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Estimation of Proinflammatory Cytokines in Platelet Concentrates Prepared by Buffy Coat Method and Prestorage Leukofiltered/Non-Leukofiltered Platelet Rich Plasma Method – A Comparative Study

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Abstract: *The platelet concentrates is one of the indispensable blood components transfused in practice. Platelet concentrates are prepared by Platelet Rich Plasma (PRP) method, Buffy Coat (BC) method and apheresis method. There was growing concern about White Blood Cells (WBCs) in blood components as WBCs were involved in pathogenesis of transfusion reactions. The reduction of WBCs in PCs prevented Febrile Non Hemolytic Transfusion Reaction (FNHTR), transmission of viruses and platelet alloimmunisation. Leukoreduction is also done to reduce the level of the cytokines released by the WBCs and thereby to reduce transfusion reactions. To compare and estimate the levels of Interleukin-6 (IL-6), Interleukin-8 (IL-8) and Tumor Necrosis Factor-α (TNF-α) in stored platelet concentrates prepared by Buffy Coat Method and Prestorage Leukofiltered/Non-Leukofiltered Platelet Rich Plasma Method at different storage time periods. During the study period of one and half years, proinflammatory cytokines IL-6, IL-8 and TNF-α were estimated on days 0, 3 and 5 of storage in 30 platelet concentrates each, prepared by Non-Leukofiltered Platelet Rich Plasma method (Non-LF PRP – PC), Buffy Coat method (BC – PC) and Leukofiltered Platelet Rich Plasma method (LF PRP – PC).These cytokines were estimated along with other parameters such as volume, platelet count and WBC count.In the present study, the WBC count in LF PRP-PCs and BC-PCs were significantly lower than Non-LF PRP- PCs. The difference in WBC count between BC-PCs and LF PRP-PCs was much less in comparison to the difference between Non-LF PRP-PCS and LF PRP-PCs. The level of proinflammatory cytokines IL-6, IL-8 and TNF-α on days 0, 3 and 5 of storage in Non-LF PRP-PCs showed a significant increase compared to BC-PCs and LF PRP-PCs. However, the difference between BC-PCs and LF PRP-PCs was considerably lower than the difference between Non-LF PRP-PCs and LF PRP -PCs. There was a statistically significant correlation between the WBC count in PCs and cytokine levels on different storage periods.*
Our study on estimation of the levels of Proinflammatory Cytokines suggests the usage of platelet concentrates prepared by Buffy Coat method to prevent FNHTR, the most frequent complication caused by increased levels of Cytokines. However, since alloimmunisation and platelet refractoriness cannot be prevented by Buffy Coat method, prestorage leukofiltration may be considered for selective cases.

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I. Introduction

Blood Transfusion Services constitute a crucial part of healthcare delivery system. Adequate and safe supply of blood and blood components is essential, to enable a wide range of critical care procedures to be carried out in hospitals.[1] The process of blood transfusion has evolved from whole blood transfusion to the present day component transfusion. The platelet concentrates (PCs) is one of the indispensable blood components transfused in practice.[2]A single unit of PC prepared by PRP method should contain at least 5.5 X 10^{10} platelets according to the Directorate General of Health Services (DGHS), American Association of Blood Banks (AABB) and Food and Drug Administration standards.[3,4] There was growing concern about White Blood Cells (WBCs) in blood components as WBCs were involved in pathogenesis of transfusion reactions.Buffy Coat (BC) method was introduced in Europe to reduce the untoward complications of WBCs in Platelet Concentrates. BC preparation process in comparison to the PRP manufacturing process, produces a platelet product with an approximately equivalent platelet content with reduced number of leukocytes.[5,6]Platelet transfusions play a central role in therapeutic regimens for patients with hematologic/oncologic diseases who develop severe thrombocytopenia either in the course of their disease or following cytostatic therapy. In spite of its medical importance, transfusion of PCs can elicit serious side-effects, and hence modifying platelet components further to alleviate potential complications is essential.[7] Like other blood components, platelet transfusions have

achieved a high degree of safety as far as transmission of viral diseases is concerned. However, transfusion of platelet concentrates was accompanied by a high frequency of febrile and anaphylactoid reactions.[8] The cause for these reactions were chiefly attributed to the amount of WBCs present in the platelet concentrates and the cytokines released by these WBCs. Febrile Non-Hemolytic Transfusion Reactions (FNHTRs) are a frequent complication of Red Blood Cell (RBC) and platelet concentrates transfusion. Incidence rate of FNHTR ranges from 0.12 to 0.5 percent for transfusion of non-leukoreduced RBCs[9] and between 1.7 and 31 percent for non-leukoreduced platelets.[10]Proinflammatory cytokines released from White Blood Cells (WBCs) present in the plasma of stored platelet concentrates are the main cause of FNHTRs associated with transfusion of PCs. IL-6, IL-8 and TNF-α generated during storage of platelet concentrates are the important cytokines responsible for FNHTRs.[11,12] The reduction of WBCs in PCs prevented FNHTR, transmission of viruses and platelet alloimmunisation.[13] Leukoreduction is also done to reduce the level of the cytokines released by the WBCs and thereby to reduce transfusion reactions.The need for leukoreduction led to the development of leukocyte reduction filters and cytapheresis techniques. The evolution of poststorage filtration to pre storage filtration was not without controversy but currently prestorage filtration method is preferred than poststorage filtration for leukoreduction.[14] The present study was undertaken to investigate whether prestorage leukoreduction methods reduces the level of proinflammatory cytokines in platelet concentrates at different storage time periods.

