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PLATELET STORAGE LESION: ROLE OF CD62 (P-SELECTIN)

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ABSTRACT
N platelets day 5: Without PAS the range was from 8.57 ± 1.12 similarly on day 5 with PAS the range was from .97 ± .54 and the p value was 0.001. **(d) For CD62 evaluation in platelets day 7:**Without PAS the range was from 9.27 ± 1.01 similarly on day 7 with PAS the range was from .77 ± .61 and the p value was 0.001.**(e) For CD62 evaluation in platelets day 10**: Without PAS the range was from 15.13 ± 2.55. Similarly on day 10 with PAS the range was from .53 ± .55 and the p value was 0.001. **Conclusion**: Cytokines such as CD62P play role in mediating transfusion reactions ·These cytokines have pyrogenic effect and can mediate inflammatory reactions.

KEYWORD: Without PAS the range inflammatory reactions.

INTRODUCTION

Platelet demand has increased over the past decade as they are not only being using to control or prevent bleeding but also being increasingly used as a source of growth factors in tissue repair, wound healing, and skin rejuvenation. Studies have shown promising results of use of topical platelet therapy in various clinical conditions. Therefore, retention of platelet functionality as much as possible is of prime importance as this may help in extension of shelf-life, and thus better inventory management.[1]

Platelets are unique blood cells that play a pivotal role in mediating hemostasis and thrombosis. Over the years, the demand for platelets is steadily rising because of their increased use in patients with thrombocytopenia and platelet function defects. The availability of platelets for transfusion is restricted by the markedly short shelf of platelets owing to risk of bacterial contamination and storage-related deterioration known as platelet storage lesion (PSL). PSL is best defined as the sum of all the deleterious changes in platelets structure and function that arise from time the blood is withdrawn from the donor to the time the platelets are transfused to the recipients. The mechanisms responsible for PSL are multifactorial and not clearly understood. Several factors including the methods of collection, processing storage and manipulation after collection can result in PSL. These lesions are associated with decreased in vivo platelet recovery, survival and hemostasis activity after transfusion.[2]

A battery of in vivo and in vitro tests has been adapted to asses PSL. Corrected count increment after platelet transfusion and bleeding time studies are commonly used for evaluating product efficacy and improved hemostasis, respectively. However, these do not reflect the true picture due to uncontrollable patient variables. Another alternative approach to measure the efficacy of product transfused is to measure the in vivo recovery and survival of fresh or stored radiolabelled or biotinylated platelets in healthy volunteers. However, these in vivo studies are expensive, time consuming, and complex to perform.[3]

MATERIALS AND METHODS

The project was approved by the institutional ethics committee. After selection of blood donor as per Drugs and Cosmetics rules 1945, the blood was collected in triple bags from donors attending various blood donation camps and donating blood in-house. Information on their age, sex, address and parameters of medical examination was recorded in donor registration from which includes the consent of the donor.

25 Platelet Concentrate (PC) were prepared namely platelet rich plasma (PRP) –PC. 25 PRP-PC were prepared from triple bags by centrifuging the bags at 20-22^{0}C at light spin 2,000 x g for 3 min. The bag with PRP and another satellite bag were centrifuged at 20-22^{0}C at heavy spin 5000 xg or 5 min. Supernatant plasma was transferred into another empty satellite bag. Approximately 50 ml of plasma was left with the platelets. PC was let undisturbed at 20-22^{0}C for 1 h., and then the platelets plasma were resuspended by gently mixing for 10 min. Platelets were stored at 20-22^{0}C under constant agitation in platelet incubator with agitator

Immediately after preparing PC, about 5ml sample was removed for quality control and cytokine measurement by ELISA method. The level of CD62 was measured in the stored platelet supernatants using commercially available assays (Bio Source International Inc., Camarillo, CA, USA) following the manufacturer's instructions.

