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Original Article:

Changes of Activation and Apoptotic Platelet Markers During Apheresis and Storage

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ABSTRACT

Background: Platelets are known to undergo, shape changes, activation, a release reaction and apoptosis/ necrosis during processing and storage. As aphaeresis may have deleterious impact on platelet achievability and functional integrity.

Methods: Platelets concentrates from 50 male volunteers obtained by COBE spectra cell separator were screened for platelet activation (CD62 and CD154) and apoptosis (Phosphatidylserine detected by Annexin V). Base line of donor samples before separation, during aphaeresis and at the third day of storage samples were collected. Platelet aggregation to ADP and collagen was performed.

Results: There was a statistically significant increase in expression of the activation markers in platelet samples at 2 different time points (during separation samples and 3rd day samples). Although the increase in Annexin V expression was slight, it was statistically significant. There was marked significant decline in the platelet aggregation. The correlations between the values of CD62, CD154 and Annexin V were detected in base line samples and were more evident in both during separation and at the 3rd day of platelets storage. Correlation between values of platelets aggregation to collagen and Annexin V was significantly relevant only in the base line samples. No other correlations were encountered between platelet aggregation and markers of activation and apoptosis during apheresis and storage.

Conclusions: Initial platelet activation which is induced by aphaeresis may have an impact on phosphatidylserine expression with no impact on aggregation function of platelets during storage.

Key Words: *Annexin V – Aphaeresis – CD154 – CD62 – Storage.*

INTRODUCTION

Platelets transfusion can be performed using either whole blood or aphaeresis product. In the case of platelet-apheresis, production of platelet concentrates (PCs) with a standard number of platelets and minimal leukocyte contamination, together with well-preserved function of platelets and minimal platelet activation, is the ultimate goal [1,2].

In spite of substantial scientific effort, mechanisms leading to the damage of platelets during their collection and storage are not fully understood [3]. Studies have suggested that platelet activation or stimulation may play an important role in the development of the platelet storage lesion.

CD62 (P-selectin) is a member of the selection family of cellular adhesion molecules, and it mediates the binding of activated platelets to neutrophils, monocytes, and endothelial cells [3]. P-selectin expression on platelet surfaces correlates with loss of viability upon transfusion of the stored platelet concentrates [4].

CD154 (CD40 ligand CD40L) is a member of the tumor necrosis family. Several important immunological phenomena are attributed to CD154 interaction with its receptor CD40 including B cell immunoglobulin class switching and maturation of dendritic cells [5,6]. CD154 has been identified on the surface of platelets [7] and its expression on platelets has a key role in inflammation and prothrombotic activity [8].

In vivo, platelet aging is associated with Phosphatidylserine (PS) exposure. Previous reports described the PS as a target molecule for the recognition and removal of activated or damaged platelets. This function is attributed

to professional phagocytes equipped with specific receptors for PS, such as the class B scavenger proteins SR-BI and CD36 [9-12]. This phenomenon means that platelets with a high PS content in the outer plasma membrane could be removed after transfusion by the reticuloendothelial system independently of their functional status [9,10,13,14]. These events may play an important role in the removal of senescent platelets from the circulation [11,12].

This study aimed to investigate the platelet alterations by detecting activation and apoptotic markers in concentrates obtained by cell separator pre and during separation and after 3 days storage in our transfusion center. Annexin V measurement using flow cytometry was employed for PS detection. We aimed also to study the effects of these changes on the platelets aggregation function.

MATERIAL AND METHODS

Cytapheresis:

Platelet concentrates were collected from 50 male volunteers who met the requirements of the American Association of Blood Bank for healthy blood donors. Segments from Platelet concentrate bags obtained by COBE Spectra blood separators (Gambro BCT Inc., Lakewood, USA) were used to obtain samples. Base line (BL) samples from donors before separation were collected. The second specimen was obtained during aphaeresis (DS) and the third in the third day of storage in platelet agitator.

Determination of Platelet CD62-P, CD154 and Annexin-V by Flow Cytometry:

To minimize and standardize platelet activation during venesection, an atraumatic procedure was used. Whole blood anti-coagulated with EDTA and the aphaeresis products were diluted 1:8 in HEPES buffer. Twenty five µl of diluted blood were directly labeled with 5µl of each CD62P-FITC, CD154-PE, CD41a Per-CP and Annexin-V APC antibodies combination. CD41a (glycoprotein IIb/IIIa complex) is specific to platelets, thus, it can be used to recognize and gate on the stained platelets. All antibodies were purchased from Pharmingen, BD, USA, except Annexin-V, which was purchased from IQ product, Groningen, Netherlands. After 20 minutes incubation at room temperature in the dark, the samples were lysed by ammonium chloride lysing solution and washed with PBS.

