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ORIGINAL ARTICLE

Time Dependent Release of Interleukin-8 and Tumor Necrosis Factor-α in Platelet Concentrate

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Abstract Contaminating white blood cells in stored platelet concentrate (PC) are the source of many pro-inflammatory cytokines. These are implicated in transfusion reactions. To study the release of interleukin (IL)-8 and tumor necrosis factor alpha (TNF-α) at different time interval in PC prepared by-platelet rich plasma (PRP) and buffy coat (BC) using different principles. Fifteen PCs were prepared by both the methods. The supernatants of PCs prepared by PRP and BC methods were collected aseptically after 1, 18, 65 and 112 h of preparation. pH, platelet and WBC counts were done. The supernatants were frozen in aliquots at −56 °C for measurement of IL-8 and TNF-α concentration using ELISA. The Mean ± SD value of WBC in PRP-PC was $7.4 \pm 3.75 \times 10^7$ and in BC-PC $3.9 \pm 2.2 \times 10^7$. The mean platelet counts were $6.05 \pm 1.94 \times 10^{10}$ and $6.54 \pm 2.18 \times 10^{10}$ respectively. The highest level of IL-8 in one hour was up to 30 pg/ml in both the type of PC. It increased up to 986 pg/ml in PRP-PC and 481 pg/ml in BC-PC at 112 h. IL-8 increased significantly during storage period of 5 days in both types of PCs (P0.000 and P0.01). TNF-α level remained low up to 18 h. The highest level was 72 pg/ml in PRP-PC and 57 pg/ml in BC-PC at 65 h. IL-8 levels significantly increased after one hour of storage and TNF-α. levels were low up to 18 h and then showed increase. The BC-PC had significantly low levels of IL-8 compared to PRP-PC (P0.0001).

Keywords Platelet concentrate · Platelet rich plasma · Buffy coat · Interleukin · Tumor necrosis factor α

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Introduction

Platelet transfusions are frequently accompanied by febrile non-hemolytic transfusion reactions (FNHTR).Cytokines such as Interleukin (IL)-1β, IL-6, IL-8 and Tumor necrosis factor (TNF)-α play a role in mediating transfusion reactions [1]. These cytokines have pyrogenic effect and can mediate inflammatory reactions [2, 3]. These are released by white blood cells (WBCs) and accumulate into the plasma of platelet concentrate (PC) during storage [4]. This study investigated the time dependent release of IL-8 and TNF-α in PC during 5 days of storage at 22 °C.

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 and quadruple 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 form which includes the consent of the donor. PCs were prepared within 6 h of blood collection.

Two types of PC were prepared namely platelet rich plasma (PRP)–PC and buffy coat (BC)–PC using two different principles for separation. Fifteen PRP-PC were prepared from triple bags by centrifuging the bags at 20–22 °C at light spin 2,000×*g* for 3 min. The bag with PRP and another satellite bag were centrifuged at 20–22 °C at heavy spin 5,000×*g* for 5 min. Supernatant plasma was transferred into another empty satellite bag. Approximately 50 ml of plasma was left with the platelets. PC was left undisturbed at 20–22 °C for 1 h, and then the platelets in

Table 1 Mean ± SD values of various parameters in PRP-PC and BC-PC

Sample	Age years	Volume ml	pH	WBC/Unit	Platelet/Unit
PRP-PC	31.2 ± 7.9	60.4 ± 5.1	7.9 ± 0.31	7.4 ± 3.75x10^{7}*	6.05 ± 1.94x10^{10}
BC-PC	31.3 ± 7.1	72.0 ± 6.4	7.9 ± 0.29	3.9 ± 2.2x10^{7}*	6.54 ± 1.81x10^{10}

't' test between WBC count of PRP-PC and BC-PC is highly significant *P* 0.0043

plasma were resuspended by gently mixing for 10 min. Platelets were stored at 20–22 °C under constant agitation in platelet incubator with agitator [5].

Buffy coat method was employed for preparation of 15 platelet concentrates using 'top and bottom' bags and an automatic component extractor Optipress II of M/S Fenwal. Blood was centrifuged at heavy spin for 8 min. After preparation of FFP and RCC on Optipress the primary bag with buffy coat and plasma in satellite bag was left hanging for about 2 h at room temperature (22°) then centrifuged at light spin at 22 °C. The supernatant plasma with platelets was slowly transferred into the empty bag using extractor [6].

Immediately after preparing PC, about 5 ml sample was removed for quality control and cytokine measurement. Total volume was determined and pH was measured on 5th day of storage using M-tronics pH meter which was standardized using standard buffers of pH 4.0, 7.0 and 9.0. Platelets and WBC of all blood components were measured on fully automated hematology analyzer Nihon Kohden. The samples of 15 PRP-PCs and BC-PCs were collected aseptically after 1, 18, 65 and 112 h of preparation and centrifuged at 3,500 rpm at 22 °C for 15 min. The aliquots of supernatant were frozen at −56 °C for measurement of IL-8 and TNF-α concentration. Cytokine analysis was done using ELISA reagents BioLegend (San Diego, CA) for Human IL-8 and TNF-α.