The use of enzyme linked immunosorbent assay (ELISA) has become very popular for the determination of cytokine levels. These assays are sensitive, rapid and monospecific due to the use of monoclonal antibodies.[1]

Whole blood was collected in 450 ml bags containing 63 ml of CPDA anticoagulant, kept at room temperature (20-24^{0}C) and PRP-PC was prepared within 6 hours of collection. The donors arm was selected and blood pressure raised by a BP cuff to locate and select a prominent vein for phlebotomy. The donor arm was prepared by cleaning the veni-puncture site at the anticubital fossa starting from center to peripheral of the selected area by spirit and betadine. The phlebotomy was done and BP set between 40-60 mm Hg with continuous pressing of the sponge ball by the donors arm in -orders to maintain optimum blood flow. The entire procedure was completed within 8 minutes under medical supervision and 450ml volume of blood collected (blood collection monitors, TERUMO PENPOL, Ltd., Trivandrum, India) in triple bags containing CPDA 1 anticoagulant (HLL, Life Care Limited., Kerala). PRP was separated from whole blood by light spin centrifugation at 1750 rpm for 11 minutes at 21^{0}C. with acceleration and deceleration curves of 5 and 4 respectively (Heraeus Kendro, Hanau, Germany 6000i).

The PC were concentrated by heavy spin centrifugation at 3940 rpm for 5 minutes at 21^{0}C with acceleration and deceleration curves of 9 and 5 respectively along with subsequent discarding of supernatant plasma.

RDPs were divided into two parts by a sterile tubing welder (Terumo TSCD, SC-201 AH, Leuven, Belgium). and one portion of RDPs will be stored in storage solution for platelets (SSP+) Macopharma India Transfusion Solution Private Limited, Span Health Care Private Limited).

8ml of PC and 10-15 ml of residual plasma was added to give a final concentration of 80% additive solution and 20% plasma. The volume of additive solution and stored platelets had a mean volume of around 50ml. PAS contains Na_{3-} citrate $2H_2$ 0:3. 18g, Na-acetate $3H_2$ O:4.42g, $NaH2PO_4$ $2H_2$ O:1.05g, Na_2HPO_4:3.05g, KCI: 0.37g, MgCl $_2$6H $_2$O:0.30g,NaCI: 4.05g. at Ph 7.2.

RDPs were placed in a platelet agitator (Terumopenpol Private Limited) with continuous agitation at 70 cycles /minutes during storage at 22 ^{0}C. 3ml of sample was taken from both the groups, (PC with and without additive solution) on day 0,3,5,7 and 10 to study the platelet indices and swirling.

The Hemoglobin count, platelet count, Mean platelet volume (MPV), platelet volume distribution width (PDW) and Platelet larger cell ration (P-LCR) were evaluated by Automated Cell Counter (SYSMEX KX-21 Sysmex Corporation Kobe, Japan).

Platelet swirling was done and scored as score 0: Turbid, Score 1: Swirling only in some part of the bag, Score 2: Clear homogenous swirling in all part of the bag and Score 3: Very clear homogenous swirling in all part of the bag.

For microbiological cultures additional 5ml samples was taken to observe bacterial contamination of PC. Aerobic and anaerobic cultures was performed on all the samples on the day, on day 0,5 and 10 using fully automated culture system, BACTEC (BACTEC system 9240, Beton Dickson And Company, United States).

At the time of initial sampling, an additional 2 mL was removed for determination of WBC and PLT counting by means of an automated analyzer (Cell-Dyn 1700, UNIPATH, Abbott Diagnostics, CA), and the volume of PRP-PCs was calculated taking the bag weight into account. For WBC counting we used Nageotte chamber methods.

Frozen aliquots of the plasma portion of stored PLT components were thawed immediately before assay at room temperature and then centrifuged for 10 minutes at 5000 rpm CD62 were performed in duplicate by commercially available ELISA (Quantikine, R&D Systems, Minneapolis, MN) and according to manufacture's protocols.