Analysis by flow cytometry was performed immediately using FACSCaliber (BD, USA) by identification of platelets according to their characteristics was determined using log forward scatter for size and log side scatter for granularity. Platelet gate was adjusted such that >95% of the particles analyzed were anti CD41a positive. The platelet activation markers and Annexin-V were expressed as geometric mean fluorescence intensity (GMFI) and as percentage of antibody positive cells. The positivity was defined as fluorescence higher than that of the isotypic control (Fig. 1).

Platelet aggregation studies:

Platelet aggregation to ADP (10µM) and Collagen (2µg/ml) had been performed using optical method of measurement of platelet aggregation (light transmitter method) by platelet aggregation profiler model PAP-4 (Bio/data corporation). The reagents were provided by Chrono-Log Company, USA.

Statistical analysis:

The data were collected, categorized and processed by using Statistical Package for Social Sciences (SPSS), version 15 software packages. The quantitative variables were expressed as mean ± standard deviation (SD) and comparison was done using paired students *t*`-test. *p*-value levels of <0.05 was considered statistically significant. Correlations between quantitative variables were done using Pearson correlation.

RESULTS

Fifty donations were studied; their product characteristics are shown in Table (1). There was a statistically significant increase in the mean values of both CD62 & CD154 (% and GMFl) in DS samples compared to BL values. These markers revealed marked rising in the 3rd day of storage samples and showed a statistically significant increase in comparison to those of BL and DS samples (Table 2).

There were minimal differences between the three samples (BL, DS, and 3rd day) in the mean value (% and GMFl) of Annexin V. However there was statically significant increase in DS samples (% and GMFl) when compared to BL samples and in 3rd day samples (% and GMFI) when compared to BL samples. When the comparison was performed between DS and

3rd day samples there was a statistically significant increase in the percentage of cells expressing Annexin V with no significant difference in Annexin V GMFl.

There was marked decline in the platelet aggregation to both ADP and collagen during separation and on the third day of storage; the difference is statistically significant (Table 2).