To compare and estimate the levels of proinflammatory cytokines IL-6, IL-8 and TNF-α in stored platelet concentrates prepared by Buffy Coat Method and Prestorage Leukofiltered/Non-Leukofiltered Platelet Rich Plasma Method at different storage time periods.

II. Material and Methods

This prospective comparative study of evaluation of three methods of platelet concentrates preparation was conducted at the Department of Transfusion Medicine, The Tamilnadu Dr.M.G.R. Medical University, Guindy and Cancer Institute Adyar (W.I.A), Chennai, after obtaining ethical clearance from the Institutional Ethical Committee of the Tamilnadu Dr. M.G.R Medical University, Chennai.

StudySamples: Samples of platelet concentrate prepared from the blood bags of voluntary blood donors

SamplingTechnique: Convenience Sampling Method.

Study Period: June 2012 – December 2013.

Samplesize:

$$n = \frac{z^2 \times (S.D)^2}{d^2}$$

z = level of significance = 5% (1.96)

S.D = Standard Deviation (27.96)

D = clinically meaningful difference (10)[16]

$$n = \frac{1.96^2 \times (27.96)^2}{10^2}$$

= 30 in each group.

Methods:

I. Preparation of Platelet concentrates by:

1. Non-Leukofiltered PRP Method (30 units)
2. Buffy Coat Method (30 units)
3. Leukofiltered PRP Method (30 units)

Platelet Concentrates were prepared from whole blood obtained from healthy voluntary donors as per AABB[4], DGHS guidelines[15].

II. Collection and Storage of Study Samples:

a) 5 ml of sample was obtained from each dedicated bag after thorough stripping of the segment with the platelet concentrate on days 0, 3 and 5. The scheduled times of sample collection for this study were as follows:

i. Day 0: Immediately after platelet concentrate preparation.

ii. Days 3 & 5: At the same time of sample collection on Day 0.

b) Then as per the manufacturer's instructions the samples were immediately centrifuged at 1500 g for 30 minutes.

c) The platelet poor supernatant plasma was stored at -80^{o}C in properly labeled aliquots in – 85^{o}CUltra Low Freezer (New Brunswick Scientific Freezer, Model No. U57085) till further assay.

d) Another 1 ml of sample from each bag was collected to measure Platelet and WBC count using Medonic CAcalibrated automatic cell count analyzer.

III. Estimation of Cytokine Levels:

Measurement of proinflammatory cytokines IL-6, IL-8 and TNF-α in platelet poor supernatant plasma was done by Quantitative ELISA technique. These cytokines were evaluated on platelet concentrates prepared by three different methods (30 bags each) on Days 0, 3 & 5 in pg/ml.

1. Human IL-6 ELISA KIT

The Diaclone IL-6 ELISA kit is a solid phase sandwich ELISA for the in-vitro qualitative and quantitative determination of IL-6 in supernatants, buffered solutions or serum and plasma samples. This assay was performed as per the manufacturer's instructions.

A. Principle

A captured Antibody highly specific for IL-6 has been coated to the wells of the microtitre strip plate provided during manufacture. Binding of IL6 samples and known standards to the capture antibodies and subsequent binding of the biotinylated anti-IL-6 secondary antibody to the analyte is completed during the same incubation period. Any excess unbound analyte and secondary antibody is removed. The Horse Radish Peroxidase (HRP) conjugate solution is then added to every well including the zero wells and following incubation excess conjugate is removed by careful washing. A chromogen substrate is added to the wells resulting in the progressive development of a blue coloured complex with the conjugate. The colour development is then stopped by the addition of acid turning the resultant final product yellow. The intensity of the coloured complex produced is directly proportional to the concentration of IL-6 present in the samples and standards. The absorbance of the coloured complex is then measured and the generated OD values for each standard are plotted against expected concentration forming a standard curve. This standard curve can then be used to accurately determine the concentration of IL-6 in any sample tested.

B. Materials required

- Microtitre plate reader fitted with appropriate filters (450nm required with optional 620nm reference filter)
- Microplate washer or wash bottle
- 10, 50, 100, 200 and 1000µl adjustable single channel micropipettes with disposable tips
- 50-300µl multi-channel micropipette with disposable tips
- Multi-channel micropipette reagent reservoirs
- Distilled water
- Vortex mixer
- Miscellaneous laboratory plastic and/or glass

C. Storage instructions

Reagent kits were stored between 2 and 8°C. Immediately after use, the remaining reagents were returned to cold storage (2-8°C).