For statistical evaluation of data, WinSTAT (version 3.1, Kalmia, Whitehall, PA) was used. To compare paired data of the PC samples during storage, we used the Wilcoxon signed-rank test for nonparametric comparison of cytokine levels at different times. Person's correlation coefficient was used to assess the effect of storage time and PLT and WBC contents in the cytokine accumulation. The association between the gene polymorphism and the cytokine accumulation during storage was evaluated using the Kruskal-Wallis test and U-test (Mann-Whitney). Hardy-Weinberg equilibrium in our donor population was tested using the chi-square test. All statistical analyses were performed using nonparametric methods. A p value below 0.05 was considered significant.

RESULTS

Estimation of CD62

(a) **For CD62 evaluation in platelets on day 0**: Without PAS the range was from 5.28 $\pm$ to 1.96. Similarly for Platelet with PAS the range was from .78 $\pm$ .58 and the p value was 0.001.

(b) **For CD62 evaluation in platelets on day 3:** Without PAS the range was from 5.60 ± 1.03. Similarly on day 3 with PAS, the range was from .75 ± .56 and the p value was 0.001.
(c) **For CD62 evaluation in platelets day 5:** Without PAS the range was from 8.57 ± 1.12 similarly on day 5 with PAS the range was from .97 ± .54 and the p value was 0.001.
(d) **For CD62 evaluation in platelets day 7:**Without PAS the range was from 9.27 ± 1.01 similarly on day 7 with PAS the range was from .77 ± .61 and the p value was 0.001.
(e) **For CD62 evaluation in platelets day 10**: Without PAS the range was from 15.13 ± 2.55. Similarly on day 10 with PAS the range was from .53 ± .55 and the p value was 0.001.

Table 1: Showing comparative analysis of CD62 with and without PAS.

DAYS	CD62 with PAS (Mean ± 2SD)	CD62 without PAS(Mean± 2SD)	P value
0 day	.78 ± .58	5.28 ± 1.96	0.001
3 day	.75 ± .56	5.60 ± 1.03	0.001
5 day	.97 ± .54	8.57 ± 1.12	0.001
7 day	.77 ± .61	9.27 ± 1.01	0.001
10 day	.53 ± .55	15.13 ± 2.55	0.001

Wilcoxon Signed Ranks Test
Statistical Analysis – The data was Collected and entered in Microsoft Excel and Coded. The data was analysed using SPSS Software version 22. The Descriptive statistics were expressed in terms of frequencies, percentages, mean, standard deviation and Median. Wilcoxon Signed Rank test was used as for comparison between groups. P value <0.05 was considered to be statistically significant.

Table 2: For CD62 (zero1, three1, five1, seven1, ten1 → with PAS and zero, three, five, seven, ten → without PAS).

Descriptive Statistics								
	N	Mean	Std. Deviation	Minimum	Maximum	Percentiles		
						25th	50th (Median)	75th
zero1	20	.78655	.580371	.022	1.884	.25250	.70350	1.27525
three1	20	.75915	.560411	.052	1.940	.32500	.63750	1.22775
five1	20	.97285	.540321	.075	1.948	.46525	.92950	1.42275
seven1	20	.77605	.619921	.045	1.950	.27725	.69550	1.07500
ten1	20	.53705	.550919	.020	1.992	.05175	.42450	1.02500
zero	20	5.28655	1.965609	2.201	8.817	3.42575	4.91800	7.03275
three	20	5.60915	1.032576	4.052	7.661	4.43300	5.89700	6.28825
five	20	8.57285	1.123917	6.481	10.335	7.84975	8.89400	9.41875
seven	20	9.27605	1.018739	7.045	10.950	8.79500	9.07950	9.83200
ten	20	15.13705	2.550516	10.046	19.320	12.56675	16.01100	17.32425

Test Statistics[a]					
	zero - zero1	three - three1	five - five1	seven - seven1	ten - ten1
Z	-3.943[b]	-3.964[b]	-3.951[b]	-3.998[b]	-3.930[b]
Asymp. Sig. (2-tailed)	.000	.000	.000	.000	.000
a. Wilcoxon Signed Ranks Test					
b. Based on negative ranks.					