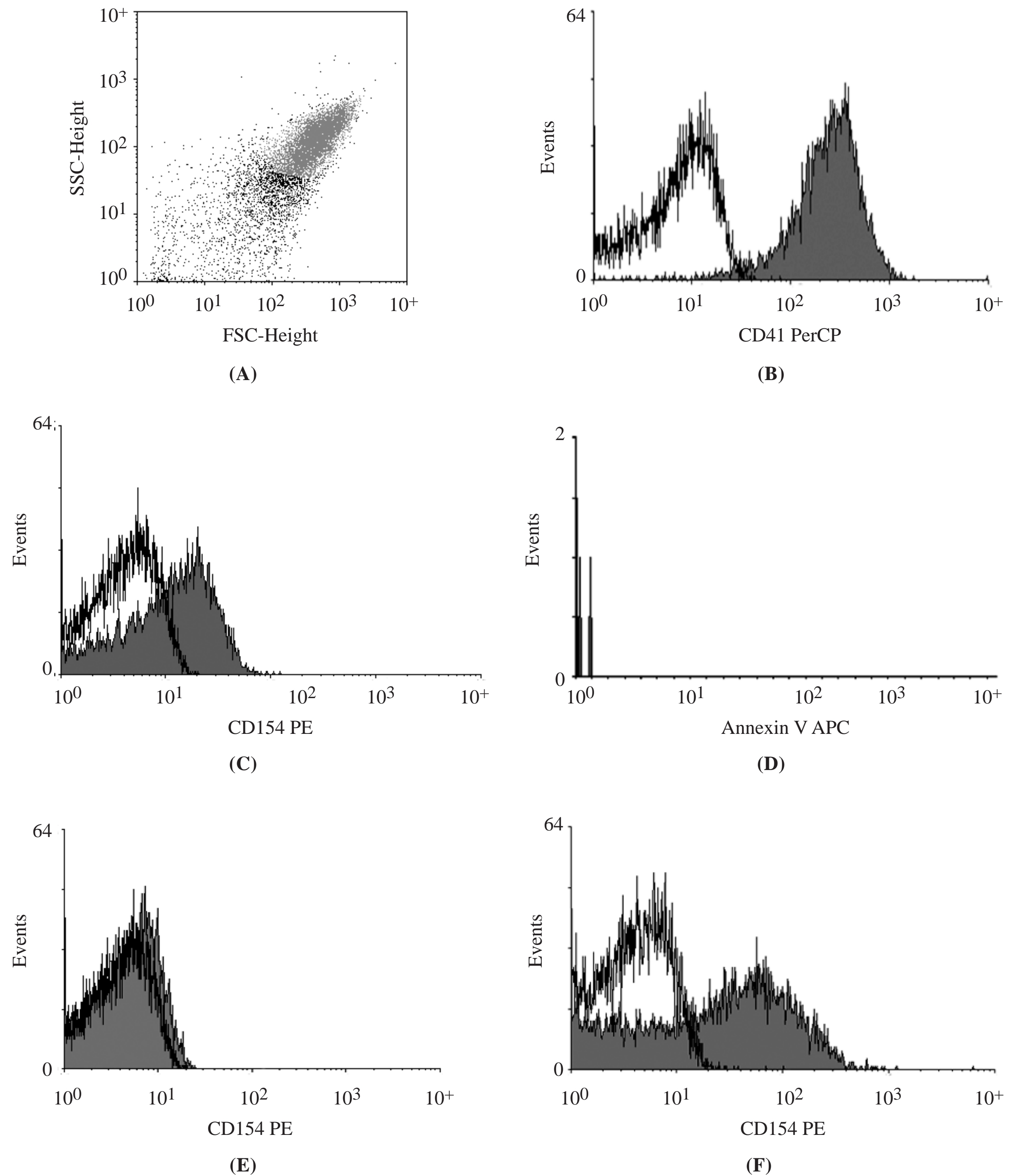

Fig. (1): Platelet gate (A) which adjusted that >95% of the particles were anti CD41a positive (B). CD62 (C) and Annexin-V (D) were expressed as geometric mean fluorescence intensity (GMFI). CD154 were expressed as GMFI in base line (E) and in the 3rd day of storage (F). The positivity was defined as fluorescence (red histogram) higher than that of the isotype control (open histogram).

Positive correlations were observed between percentage values of platelets activating markers CD62 and CD154 in BL samples (r=0.634 and p=0.02) and also between their GMFl values (r=0.684 and p=0.01). These correlations were stronger in D.S and in the 3rd day of platelets storage samples and were observed between % and GMFl of CD62 and CD154 (r=0.905, p<0.001, Fig. 2) and (r=0.919, p<0.001) respectively.

There was a positive correlation between Annexin V percentage expression between of BL and DS samples (r=0.714 and p=0.004), it was also encountered between their GMFl values (r=0.838 and p<0.001). However these correlations decreased when comparing DS and in the 3rd day of platelets storage samples (r=0.646, p=0.043) and (r=0.661, p=0.038) respectively.

On the other hand, correlations between platelets activation and apoptotic markers were performed and revealed positive relation between BL CD62 and BL Annexin V GMFl (r=0.555, p=0.03). This correlation was increased to be r=0.682, p=0.03 in the 3rd day samples. It was also detected also between CD154%, CD154 GMFl and Annexin V GMFl in the 3rd day of storage (r=0.778, p=0.01 and r=0.675, p=0.03 respectively).

Correlation between values of platelets aggregation to collagen and Annexin V in the BL samples was encountered (r=0.535, p=0.04) (Fig. 3). Positive correlations were encountered between DS platelets aggregation to ADP and platelets aggregation to collagen (r=0.688 p=0.002). This correlation was increased after platelets storage in day 3 samples (r=0.775 and p<0.001).

Table (1): Product characteristics for 50 donations.

Procedure time (min)	90±10
Total procedure volume (ml)	2500±340
Platelets volume (ml)	264±18
Platelets yield (plt/10^9)	361±72
Platelet concentration (plt/l)	1150±95

Table (2): Comparison between base line, during separation and 3rd day of storage samples in all tested markers.