D. Assay Procedure:

1. All reagents were brought to the room temperature (18^0-25^0C) before use.
2. All reagents were mixed by gentle inversion or swirling prior to use without inducing foaming.
3. The desired numbers of coated wells were secured in the holder.
4. Then 100µl of Sample, Standard, Control and zero (appropriate Standard diluent) was dispensed in duplicate to appropriate number of wells.
5. 50µl of diluted Biotinylated anti-IL-6 was added to all wells.
6. Then the plate was covered with a plastic plate cover and incubated at room temperature (18 to 25°C) for 1 hour.
7. Then the cover was removed and the plate was washed as follows:

a. The liquid was aspirated from each well
b. 0.3 ml of 1x washing solution was dispensed into each well
c. The contents of each well was aspirated
d. Steps b and c were repeated another two times

8. Then 100µl of Streptavidin-HRP solution was added to all wells.
9. Then the plate was covered with a plastic plate cover and incubated at room temperature (18 to 25°C) for 30 minutes.
10. Wash step 7 was repeated.
11. Then 100µl of ready-to-use Tetra Methyl Benzidine (TMB) Substrate Solution was added to all wells.
12. The plate was then incubated at room temperature in the dark for 12-15 minutes.
13. The reaction was stopped by adding 100 µl of stop solution (H_2SO_4) to each well.

14. The wells were then mixed gently for 30 seconds. It was also important to make sure that all the blue colour changed completely to yellow colour.
15. The absorbance value of each well was read immediately on a spectrophotometer using 450 nm as the primary wavelength.

E. Calculation of results:

a. The mean absorbance value for each set of reference standards, controls and samples were calculated. The standard curve was constructed by plotting the mean absorbance obtained from the reference standard against its concentration in pg/ml on graph paper, with absorbance on the vertical (y) axis and the concentration on the horizontal(x) axis.
b. The corresponding concentration of cytokine IL-6 was determined from the standard curve in pg/ml using the mean absorbance value for each sample.

F. Sensitivity: Minimum detectable dose of IL-6 was less than 2 pg/ml by using Diaclone IL-6 ELISA kit.

2. Human IL-8 ELISA KIT

The same procedure for estimation of IL-6 was repeated with specific reagents for IL-8 in crucial steps.
Sensitivity: Minimum detectable dose of IL-8 was 17 pg/ml by using Diaclone IL-8 ELISA kit.

3. Human TNF - α ELISA KIT

The same procedure for estimation of IL-6 & IL-8 was followed with specific reagents for TNF - α in crucial steps, except the incubation time of 3 hours in step 4.
Sensitivity: Minimum detectable dose of IL-8 was less than 8 pg/ml by using Diaclone TNF - α ELISA kit

Automated cell count analyzer

1 ml of sample collected separately was fed into the Medonic CA620 calibrated automated cell count analyzer to measure Platelet and WBC count.Measuring principle of automated analyzer is based on the detection accomplished using impedance principle and occurs in the orifice of the transducer. Blood is diluted to 1: 400 for WBC and haemoglobin and 1:40000 for RBC and platelet through a precise shear valve system. Two separate measuring chamber and transducer are used one for RBC and platelet and one for WBC and haemoglobin.Since, the number of WBCs in leukofiltered PCs was below minimum detectable level by the automated cell count analyser, in these samples WBC count was done by improved Neubauer counting chamber.

Statistical Analysis :

1. Data entry was done in EXCEL and analysis was done using SPSS software version 11.
2. Quantitative data was given in mean and standard deviation.
3. ANOVA was used to compare three methods.
4. Multiple comparison test (TUKEY HSD Test) was used for comparison between the three groups.
5. P value <0.05 is considered to be significant.

IV. Results

During the study period, proinflammatory cytokines IL-6, IL-8 and TNF-α were measured on days 0, 3 and 5 of storage in the following platelet concentrates:

1. Non-Leukofiltered Platelet Rich Plasma Platelet Concentrate (Non-LF PRP – PC) - 30 units.
2. Buffy Coat Platelet Concentrate (BC – PC) - 30 units.
3. Leukofiltered Platelet Rich Plasma Platelet Concentrate (LF PRP – PC) - 30 units.

These cytokines were estimated along with other parameters such as volume, platelet count and WBC count.
The mean volume of PCs prepared by Non-LF PRP method, BC method and LF PRP method were 63 ml, 61 ml and 61 ml respectively.

Table 1: Platelet Count in PCs prepared by three different methods on Days 0, 3 & 5 of Storage

PCs prepared by three different methods	Platelet Count (x 10^9/L)						P value (between days)		
	Day 0		Day 3		Day 5				
	Mean (SD)	95% CI (Confidence Interval)	Mean (SD)	95% CI (Confidence Interval)	Mean (SD)	95% CI (Confidence Interval)	Day 0 Vs Day 3	Day 3 Vs Day 5	Day 0 Vs Day 5
Non-LF PRP-PC	934.07 (79.2)	904.49 -963.64	908.97 (77.63)	879.98 - 937.95	878.77 (75.24)	850.67 - 906.86	0.67	0.45	0.56

BC- PC	951.33 (81.07)	921.06 -981.6	918.33 (77.1)	889.54 - 947.12	875.77 (79.66)	846.02 - 905.51	0.59	0.48	0.53
LF PRP-PC	931.27 (84.99)	899.53 - 963.0	897.67 (80.78)	867.50 - 927.83	861.97 (79.32)	832.35 - 891.58	0.62	0.53	0.56

Comparing the platelet counts on each of the storage time periods (viz Days 0, 3 and 5), there is no significant change between the three methods of preparation.