Results

All donors were male and in the age group 31.2 ± 7.9 years. pH was above 6 in all platelet concentrates. Mean values of various hematological parameters of PC are presented in Table 1. Statistical analysis using "*t*" test revealed significantly higher WBC count (P0.0043) in PRP-PC compared to BC-PC. Platelet counts compared in PRP-PC and BC-PC were comparable.

IL-8

Table 2 shows gradual increase in levels of IL-8 in PRP and BC-PC with respect to storage time. The lowest detection limit of the assay was 8 pg/ml, therefore the first range selected was <8 pg/ml. In both the types of PC the level was below the detection limit in more than half of the samples collected after 1 h of preparation. The highest level of IL-8 in this category was up to 30 pg/ml. In PRP-PC the level increased up to 193 pg/ml in 18 h and 70 pg/ml in BC-PC. At 65 h the level was almost six times higher than in 1 h and was >100 pg/ml in 12 units in PRP-PC and five units in BC-PC. The level increased continuously up to 986 pg/ml in PRP-PC and 481 pg/ml in BC-PC at 112 h. No sample had >500 pg/ml concentration in BC-PC. An increase was seen in the levels of IL-8 during storage period of 5 days in both types of PCs. Comparison between mean values of IL-8 in PRP-PC and BC-PC by '*t*' test on different storage shows significant high levels in PRP-PC at 65 and 112 h compared to BC-PC (P0.0001).

TNF-α

The observed range of TNF-α at 1 h of storage was 0–3 pg/ml which was considered as normal range and for further analysis the range >3 pg/ml was selected. As shown in Table 3 TNF-α was in normal range in 93 % units at 1 h in PRP-PC and 40 % units at 112 h. The level increased above 11 pg/ml at 65 h in both PCs. The highest level was 72 pg/ml in PRP-PC and 57 pg/ml in BC-PC at 65 h of storage. The level was low at 112 h with maximum 26 pg/ml in PRP-PC and 45 pg/ml in BC-PC. Comparison between mean values of TNF-α in PRP-PC and BC-PC by '*t*' test at different time of storage shows no significant difference in two methods but there is significant increase from 1 to 112 h in PRP-PC (P0.046) and BC-PC (P 0.0256).

Discussion

In preliminary study interleukin 1β and IL-6 were also considered as they are pyrogenic and involved in many febrile transfusion reactions. IL-1β was not detected in BC-PC and small amount was found in PRP-PC. IL-6 was considered in some single donor platelets but was below the detection limit 2 pg/ml.The maximum IL-6 level in SDP was below 20 on 5th day of storage. Thus IL-6 was not analysed further and they were not included in detailed study.

IL-8 and TNF-α were selected as they are the main proinflammatory cytokines involved in febrile reactions and induce the secretion of cytokines by other cells. IL-8 is

Table 2 IL-8 Levels in 15 PRP and BC-PC with respect to storage time

IL-8 Level pg/ml range		1 h n	18 Hrs. n	65 Hrs. n	112 Hrs. n
PRP	< 8	9	6	0	0
	8–100	6	6	1	0
	101–500	0	3	12*	2
	501–1,000	0	0	2	13
	Mean ± SD	9.26 ± 11.17	48.70 ± 65.80	307.72 ± 196.97	731.61 ± 204.22
BC	< 8	12	9	7	0
	8–100	3	6	3	3
	101–500	0	0	5*	12
	501–1,000	0	0	0	0
	Mean ± SD	7.9 ± 14.01	14.6 ± 21.97	58.45 ± 70.17	247.11 ± 139

* Significantly higher number of units showed the values in the range 101–500 in PRP-PC compared to BC-PC by $\chi 2$ test (P 0.005) at 65 h

Table 3 TNF—α Levels in 15 PRP and BC-PC with respect to storage time

TNF—α level pg/ml range		1 h n	18 h n	65 h n	112 h n
PRP	0–3	14	11	13	6
	>3	1	4	2	9
	Mean ± SD	0.63 ± 2.44	2.33 ± 4.39	5.65 ± 18.83	9.3 ± 10.63
BC	0–3	11	13	10	8
	>3	4	2	5	7
	Mean ± SD	1.08 ± 1.77	0.29 ± 0.83	6.01 ± 14.96	8.97 ± 12.84

the cytokine which is a marker to monitor leucoreduction as it is mainly secreted from leukocytes [4].

The pH was measured on 5th day of storage platelets and was above six in all the PCs which shows that platelet integrity was not greatly disturbed by preparation and storage condition. The measurement of pH is usually included in all studies of PC which is necessary to assess platelet quality. Platelets are stored in plasma and in platelet metabolism when the lactate concentration increases the pH falls from 6.8 to 6.2 resulting in progressive swelling, disc to sphere transformation, agglutination of platelets and lysis which may release granules resulting in high cytokines [7].

