

A NOVEL TECHNIQUE FOR PREPARING IMPROVED BUFFY COAT PLATELET CONCENTRATES

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ABSTRACT. We evaluated *in vitro* platelet function of platelet concentrates stored at 22 C for 5 days prepared either by the conventional pelleting procedure or platelet concentrates prepared from buffy coats by utilizing a novel bucket designed to support a suspended bag. For platelet concentrates from buffy coat, whole blood was centrifuged at 3,000 x g for 13 min, with all but 30cc of the cell poor plasma transferred to a satellite bag, followed by a second centrifugation at 170 x g for 5 min utilizing our novel centrifugation device. For pelleted platelets, whole blood was centrifuged at 2,000 x g for 3 min, platelet rich plasma removed, centrifuged, and the pellet resuspended in plasma. Leukocyte contamination in buffy coat platelet concentrates was reduced by 95% (p<0.001) in comparison to pelleted platelets. Further, platelets from buffy coat platelet concentrates demonstrated significantly enhanced ADP-induced aggregation, increased recovery from hypotonic shock, higher morphology scores, and reduced GMP-140 expression in comparison to pelleted preparations. No differences in O₂ consumption, CO₂ production, pH and total ATP were observed between the two types of preparations at day 5 of storage. Our results indicate that platelet concentrates from buffy coat, prepared by a suspended storage bag centrifugation technique, are superior with respect to *in vitro* platelet function when compared to pelleted platelets.

Key words: platelet storage, buffy coat, leukocyte contamination.

INTRODUCTION

Standard approaches used to prepare platelet concentrates involve the process of high speed centrifugation followed by pelleting. Previous reports have demonstrated that platelets from these preparations have impaired *in-vitro* agonist-induced platelet aggregation (1), as well as a substantial increase in the secretion of platelet α -granular contents (2). Additionally, platelets from concentrates prepared by the pelleting process have been shown to have impaired recovery from hypotonic stress (3), an *in vitro* indicator of platelet viability *in vivo* (4). Further impairing the effectiveness of transfused platelets, platelet

deterioration during early stages of storage results in reduced *in vitro* platelet function (5) and diminished *in vivo* hemostatic effectiveness after transfusion (6). A number of *in vivo* studies have documented functional impairment and reduced survival of platelets procured by the pellet technique and stored for long periods of time (7, 8).

Platelet concentrates can be obtained from either pelleting platelets in platelet rich plasma (PRP) or by isolating the fraction of the buffy coat layer. For the latter, after initial high speed centrifugation of whole blood, cell free plasma is removed and the buffy coat is collected in a separate bag. The platelet concentrates are next

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isolated from the buffy coat by low speed centrifugation. Currently, in designs of others, the bag containing the buffy coat is placed in inflexible compartments within the centrifuge bucket for an upright orientation of the storage bag (9). Hence, the buffy coat technique avoids platelet pelleting, a procedure known to significantly reduce platelet viability during storage (10).

For the present study, platelet concentrates were obtained either from the pelleting process or by buffy coat separation. For buffy coat derived platelet concentrates, a bucket was designed such that bags of platelet concentrate could be suspended by metal rods within the bucket, resulting in free mobility of the bag during centrifugation, allowing for a superior separation of cellular components. The two types of platelet concentrates were stored for 5 days and platelets from each were compared for their response to ADP-induced aggregation and their degree of GMP-140 expression, a membrane antigen expressed upon platelet α -granule secretion (11). Further, leukocyte contamination, platelet morphology, and platelet metabolic parameters were compared using these two methods of platelet concentrates preparation.