Table 2: Comparison of platelet counts in platelet concentrates prepared by three different methods on different storage time periods

PCs prepared by three different methods	Platelet count(x 10^9/L) Mean (SD)		
	Day 0	Day 3	Day 5
Non-LF PRP – PC	934.07 (79.2)	908.97 (77.63)	878.77 (75.24)
BC- PC	951.33 (81.07)	918.33 (77.1)	875.77 (79.66)
LF PRP-PC	931.27 (84.99)	897.67 (80.78)	861.97 (79.32)
P values			
Non-LF PRP–PC Vs BC-PC	0.693	0.889	0.988
BC-PC Vs LF PRP-PC	0.610	0.567	0.773
Non-LF PRP–PC Vs LF PRP-PC	0.990	0.843	0.684

➢ Comparing the platelet counts on each of the storage time periods (viz Days 0, 3 and 5), there is no significant change between the three methods of preparation.

Figure 1: Comparison of platelet counts in platelet concentrates prepared by three different methods on different storage time periods

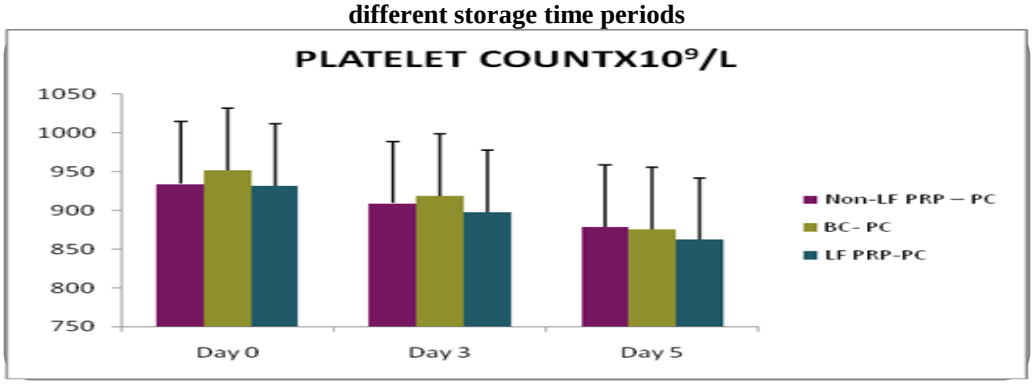


Table 3: WBC count in PCs prepared by three different methods onDays 0, 3 & 5 of Storage

PCs prepared by three different methods	WBC count(x 10^9/L) Day 0 Mean (SD)	Day 0 95% CI (Confidence Interval)	Day 3 Mean (SD)	Day 3 95% CI (Confidence Interval)	Day 5 Mean (SD)	Day 5 95% CI (Confidence Interval)	P value (between days) Day 0 Vs Day 3	Day 3 Vs Day 5	Day 0 Vs Day 5
Non-LF PRP-PC	5.41 (0.76)	5.12 - 5.69	4.91 (0.72)	4.64 - 5.18	4.36 (0.72)	4.09 - 4.63	0.59	0.49	0.61
BC- PC	0.41 (0.21)	0.33- 0.49	0.30 (0.15)	0.25 - 0.36	0.21 (0.10)	0.17 - 0.25	0.52	0.53	0.69
LF PRP-PC	0.08 (0.04)	0.06 - 0.09	0.06 (0.02)	0.05 - 0.07	0.05 (0.01)	0.06 - 0.09	0.55	0.49	0.64

➢ In platelet concentrates prepared by each of the three different methods, there is no significant change in the WBC count between days 0, 3 and 5.

Table 4: Comparison of WBC Count in Platelet Concentrates prepared by three different methods on different storage time periods

PCs prepared by three different methods	WBC count(x 10^9/L) Mean (SD)		
	Day 0	Day 3	Day 5
Non-LF PRP – PC	5.41(0.76)	4.91 (0.72)	4.36 (0.72)
BC- PC	0.41 (0.21)	0.30 (0.15)	0.21 (0.10)

LF PRP-PC	0.08 (0.04)	0.06 (0.02)	0.05 (0.01)
P values			
Non-LF PRP–PC Vs BC-PC	0.000	0.000	0.000
BC-PC Vs LF PRP-PC	0.016	0.043	0.031
Non-LF PRP–PC Vs LF PRP-PC	0.000	0.000	0.000

- There is a significant 2 log reduction in WBC Count between Non-LF PRP-PCs and LF PRP-PCs
- There is a significant 1 log reduction in WBC Count between Non-LF PRP-PCs and BC-PCs
- There is a significant 1 log reduction in WBC Count between BC-PCs and LF PRP-PCs

Figure 2: Comparison of WBC Count in Platelet Concentrates prepared by three different methods on different storage time periods