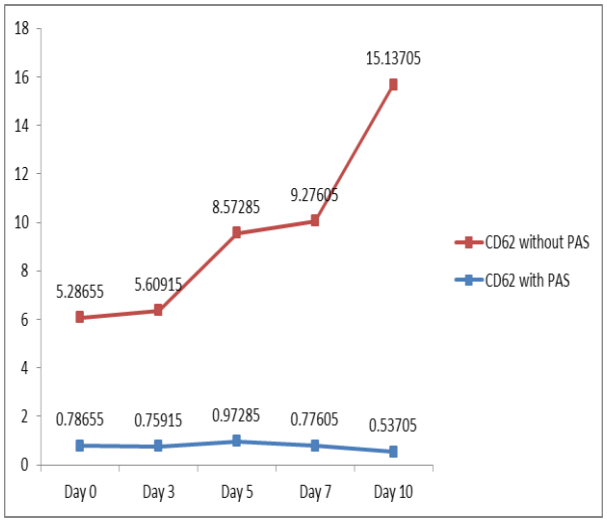

Figure 1: showing comparative study of CD62 with and without PAS.

DISCUSSION

Preservation of platelet structure, composition, and function during preparation and storage is one of the primary goals of transfusion medicine practice. Technical advances in the form of introduction of automated cell separators, optimized centrifugation procedures, improved storage containers, leucoreduction, and platelet additive solution CD62 has greatly facilitated the collection and storage of platelets. Despite these advances, CD62 is still a matter of concern. Platelets get activated following exposure to foreign surface, trauma, low pH, agonist (thrombin, ADP), and shear stress.[4] Upon activation, the platelets lose their discoid morphology and become more spherical with multiple pseudopods. Conformational changes in GPIIb/IIIa complex expose bindings sites for adhesive proteins (fibrinogen, vWF) resulting in platelet aggregates. Activation further stimulates release of granular contents and expression of sequestered membrane proteins on outer surface. Release of these granular contents at the site of injury is indispensable for platelet function as the contents not only mediate the recruitment of leucocytes and platelets, promote clotting but also provide immunity against infection and contribution to wound healing. Moreover the presence of these contents in storage medium can lead to various transfusion reactions. Expression of negatively charged phospholipids (phosphatidyl serine and phosphatidyl ethanolamine) increases on the outer leaflet of activated platelets, providing a surface for the prothrombinase complex(X-Va), thereby contributing to procoagulant activity.[5]

During preparation and storage, PC undergoes metabolic and morphologic changes that leave undesirable effect on platelet structure and functions, causing poor outcomes after transfusion. The aim of this study was to evaluate morphological and biochemical alternations in PC during storage at 22oC, as a program of QC in IBTO centers. According to international standards, CPD-A1 is used as anticoagulant in bags, and PC is prepared by PRP method, the commonest PC preparation method in the world. The mean volume of PC per bag in the three centers was 57ml and the mean platelet count was 5.8×10.[10.6] Although smaller volume does not affect platelet functions, reduced plasma volume in PC, causing decrease in platelets movements in bags, increases the incidence of direct contacts of platelets to each other. These contacts leads to activation and aggregation of platelets before transfusion, and consequently aggregated platelets filtrate by transfusion sets and undesirable platelet count will achieve in patients after transfusion. This condition increases the demand for more PC, and increases the incidence of adverse effect of platelet transfusion like being refractory to platelet transfusion. The swirling score, done as a rapid inexpensive quality control measure, was assessed in our study. Swirling has a direct correlation with pH and function of platelets. Functional platelets have a discoid morphology, which allows them in this form appearance waves or hurricane can be seen with the naked eye. For evaluation of temperature effects on swirling of platelets in PC, Gabriele and colleagues compared swirling at 4°C and 22°C. Their results showed that swirling at 4°C was negative, but it was positive at 22°C. In our study, swirling was positive in PC during storage that indicates its preparation and storage was done in a good condition in these centers.[7] Over the years, improvements in donor screening has nearly eliminated hepatitis B virus, hepatitis C virus and HIV from many countries' blood supply. As the transfusion-transmission rates of these agents decreased, platelet bacterial contamination assumed a new noticeable role as the most frequent infectious risk of transfusion. Unlike other products such as red cell and FFP, while room temperature storage allows transfused platelets to circulate *in vivo*, it has the negative side of promoting bacterial growth. Because of this risk, platelet storage is ordinarily limited to only 5 days, making platelet inventory management extremely challenging.[8]