Markers	Base line	During Separation	3rd day	p value		
				A	B	C
CD62%	1.7±1.7	43.4±16.1	64.3±23.2	0.000	0.000	0.01
CD62 GMFl	3.5±0.47	25.8±34.6	71.2±66.7	0.011	0.011	0.04
CD154%	0.18±0.12	23.8±14.8	47.6±11.4	0.00	0.000	0.04
CD154 GMFl	2.7±0.28	9.9±8.6	19.6±14.5	0.011	0.05	0.04
Annexin V %	0.3±0.17	0.5±0.24	1.0±0.46	0.00	0.001	0.008
Annexin V GMFl	3.15±0.47	3.4±0.6	3.4±0.65	0.00	0.006	0.23
ADP %	67.0±6.7	39.0±4.8	9.0±4.19	0.00	0.000	0.000
Collagen %	60.0±3.4	32.0±5.8	7.0±3.85	0.00	0.000	0.000

GMFI: Geometric mean fluorescence intensity; Quantitative variables are expressed as mean ± standard deviation.
A = p-values when base line group compared with DS group.
B = p-values when DS group compared with 3rd day group.
C = p-values when base line group compared with 3rd day group.

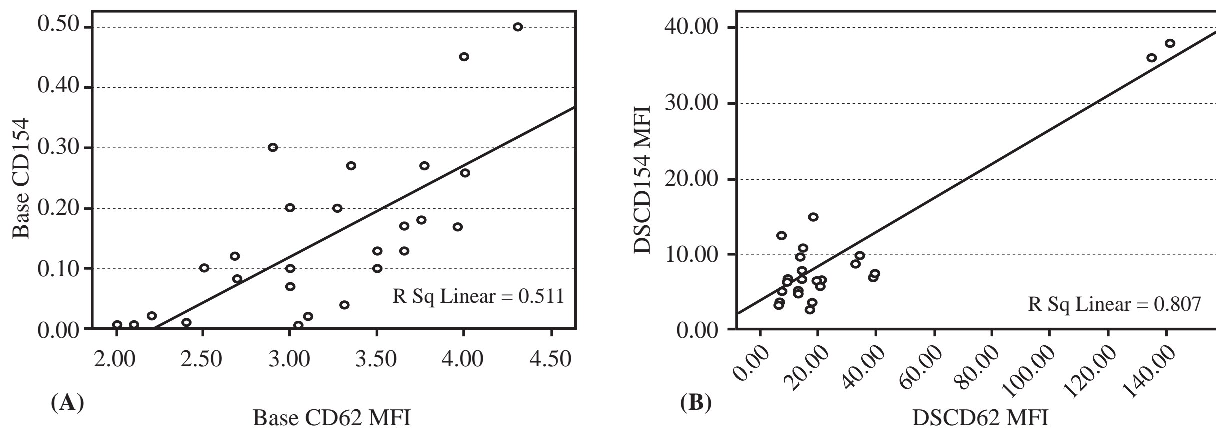

Fig. (2): Correlations between CD62 and CD154 GMFI in base line samples (A) and during separation samples (B).

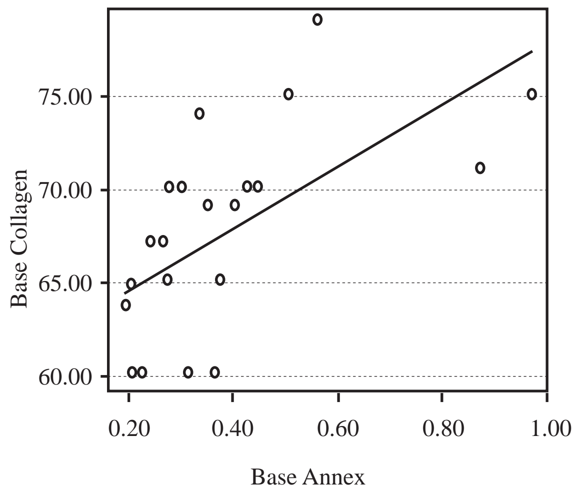

Fig. (3): Correlation between Annexin V % expression and aggregation to collagen in base line samples.

DISCUSSION

The present study was performed to analyze the degree of antigenic alterations in platelets caused by aphaeresis and storage of platelets concentrates, prepared with continuous cell separators. The results of this work demonstrated that platelets activation which was detected by flow cytometric analysis of CD62-P and CD154 begins early during separation and increases during the storage.