MATERIALS AND METHODS

Preparation of Platelet Concentrates

Platelet concentrates, purchased from a community blood bank, were obtained from healthy volunteers who had denied taking any medications for at least 48 hours. For platelet concentrates obtained by the pelleting technique, twelve individual units of blood were drawn into quadruple blood bags, one containing CPDA-1 as an anticoagulant (CLX, Cutter Biological, Berkeley, CA). Whole blood (450 ml) was centrifuged at 2,000 x g for 3 min (Sorval RC3B, Newton, CT) at 22 C. The supernatant, platelet-rich plasma, was transferred to a satellite container and centrifuged at 2,000 x g for 15 minutes at 22 C to obtain a platelet pellet. All but

60 ml of platelet-poor plasma was transferred to another satellite container while the primary bag was allowed to equilibrate for 1 hour before resuspension. For platelet concentrates obtained by the buffy coat technique, 12 units of CPDA-1 anticoagulated whole blood (450 ml) were collected into quadruple blood bags (CLX, Cutter Biological, Berkeley, CA) and centrifuged at 3,000 x g for 13 min at 22 C. Cell poor plasma was transferred to a satellite bag while a small portion of the plasma remained covering the buffy coat (about 30 ml). The whole layer of buffy coat and about 20 ml of the upper portion of red cells were transferred to a separate container. The buffy coat within the bag which had its additional attached satellite bag, was gently centrifuged, 170 x g for 5 min at 22 C. This second centrifugation was performed with a device consisting of a free hanging bag within a bucket, which allowed for a clear resolution between the platelet concentrate and the remaining cellular components (Figure 1).

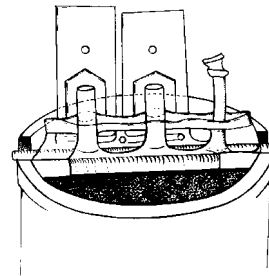


Figure 1. Depiction of the suspension device used for buffy coat preparations. Opposing metal rods are fixed securely in slots, allowing for the suspension of the concentrate bag within the centrifuge bucket. Plastic buckets were utilized in this study, with slots cut in the rim of the bucket to a depth of 2 cm.

Storage and Sampling

All platelet concentrates were stored in 300 ml satellite containers, suitable for storage at 22 ± 2 C on a horizontal flat bed shaker at 30 cycles/minute (Stovall Life Science, Inc. Greensboro, NC, USA). The platelet count was measured using a counting chamber and light microscopy. Leukocyte contamination was evaluated on the first storage day with the Nageotte chamber at a 1:10 dilution with 0.3 percent acetic acid. Platelet counts were determined by cell counter (Sysmex-K-100 cell analyzer, Baxter Diagnostic Inc, McGraw Park, IL). For the studies described below, samples

were removed under sterile conditions using a sampling site coupler (Fenwal, Deerfield, IL, USA) on days 1, 3, and 5.

Assessment of Platelet Morphology

Platelet morphology was evaluated in wet preparations under light microscopy using the scoring method of Kunicki et al (12). The cells were categorized into four morphological populations: discoid, spherical, dendrites and balloons. At least 100 cells were examined and differentiated. The number of platelet morphological forms was given a scoring factor as follows: for discoid cells the factor was 4, round cells received a factor of 2, dendritic cells were given 1 and balloons received a factor of 0. Scanning electron microscopic (SEM) preparations were carried out using modifications of the technique as described by Saniabade et al (13). Briefly, platelets obtained by the buffy coat or pellet technique were fixed for 2 hours at room temperature with 2.5% glutaraldehyde, at pH 7.3, placed on Nucleopore polycarbonate membrane filters (Nucleopore, Pleasanton, CA), 0.4 μm in diameter, and then dehydrated by successive 5 minute incubations with 30%, 50%, 70%, 90% and finally three times with 100% ethanol. The samples were dried with CO_2 by the critical point method. The dried samples were sputter coated with 20 μM gold:palladium (60:40) (Hummer V, Technics, San Jose, CA) and observed in a PSEM 500 scanning electron microscope (Phillips, Ltd). For each sample, chambers containing monolayers of cells were analyzed by counting all cells in the chamber and calculating the percentage of discoid, spheroidal and bipolar forms.