The platelet count was more in BC-PC and WBC were more in PRP-PCs. The WBC count in PRP-PC was $7.4 \pm 3.75 \times 10^7$ and in BC-PC was $3.9 \pm 2.2 \times 10^7$ which was significantly lower P0.0043. The WBC count is comparable with study of Chaudhary et al. [8] where the WBC count was $6.3 \pm 3.1 \times 10^9$/l in PRP-PC. WBC count in PC also is important in view of Alloimmunization to platelet antigens. There is role of HLA class I antibodies in refractoriness to platelet transfusions. There are many leucocytes in the PC that evoke the formation of antibodies against histocompatibility antigens [9–11].Formation of such antibodies occur if the platelets are contaminated with leukocytes but as suggested by Gouttefangeas et al. [12] platelets alone are capable of inducing a secondary immune response. Evidence shows that to avoid primary immunization the total number of leucocytes transfused in red cell or platelet concentrate must be $<5 \times 10^6$ [13–15].

Role of leucocytes in cytokine release has been understood since long. Fujihara et al. [1] suggested that cytokine generation is due to new synthesis and release of cytokines from residual WBCs. According to them the release of cytokines from dead WBCs is also possible as they showed that IL-8 mRNA levels increased from Day 1 to Day 8 using semi quantitative RT-PCR. Heddle et al. [16] demonstrated that if WBC counts are reduced to a concentration lower than 5×10^6/unit the cytokine release is reduced . Different studies have adopted different techniques for leukoreduction. Earlier it was buffy coat removal, filtration, then came automated extractors like Optipress and as the technology grew different methods were available for preparation of different components. In light of this the present study incorporated the comparison of different PCs prepared using different methods.

As suggested by Davenport [17] many factors activate WBC to generate cytokines during storage like activated complement components, thrombin or by cytokines released from damaged WBC or by non-biological surfaces of plastic containers. The measure of sCD40L a pro-inflammatory mediator released by leukocytes and platelet upon activation indicates that WBC is activated and cytokines are synthesized due to which the levels increase from

0 day to 5th day [17]. Thus there is an increase seen in the level of IL-8 and TNF-α during storage. This study focuses on WBC level and cytokine levels which proves that cytokine concentration is directly related to WBC content and storage time. As demonstrated by Wadhwa et al. [18] cytokines have the potential to mediate some of the adverse effects of platelet transfusions by inducing the secretion of other cytokines from target cell types or by synergizing with other cytokines. It increases further by activated platelets which upon transfusion may induce WBC activation and/or secretion of pro inflammatory cytokines and strengthen inflammatory reactions.

It has been reported that cytokine levels are more in PC stored at 22 °C than in red cells stored at 4 °C because higher temperature may allow greater metabolic activity of cytokine producing WBCs. Heddle et al. [16] demonstrated this by separating cellular and plasma or supernatant portion of PCs. It was shown that most reactions to platelets were caused by WBC derived cytokines that accumulate in the plasma portion of the component. As an alternative to WBC reduction they adopted the depletion of plasma from platelet to remove the cytokines. They removed most of the plasma before transfusion and showed that removal of supernatant plasma from platelet before transfusion is an effective way to reduce the frequency and severity of reactions.

In preliminary study the observed range of IL-8 was up to 0–12 pg/ml in WB which was considered as normal range as the samples were of healthy blood donors. The range of IL-8 observed after 1 h of preparation of PC showed the levels in the range of 8–100 pg/ml in many samples which suggests that they must be released during preparation of PC which continuously increased during storage since the levels were increased immediately after preparation. Comparison of cytokine levels between PRP and BC method shows that IL-8 levels are higher in PRP-PC. Analyzing the time dependent level it was found that IL-8 level increased significantly P0.0001 at 65 h in PRP-PC and BC-PC. As studied by Cardigan [19], detection of IL-8 predominantly reflects leucocyte contamination thus leucocytes being more in PRP-PC it justifies the findings of low IL-8 levels in BC-PC which have less WBC contamination. Flegel estimated the cytokine levels in PC prepared by BC method by a large scale production process. The WBC count in these PCs was $<1 \times 10^9$/L and were almost free of cytokines during 5 days storage [20]. Similarly, Muylle suggested that BC method is preferable for production of PCs as cytokine levels are low in BC-PC and there is less frequency of reactions [21].

TNF-α levels increased after 18 h of storage which was lower in BC-PC compared to PRP-PC. After 65 h of storage levels went together in both types of PC. A peak level of TNF-α is seen at 65 h in BC-PC and is less than PRP-PC at 112 h. It may be due to short half life [8]. Similar to Bayraktarogglu et al. [4] it was observed that TNF-α also increased from 1 to 112 h in both PC but more in PRP-PC. Statistically significant difference has been observed in this study between BC-PC and PRP-PC cytokine levels. The reason is low levels of contaminating WBCs in BC-PC which are the main source of cytokines as demonstrated by Aye et al. [2]. The results in this study indicate that there may be active synthesis of cytokine by activated WBCs at 22 °C storage temperature as levels start increasing after 1 h.

Conclusion

In conclusion, this study shows that IL-8 levels significantly increased after 1 h of storage and TNF-α. levels were low up to 18 h and then showed increase but not as marked as IL-8. The BC-PC had significantly low levels of IL-8 compared to PRP-PC. This shows that the method of preparation and degree of leucocyte contamination are the critical factors that influence the cytokine levels in PC.

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