also similar to Chao et al's with respect to the PF3 activity of IPM.[4]

Other study by Graham SS et al. described that infusible platelet membranes (IPMs) prepared from fresh or outdated human platelets are capable of correcting prolonged bleeding time in thrombocytopenic rabbits. They also claimed that function of the GPIb/IX/V receptor which is binding to von Willebrand factor in a modular manner, is partially retained.[18] In another study by Alemany et al., addition of nonviable platelet preparations to thrombocytopenic blood always promoted a statistically significant increase in deposition of fibrin on subendothelium, but lyophilized platelets retained some ability to interact with the subendothelium. Their flow cytometry studies demonstrated that GPIb, GPIIIa and P-selectin are present on lyophilised IPM[19] and these molecules are essential for the hemostatic function of IPM.[20]

Interestingly, coagulation-dependent mechanisms may not require the participation of a great number of intact platelets, but may depend on the expression of phospholipids in the appropriate conformation on the external side of platelet membrane.[21]

In general, our results showed concordant results with other studies in terms of hemostatic activity of IPM both in-vitro[4, 17-20] and in-vivo.[4-6, 22, 23]

Conclusion

It may be concluded that IPM as a platelet substitute retains hemostatic properties to some extent. It should be mentioned that not all the properties of platelets necessarily have to be provided by a platelet substitute. Platelet substitutes such as IPM may be able to replace certain aspects of platelet function and may be appropriate in specific clinical situations. However, further investigation will be needed to define the exact role of IPM in the management of patients with thrombocytopenia.

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Conflict of Interest: None declared.

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