One of the most important changes in PC during storage is platelet activation, whereby the function and surveillance of platelets will decline. One of useful platelet activation markers is CD62P. Platelet surface CD62P is used as a marker for the detection of circulating degranulated platelets in clinical settings. The expression of CD62P has been shown to be inversely correlated with recovery of platelets and platelet count increment, because of triggering fast CD62-mediated platelet clearance.[9]

Exposure to shear stress, as in centrifugation, during component separation and agitation while storage not only activates platelets but can also cause their lysis and calpain activation. Platelets lysis leads to discharge and accumulation of cytosolic lactate dehydrogenase CD62 and granular contents. Calpain (protease) activation results in degradation of cytoskeletal proteins such as actin, talin, and actin binding protein generating platelet

microvesicles.[10] Microvesicle formation leads to decrease in mean platelet volume (MPV) and also required for e ach aspect of platelet function and is generated via oxidative and glycolytic pathways. Glycolysis is upregulated under low oxygen conditions.[11] Lactate produced via glycolysis is buffered primarily by plasma bicarbonate. Progressive lactate production and bicarbonate exhaustion during storage results in deleterious in pH augmenting platelet damage. Platelet apoptosis occurring simultaneously during storage further contributes to loss of mitochondrial function, cytoskeletal damage, and surface expression of phosphatidly serine. In addition, consumption of nutrients by platelets and contaminating leucocytes and accumulation of metabolic end products such as cellular debris, activated clotting factors, proteolytic enzymes, and cytokines in plasma also adversely affect the functional integrity of platelets.[12]

Biochemical test like decrease in pH, pO_2 LDH accumulation, glucose consumption, and ATP depletion are employed to assess platelet viability. Aging has also been quantified by in vitro tests measuring changes in morphology of platelets. Swirilng phenomenon, decrease in MPV, reporting of the percentage of discoid forms, and Kunicki morphological score have been used for measuring the alterations in the discoid morphology. Extent of shape change in response to an agonist and increase in hypotonic shock response provide more objective measure of morphological changes.[13]

Platelet activation markers such as release of specific granular contents into plasma (β thromboglobulin, platelet factor 4, and RANTES), changes in GP expression on platelet surface (GPIb, GPIIb, and GPIIIa), expression of sequestered granular membrane proteins (P-selectin –CD62P, CD63 and CD40L), and phosphatidl serine (determined by Annexin V binding) on surface occurring during processing and storage have been reported. Tormey and Stack observed diminution of secretory capacity of platelets during processing and storage using cytokines (RANTES) release assay. Wenzel et al., used soluble CD40 ligand (sCD40L) to study the effect of storage on platelet viability by monitoring sCD40L levels in storage medium. They noticed an increase in sCD40L concentration and decrease in platelet release capacity with an increase in duration of storage.[14]

However, the second reason for the limitation on platelet shelf life remains. Over the storage period, platelets begin to show evidence of a loss of quality that raises concerns about the efficacy of a transfusion that would be performed with such a product, Collectively, the loss of platelet quality over storage is known as the platelet storage lesion, or the platelet storage deficit. Our current understanding of the basis of the storage lesion and the significance of ongoing research in this area is the topic of this article.[15]

Other research developments will also ultimately have an impact on the evolution of the storage lesion. One interesting area is research to improve the processing and storage of components. This effort will lead to the consideration of the impact of the processing equipment and the storage container rather than the additive solution. The storage of platelets in a flexible plastic container is less than ideal for product inventory management, and the requirements for narrowly controlled storage temperature of 20 to 24 ^{0}C under conditions of agitation further complicate the storage of platelet concentrates. The issues of container biocompatibility have received little attention to date, but may actually be one of the potential sources for the ongoing stimulus for storage lesion development post processing.[16]