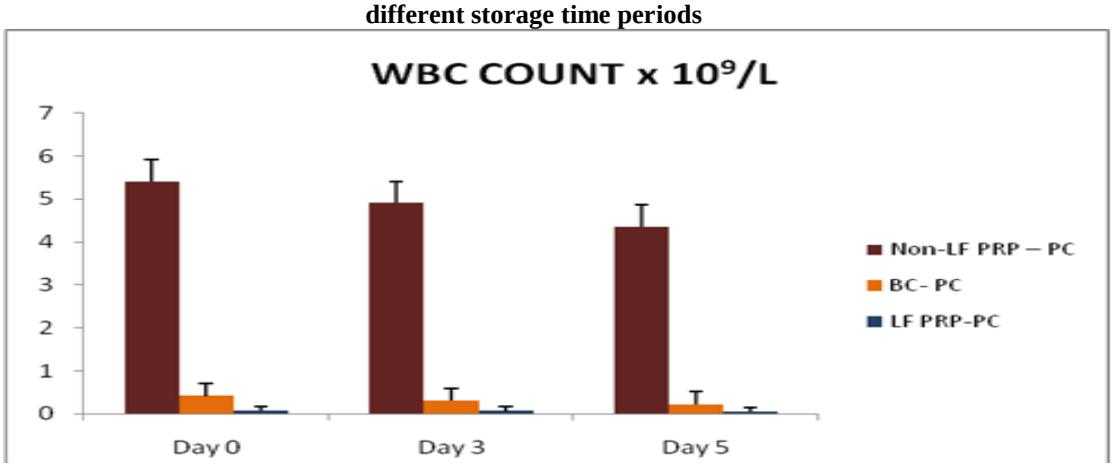


Table 5: IL-6 level of Platelet Concentrates Prepared by three different methods on Days 0, 3 and 5 of storage

PCs prepared by three different methods	IL-6 level (pg/ml)						P value (between days)		
	Day 0		Day 3		Day 5				
	Mean (SD)	95% CI (Confidence Interval)	Mean (SD)	95% CI (Confidence Interval)	Mean (SD)	95% CI (Confidence Interval)	Day 0 Vs Day 3	Day 3 Vs Day 5	Day 0 Vs Day 5
Non-LF PRP – PC	5.78 (1.38)	5.26 - 6.30	91.58 (16.32)	85.49 - 97.67	176.05 (30.26)	164.75- 187.34	0.000	0.000	0.000
BC- PC	5.41 (0.8)	5.11 - 5.71	19.27 (5.26)	17.30 - 21.23	76.3 (12.24)	71.72 - 80.87	0.000	0.000	0.000
LF PRP-PC	3.64 (0.54)	3.44 - 3.84	3.79 (0.55)	3.59 - 4.00	3.90 (0.53)	3.44 - 3.84	0.643	0.593	0.621

- There is a significant increase in IL-6 values between days 0, 3 and 5 in PCs prepared by Non-LF PRP method and BC method.
- However, there is no significant increase in IL-6 values between days 0, 3 and 5 in PCs prepared by LF-PRP method.

Table 6: Comparison of IL-6 level in Platelet Concentrates prepared by three different methods on different Storage time periods

PCs prepared by three different methods	IL-6 level (pg/ml) Mean (SD)		
	Day 0	Day 3	Day 5
Non-LF PRP – PC	5.78 (1.38)	91.58 (16.32)	176.05 (30.26)
BC- PC	5.41 (0.8)	19.27 (5.26)	76.30 (12.24)
LF PRP-PC	3.64 (0.54)	3.79 (0.55)	3.90 (0.53)
P values			
Non-LF PRP-PC Vs BC-PC	0.543	0.000	0.000
BC-PC Vs LF PRP-PC	0.373	0.000	0.000
Non-LF PRP-PC Vs LF PRP-PC	0.316	0.000	0.000

- There is no significant difference in IL-6 values in PCs prepared by three different methods on Day 0.
- There is a marked difference in IL-6 values between Non-LF PRP-PCs and LF PRP-PCs, BC-PCs and LF PRP-PCs & Non-LF PRP-PCs and BC-PCs on Days 3 & 5 of storage

Figure 3: Comparison of IL-6 level in Platelet Concentrates prepared by three different methods on different storage time periods

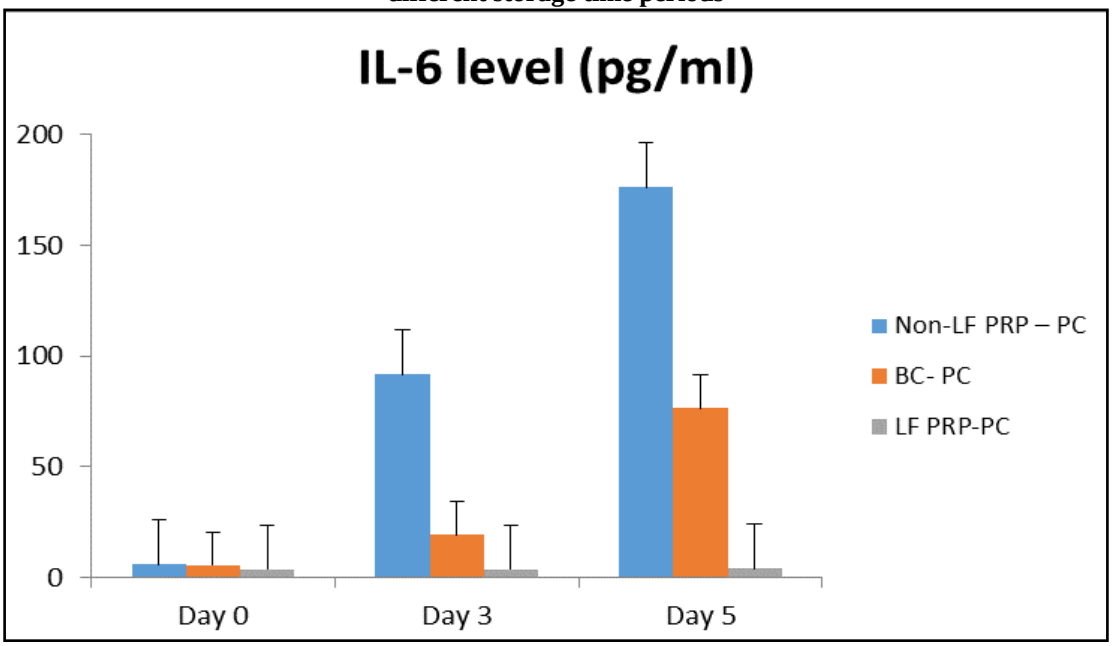


Table 7: IL-8 Level in the Platelet Concentrates Prepared by three different methods on Days 0, 3 and 5 of storage