Platelet Aggregation

For platelet aggregation studies, the platelet samples were diluted with autologous plasma to a concentration of $400 \times 10^3/\mu\text{l}$. ADP, at a final concentration of 20-40 μM , was added to 0.480 ml of PRP. Aggregation was recorded on a dual-channel aggregometer (Payton Scientific,

Buffalo, NY, USA).

Measurements of Platelet Metabolic Parameters

Levels of ATP in platelet concentrates were determined using an enzyme linked assay (Sigma Diagnostic, St.Louis, MO, USA). This assay uses phosphoglyceric phosphokinase (PGK) and glyceraldehyde phosphate dehydrogenase (GAPD) in a coupling reaction of specific phosphorylations requiring ATP with oxidative-reduction systems involving NAD/NADP linked enzymes. Data were expressed as $\mu\text{M}/10^{11}$ platelets. Hypotonic Shock Response (HSR) was measured spectrophotometrically at 610 nm (LKB Model 4053, Sweden) according to Valeri et al (14). Percent recovery after hypotonic stress was calculated after 15 minutes of undisturbed incubation. CO_2 production, O_2 consumption, and pH were measured on a blood gas analyzer (ABL model 30 Radiometer, Copenhagen, Denmark) at 37 C and automatically recalculated at 22 C.

Platelet GMP-140 expression

Samples from platelet concentrates were analyzed in a flow fluorocytometer (FACStar, Becton Dickinson, Palo Alto, CA) as described previously (15). Platelet preparations were fixed in 1 ml of 1% paraformaldehyde for at least 2 hours, washed three times with phosphate-buffered saline, pH7.4 and resuspended in phosphate-buffered saline supplemented with 2% newborn calf serum. Platelets were incubated for 20 min in the dark with murine monoclonal antibody to GMP-140 (CD62; Becton Dickinson, Palo, Alto, CA), extensively washed in phosphate buffered saline, followed by incubation with secondary GAM-FITC antibody of the same isotype. After washing, samples were immediately evaluated on a flow cytometer which was calibrated for log mode fluorescence and light scatter with 10,000 events per sample measured. Control specimens were processed as above, but incubated with an primary irrelevant monoclonal IgG.

Statistics

Statistical analysis was performed using the Student's paired t-test, comparing buffy coat

values with those of pelleted preparations. All data are expressed as a mean \pm standard deviation.

TABLE 1.

COMPARISON OF FUNCTIONAL, MORPHOLOGICAL AND METABOLIC PARAMETERS OF BUFFY COAT AND PELLETTED PREPARATIONS DURING 5 DAYS OF STORAGE AT ROOM TEMPERATURE										
	DAY 1			DAY 3			DAY 5			N
	Buffy coat	Pelleted	p-value	Buffy coat	Pelleted	p-value	Buffy coat	Pelleted	p-value	
Platelet Count (x 10 ⁹ /ml)	945 \pm 110	620 \pm 86	NS	952 \pm 101	632 \pm 74	NS	932 \pm 93	540 \pm 72	NS	24
Leukocyte Count (x10 ⁶)	0.37 \pm 0.12	7.23 \pm 3.2	<0.001							24
ATP (μ mol/10 ¹¹ cells)	7.80 \pm 1.56	7.32 \pm 0.5	NS	NT	6.98 \pm .46		5.62 \pm 0.98	5.72 \pm 0.34	NS	24
Maximum Aggregation (%)	66 \pm 7.8	46.6 \pm 13.9	<0.001	37.2 \pm 16.5	30.0 \pm 10.7	NS	38.7 \pm 14.9	22.6 \pm 8.5	<0.009	24
pH	7.59 \pm 0.075	7.42 \pm 0.035	NS	NT	7.64 \pm 0.16	NS	7.41 \pm 0.14	7.41 \pm 0.24	NS	24
pO ₂ (mm Hg)	21.0 \pm 14	31.3 \pm 5.5	<0.05	NT	48.1 \pm 16.2		34.6 \pm 12.9	43.3 \pm 19.5	NS	24
pCO ₂ (mm Hg)	13.0 \pm 0.91	21.0 \pm 1.9	<0.001	NT	9.2 \pm 1.8		7.2 \pm 1.2	7.2 \pm 2.4	NS	24
GMP-140 (% positive cells)	11.4 \pm 4.61	20.0 \pm 0.98	<0.017	21.1 \pm 6.8	32.5 \pm 3.3	<0.031	30.0 \pm 4.3	37.6 \pm 4.0	<0.05	12