A number of authors have demonstrated that platelet activation markers increase during storage of PCs, they suggested some factors which may explain the increase in CD62-P expression such as the manner of agitation, the conditions available in container and the number of polymorphonuclear leukocytes in platelet concentrates **[15,16]**. Other authors **[17]** recognized this increase immediately after collection and after storage compared to pre-donation level in blood donors; they concluded that these changes are may be due to change in pH as a result of acid production which lead to decreased metabolic activity, function and viability of platelets **[18]**. The increased expression of CD62-P during separation may reflect aphaeresis technology; platelets are exposed to both physical forces and artificial surfaces **[19,20]**.

CD154 can activate adherent polymorphnuclear leukocytes and is associated with transfusion-induced acute lung injury **[21]**. Some authors have provided evidence that CD154 is implicated in adverse platelets transfusion reactions **[22]**. Others concluded that CD154 expression on platelets has a key role in inflammation and prothrombotic activity **[8]**. Our findings on platelets changes during and after apheresis are consistent with Kaufman et al. **[23]** who found high level of both membranous and soluble CD154 in stored PCs and provided additional evidence that this increase begins early during separation and increases during storage. This increase is affected by the same factors affecting CD 62-P expression as the current study showed significant positive correlations between the two markers at the three different time points of sampling (pre, during apheresis and after storage).

It is so important to detect the possible links between activation of platelets and their apoptosis, Therefore, in this study, platelet viability was tested by detecting the degree of PS expression on the platelets surface. It showed a minimal increase during separation and storage. There was a significant increase in the percentage of cells expressing Annexin V in 3rd day samples when compared to DS with no significant difference in Annexin V GMFl. Percentage of positive platelets is a qualitative marker to assess the degree of platelet activation but GMFI is a quantitative one **[24]**. It can be concluded that the increase in expression of Annexin V is more during separation and in the storage it is very limited to few platelets. It is possible that enhancement of PS exposure during separation is generated by the stress to which platelets are exposed during the aphaeresis procedure.

On the other hand, correlations were found between the percentage and GMFI of platelets positive for Annexin V during apheresis and the same parameters on third day of storage. These results disagree with Lai et al. **[25]** study who found a correlation between Annexin V value in the 3rd and 5th day of storage with no correlation between the same parameter immediately after apheresis and after storage. This may be attributed to the small size of samples of their study (twenty seven single donor PCs).

Other correlations were found between markers of activation and Annexin V in the 3rd day of storage. Some authors found similar results between P-selectin and Annexin V in PCs collected by continuous apheresis and concluded that the level of P-selectin closely reflects cell damage **[18,26]**. Additionally the results of this

work can suggest that CD154 may reflect cell damage also, and this effect takes some time to be observed as the correlations appear in the 3rd day of storage not during apheresis. To the best of our knowledge no such relations were reported between CD154 and Annexin V before.

A platelet aggregation testing to ADP and collagen results are in good agreement with earlier literatures. It was reported that aggregation response to ADP and collagen decreased gradually from the time of separation of PCs to the storage time in the platelets shaker for three days [27-29]. Sloand et al. [30] has also compared the platelets aggregation activity to ADP and collagen and postulated that ADP and collagen induced platelet activity of the PCs on the 5th day is much lower than normal. Various authors [31-34] demonstrated impaired platelets function through investigation of platelets aggregation with thrombin and collagen. Findings of the current work are in concordance with the results which suggest that PCs should be used as soon as possible, since there is a loss of platelet functions [34].

In the present work, a significant correlation was detected between platelets aggregation to collagen and Annexin V value in BL. This can be explained by the fact that PS in platelets exerts a procoagulant effect, accelerating the enzymatic cleavage of coagulation proteins (e.g., prothrombin) on the platelet membrane. This event triggers the formation of the fibrin clot and the stabilization of the platelet thrombus [35]. On the other hand, no other correlations could be detected between platelet aggregation and markers of activation and apoptosis. This means that no one of these factors alone can affect the function of platelets collected by apheresis. Neiva et al. [36] found a correlation between pH and aggregation and between PCo_2 and aggregation. Considering that many variations in the composition of platelets membrane can be involved in aggregation, this area needs further work.

It could be concluded that initial platelet activation which is induced by aphaeresis has an impact on PS expression with no impact on aggregation function of platelets during storage.

Conflict of interest statement:

All authors declare that they have no conflict of interest.

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