The ability to create a platelet product for transfusion purposes has existed for more than 50 years, and has contributed to fundamental changes in the practice of transfusion medicine. At the same time, this product still presents one of the major challenges to the blood bank, owing not to an inability to prepare a platelet components but because of the limitations of storing platelets under standard blood bank conditions. Depending on the jurisdiction, platelets have a maximum storage time of 3 to 7 days.[17] In countries with a 5-day storage limit, it is estimated that upwards of 30% of the platelet inventory is discarded either by the blood supplier or the hospital blood bank. With the constraints imposed by the time to complete donor testing and shipment of product, platelet products are often received by hospital blood banks with a remaining shelf life limited to hours, further challenging inventory management practices and at times contributing to platelet shortages. This platelet storage constraint represents a significant waste of resources and is a focus of significant research effort.[18]

Apart from these, our study proposed to make suitable interventions to extend the shelf life of the platelets beyond 5 days by exploring the feasibility of utilizing platelet additive solutions (PAS).The results of our study conclusively proves the beneficial affects of PAS as there is no deleterious effect of cytokines release and subsequent platelet activations following the incorporation of additive solution on day 5, 7 and 10.

One of the important objectives of the study is to improve the quality of platelet and to explore the feasibility of extending the shelf life of platelet beyond 5 days. The results obtained highlight the fact that the objective can be met in different ways by adhering to simple but effective steps will incudes the following:

During collection and storage the quality of platelet can be improved by well standardized collection and storage methods which will prevent platelet-platelet and platelet-bag adhesion and the subsequent platelet activation with

the release of cytokines, which will ultimately, led to formation of platelet storage lesion.

Adequate amount of blood (450ml) should be collected under aseptic precaution either in triple or quadruple bag with or without SAGM in blood collection monitor. The volume collected should be adequate, and blood collection monitor should be calibrated to give a proper reading in order to prevent over collection (excess) or under collection (less) correct volume of blood collection will ensure adequate platelet yield having better quality parameters.

Adequate amount of blood collected should have a minimum resting phase of 8 hours which will.

Allow the RBCs to settle down plasma to shift in the supernatant layer and thereby preventing RBC contamination of the platelets and also improving the platelet yield having better quality parameters, which in turn, which enable better patient care.

The platelets so prepared should be stored in a platelet agitator at 22 0C continuous, regular horizontal movements for a period of 5 days which will prevent contact activation of platelet and platelet-bag interaction and prevent the subsequent release of cytokines ultimately leading to the formation of platelet storage lesions.

All the above mentioned measures have been adhered to during the course of the study to improve the quality of the platelet and prevent activation and release of cytokines to fulfill the objectives.

One of the objectives of my study was to take effective measures to extend the shelf-life of platelets beyond 5 days without compromising the basic quality parameters of platelets including their functional integrity. The suggested innovation techniques for extending the shelf life of platelets should be safe, simple, economical and reproducible. Keeping in view the above mentioned requirements, our study conclusively proves that platelets additive solution (PAS) fulfills all the essential criteria to act as an effective interventional step for extending the shelf-life of platelets while maintaining their functional integrity as well.

Our study reveals a new light on the role of cytokines and their various mechanism of action with regard to the quality parameters and transfusion reaction along with it's preventive by the optimum utilization of PAS.

However, more studies with including of clinical trials are required for further confirmation.

CONCLUSION

In conclusion, this study shows that CD62P levels significantly increased in stored platelets without PAS as compared to the platelets with PAS.

Platelet transfusions are frequently accompanied by febrile non-hemolytic transfusion reactions (FNHTR). Cytokines such as CD62P play role in mediating transfusion reactions. These cytokines have pyrogenic effect and can mediate inflammatory reactions. These are released by white blood cells (WBCs) and accumulate into the plasma of platelet concentrate (PC) during storage. This study highlights the time dependent release of CD62P during 5 days of storage of PC at 22 ^{0}C.

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