PCs prepared by three different methods	IL-8 level (pg/ml)						P value (between days)		
	Day 0		Day 3		Day 5				
	Mean (SD)	95% CI (Confidence Interval)	Mean (SD)	95% CI (Confidence Interval)	Mean (SD)	95% CI (Confidence Interval)	Day 0 Vs Day 3	Day 3 Vs Day 5	Day 0 Vs Day 5
Non-LF PRP-PC	20.53 (7.85)	17.60 - 23.46	528.15 (69.78)	502.09 - 554.2	812.02 (79.95)	782.16 - 841.87	0.000	0.000	0.000
BC- PC	18.81 (7.05)	16.17 - 21.44	250.7 (60.72)	228.02 - 273.37	345.85 (54.52)	325.50 - 366.21	0.000	0.000	0.000
LF PRP-PC	21.87 (6.85)	19.31 - 24.42	20.1 (6.56)	17.65 - 22.55	17.99 (6.77)	15.46 - 20.52	0.673	0.593	0.521

- There is a significant increase in IL-8 values between days 0, 3 and 5 in PCs prepared by Non-LF PRP method and BC method
- However, there is no significant increase in IL-8 values between days 0, 3 and 5 in PCs prepared by LF-PRP method.

Table 8: Comparison of IL-8 Level in Platelet Concentrates prepared by three different methods on different Storage time periods

PCs prepared by three different methods	IL-8 level (pg/ml) Mean (SD)		
	Day 0	Day 3	Day 5
Non-LF PRP-PC	20.53 (7.85)	528.15 (69.78)	812.02 (79.95)
BC-PC	18.81 (7.05)	250.7 (60.72)	345.85 (54.52)
LF PRP-PC	21.87 (6.85)	20.1 (6.56)	17.99 (6.77)
P values			
Non-LF PRP-PC Vs BC-PC	0.627	0.000	0.000
BC-PC Vs LF PRP-PC	0.901	0.000	0.000
Non-LF PRP-PC Vs LF PRP-PC	0.367	0.000	0.000

- There is no significant difference in IL-8 values in PCs prepared by three different methods on Day 0.
- There is a marked difference in IL-8 values between Non-LF PRP-PCs and LF PRP-PCs, BC-PCs and LF PRP-PCs & Non-LF PRP-PCs and BC-PCs on Days 3 & 5 of storage.

Figure 4: Comparison of IL-8 Level in Platelet Concentrates prepared by three different methods on different Storage time periods

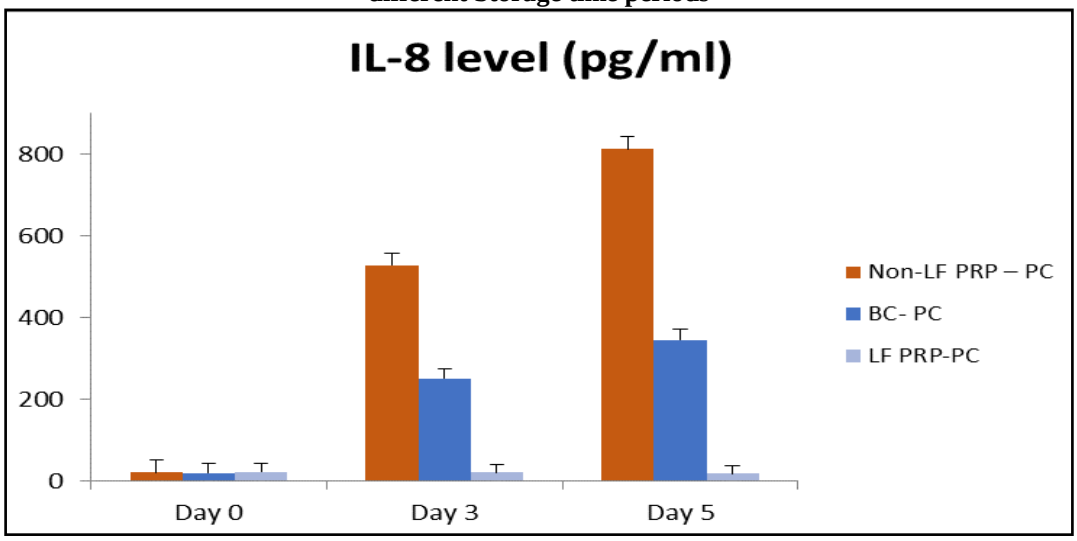


Table 9: TNF-α Level in Platelet Concentrates Prepared by three different methods on Days 0, 3 and 5 of Storage