Data is expressed as mean \pm SD; NT: not tested; NS: not significant.

RESULTS

The mean platelet concentration of buffy coat platelets was nonsignificantly increased in comparison to the concentration of platelets in pelleted preparations (Table 1). However, leukocyte contamination in platelet concentrates prepared from buffy coat was 95% (p<0.001) less than leukocyte contamination in pelleted preparations.

Assessment of platelet metabolic integrity was evaluated by measuring pH, pO₂, pCO₂ production and ATP concentrations. Buffy coat and pelleted platelet preparations demonstrated no significant changes in pH throughout the period studied (Table 1). On day 1 of storage, O₂

consumption and CO₂ production in pelleted platelets were increased by 47% (p <0.05) and 61% (p <0.001), respectively, in comparison to platelet concentrates of buffy coat. No significant differences were observed between the two preparations with respect to platelet ATP concentration (Table 1). As depicted in Table 1, a 42% (p<0.001) enhancement in 20 μ M ADP-induced maximum aggregation was observed in platelets prepared from buffy coat platelet concentrations in comparison to maximum aggregation of pelleted platelets stored for 1 day. Similarly, a 71% (p<0.009) enhancement was observed in 20 μ M ADP-induced maximum aggregation of buffy coat platelets in comparison to pelleted platelets aggregated with 40 μ M ADP at day 5.

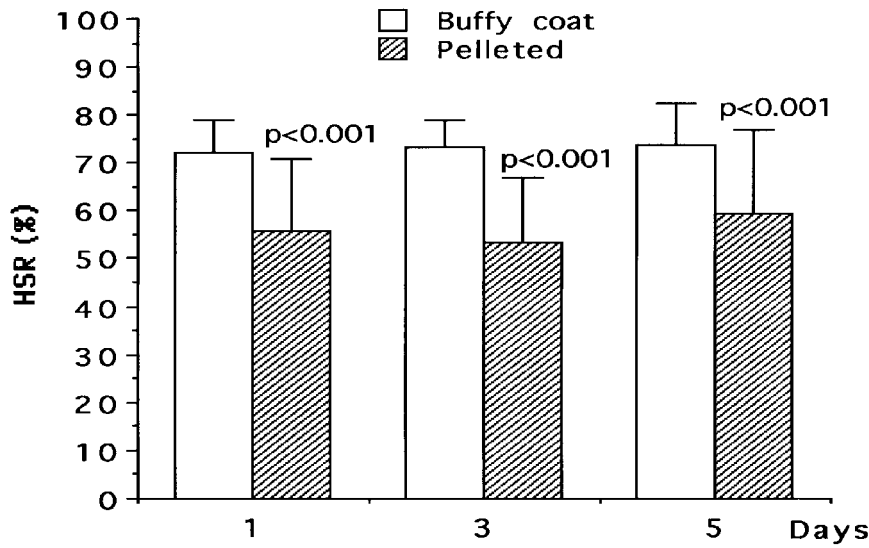


Figure 2. Hypotonic Shock Response (HSR) in buffy coat and pelleted platelet concentrates during 5 days of storage at room temperature. Platelet concentrate samples were subjected to hypotonic stress on days 1, 3, and 5, as described in Methods. p-values are paired Student's t-test comparing values in buffy coat preparations to those of pelleted preparations.