PCs prepared by three different methods	TNF-α level (pg/ml)						P value (between days)		
	Day 0		Day 3		Day 5				
	Mean (SD)	95% CI (Confidence Interval)	Mean (SD)	95% CI (Confidence Interval)	Mean (SD)	95% CI (Confidence Interval)	Day 0 Vs Day 3	Day 3 Vs Day 5	Day 0 Vs Day 5
Non-LF PRP – PC	8.17 (1.60)	7.57 - 8.77	34.65 (7.20)	31.97 - 37.34	168.38 (38.32)	154.07 - 182.68	0.000	0.000	0.000
BC- PC	8.27 (1.36)	7.76 - 8.8	17.88 (4.19)	16.31 - 19.44	60.17 (10.51)	56.24 - 64.09	0.012	0.000	0.000
LF PRP-PC	8.59 (1.08)	7.70 - 8.42	8.45 (1.08)	8.05 - 8.86	8.06 (0.96)	8.19 - 9.0	0.643	0.587	0.465

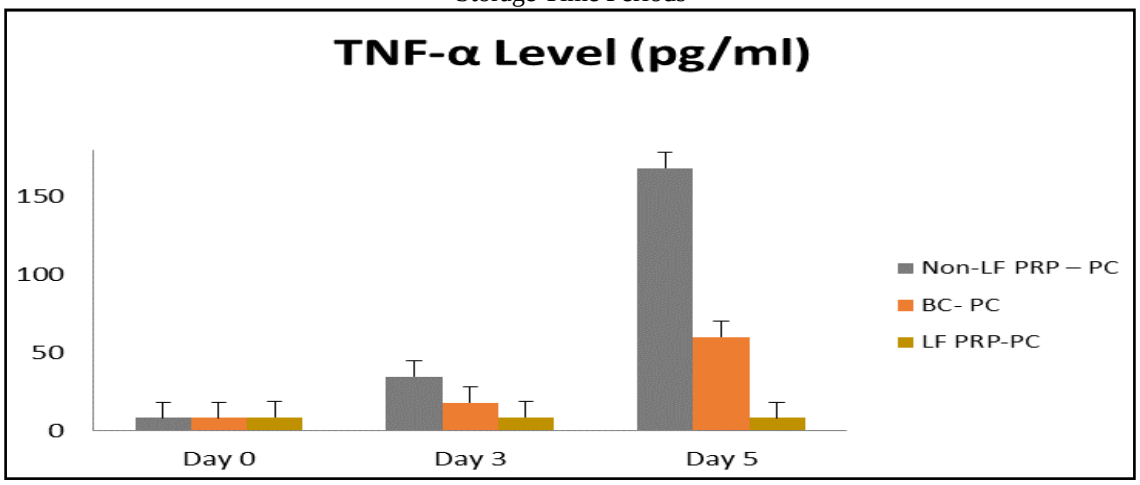
- There is a significant increase in TNF-α values between days 0, 3 and 5 in PCs prepared by Non-LF PRP method and BC method.
- However, there is no significant increase in TNF-α values between days 0, 3 and 5 in PCs prepared by LF-PRP method.

Table 10: Comparison of TNF-α Level in Platelet Concentrates prepared by three different methods on different Storage Time Periods

PCs prepared by three different methods	TNF-α level (pg/ml) Mean (SD)		
	Day 0	Day 3	Day 5
Non-LF PRP – PC	8.17 (1.60)	34.65 (7.20)	168.38 (38.32)
BC- PC	8.27 (1.36)	17.88 (4.19)	60.17 (10.51)
LF PRP-PC	8.59 (1.08)	8.45 (1.08)	8.06 (0.96)
P values			
Non-LF PRP-PC Vs BC-PC	0.961	0.000	0.000
BC-PC Vs LF PRP-PC	0.824	0.000	0.000
Non-LF PRP-PC Vs LF PRP-PC	0.944	0.000	0.000

- There is no significant difference in TNF-α values in PCs prepared by three different methods on Day 0.
- There is a marked difference in TNF-α values between Non-LF PRP-PCs and LF PRP-PCs, BC-PCs and LF PRP-PCs & Non-LF PRP-PCs and BC-PCs on Days 3 & 5 of storage.

Figure 5: Comparison of TNF-α Level in Platelet Concentrates prepared by three different methods on different Storage Time Periods



V. Discussion

In the present study, 30 platelet concentrates prepared by each of the three different methods: Non-LF PRP, BC and LF PRP methods were analysed for proinflammatory cytokines: IL 6, IL 8 and TNF-α on Days 0, 3 and 5 of storage.

In addition, to find out the amount of leukoreduction in BC-PCs and LF PRP-PCs, WBC count on Days 0, 3 and 5 was done and compared with the WBC count obtained from Non LF PRP-PCs. To assess the adequacy of platelets in platelet concentrates prepared by these three different methods, platelet count was also done on days 0, 3 and 5 of storage.

The amount of leukoreduction plays a vital role on the amount of cytokines released in to the platelet concentrates stored up to 5 days at 22±2 ^{0}C. In our study the number of leukocytes on Day 0 in platelet concentrates prepared by Non-LF PRP-PCs, BC-PCs and LF PRP-PCs were 5.4 ± 1.4 x 10^9/L, 0.4 ± 0.4 x 10^9/L and 0.08 ± 0.08 x 10^9/L respectively. This corresponds to mean WBC count of 3.2 x 10^8/ unit for Non-LF PRP-PCs, 2.4 x 10^7/unit for BC-PCs and 4.8 x10^6/unit for LF PRP-PCs. The WBC count of Non-LF PRP-PCs and LF PRP-PCs were within the normal standards prescribed by AABB[4]. With reference to BC-PCs, the WBC count observed in our study is well within normal range prescribed by the council of Europe.

In our study, there is a significant 2 log reduction in WBC Count between Non-LF PRP-PCs and LF PRP-PCs, there is a significant 1 log reduction in WBC Count between Non-LF PRP-PCs and BC-PCs and there is a significant 1 log reduction in WBC Count between BC-PCs and LF PRP-PCs.

On Days 3 and 5, the number of leukocytes in PCs prepared by Non-LF PRP-PCs, BC-PCs and LF PRP-PCs were similar to studies by Muyelle et al.,[17] Aye et al[18] and Day 5 values by Chaudhary et al[16].

There are a few frequently occurring complications which could be prevented by prestorage leukofiltration. However, these complications cannot be prevented by pretransfusion bed side filters because of the accumulation of cytokines in various blood components following storage. Hence, our study tried to find out the correlation between the amount of leukoreduction and cytokines in platelet concentrates prepared by buffy coat method and leukofiltered platelet rich plasma methods. The proinflammatory cytokines studied were IL-6, IL-8 and TNF-α. These cytokines were studied on Days 0, 3 and 5.

On day 0, IL-6, IL-8 and TNF-α levels in platelet concentrates prepared by Non-LF PRP method were 5.78 pg/ml, 20.53 pg/ml and 8.17 pg/ml respectively. These values had increased significantly on days 3 and 5 of storage.

On day 0, IL-6, IL-8 and TNF-α levels in platelet concentrates prepared by BC method were 5.41 pg/ml, 18.81 pg/ml and 8.27 pg/ml respectively. These values had shown considerable increase on days 3 and 5 of storage. However, these values were significantly lower than platelet concentrates prepared by Non-LF PRP method.

On day 0, IL-6, IL-8 and TNF – α levels in platelet concentrates prepared by LF-PRP method were 3.64 pg/ml, 21.87 pg/ml and 8.59 pg/ml respectively. These values remained almost same on days 3 and 5 of storage. This is in stark contrast to the values obtained in platelet concentrates prepared by Non-LF PRP method and BC method. However, the margin of difference in values between BC-PCs and LF PRP-PCs are much lower than the difference between Non-LF PRP-PCs and LF PRP-PCs.

There are several studies claiming the importance of prestorage leukofiltration, mainly prevention of FNHTR and alloimmunization. However, at least 3 log reduction is essential for the prevention of alloimmunization which could be achieved only by prestorage third generation leukofilters.[19] The cost factor is

the main hindrance to implement universal leukofiltration in developing countries, which could be circumvented by utilizing platelet concentrates prepared by BC method. However, BC method could yield PCs with only 1 log reduction compared to the PCs prepared by Non-LF PRP method, which could prevent only FNHTR.[20]

In our study, there is a statistically significant correlation between the WBC count and cytokine levels in PCs at different storage time periods. This is in concordance with other studies by Muyelle et al.,[17] and Flegel et al.,[21] who demonstrated that no active cytokine synthesis was noted when the WBC count was below 3 x 10^9/ L and ≤1 x 10^9/L respectively and this could be related to the preparation method of PCs.

Studies by Aye et al.,[18] Mojgan Shaiegan et al.,[22] and Chaudhry et al.,[16] had reported almost similar findings obtained in the present study. However, a study by Muyelle et al.,[17] reported that the amount of leukoreduction and the levels of cytokines IL-6 & TNF-α in BC-PCs and LF PRP-PCs were almost similar.

VI. Conclusion

In our study on estimation of the levels of Proinflammatory Cytokines IL-6, IL-8 and TNF-α in Platelet Concentrates prepared by Prestorage Leukofiltered Platelet Rich Plasma Method and Buffy Coat Method in comparison to Platelet Concentrates prepared by Non-Leukofiltered PRP method on different storage periods revealed leukoreduction and corresponding reduction in cytokine levels on par with similar studies done earlier.

Unlike developed countries wherein Universal Leukoreduction is a norm, in a country like India, considering the cost factor of the leukofilter, depending on the need for leukoreduction, either Platelet Concentrates prepared by Buffy Coat method or Prestorage Leukoreduction can be used selectively. Since for the prevention of FNHTR leukoreduction level by BC method is satisfactory, PCs prepared by this method comply with the expected clinical outcome. For the prevention of alloimmunization, platelet refractoriness and prevention of CMV transmission, the required leukoreduction is achieved only by platelet concentrates prepared by prestorage leukofiltration.

Hence our study has reiterated the facts revealed by other studies with respect to the cytokine levels in Platelet Concentrates prepared by BC and Prestorage LF PRP methods in comparison to PCs prepared by Non-LF PRP method.

If facilities are available, platelet concentrates with expected leukoreduction can be prepared by apheresis method of collection. This method has added advantages of exposing recipient to less number of donors in comparison to PCs prepared by PRP and BC methods.

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