Platelet concentrates prepared from buffy coat demonstrated a 31% ($p < 0.001$), 33% ($p < 0.001$) and 24% ($p < 0.001$) enhancement in recovery from hypotonic stress in comparison to pelleted platelet concentrates at days 1, 3, and 5,

respectively (Figure 2). Throughout the storage period, the recovery from hypotonic stress for each preparation did not vary with day of storage.

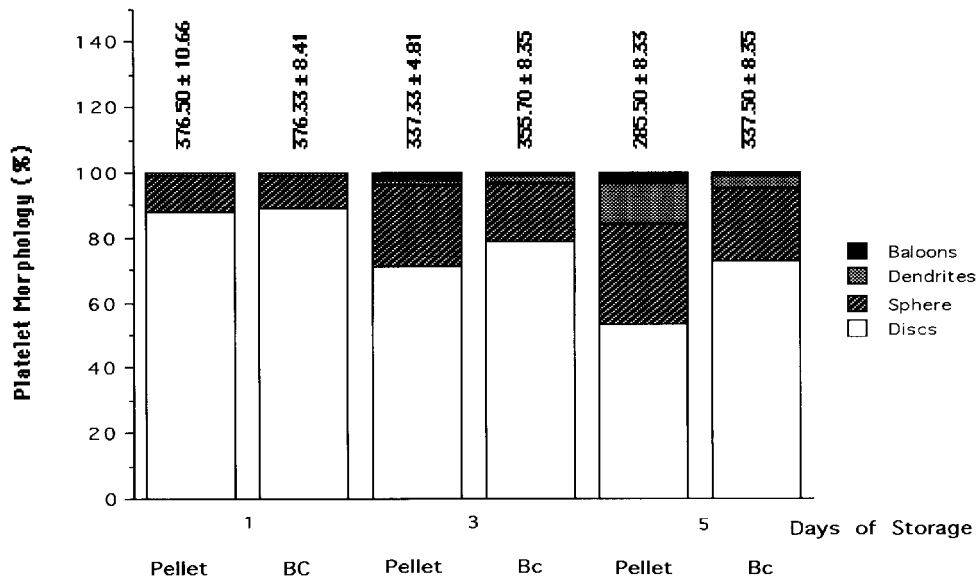


Figure 3. Light microscopic differential and morphology scores of pelleted and buffy coat platelet preparations during storage at room temperature. Morphological scores were evaluated as described in Methods. Maximal scoring was assigned a value of 400. At day 5 of storage a significant decrease in discoid forms with an increase in spheroidal and dendrite forms were observed in pelleted platelet preparations in comparison to those of buffy coat ($p < 0.001$).

Results of platelet morphological scoring using light microscopy are illustrated in Figure 3. No differences were observed between platelet morphological scores of platelet concentrates from buffy coat and pelleted platelets on day 1. At days 3 and 5 of storage, the morphology scores for both sets of platelet concentrates progressively decreased and were consistently lower for the pelleted platelets. At day 5, a 15% decrease ($p < 0.001$) in morphology score was observed in platelet concentrates of pelleted platelets in comparison to those of buffy coat preparations. At day 5 of storage, a 2-fold increase ($p < 0.001$) in dendrites and balloon forms was observed in pelleted platelets in comparison

to buffy coat preparations. Similar results were obtained by analysis using electron microscopy. As illustrated in Figure 4A and C, platelet specimens derived from buffy coat preparations (day 1) were composed of discoid morphological forms (4A) in contrast to pelleted platelet preparations (4C) which illustrate platelet aggregates and spheroidal platelet forms. Scanning electron microscopic evaluation at day 5 demonstrated the presence of spheroidal and discoid platelet morphological forms in buffy coat platelets preparation (4B), whereas platelet concentrates from pelleted preparations demonstrated dendritic forms and large platelet aggregates (4D).

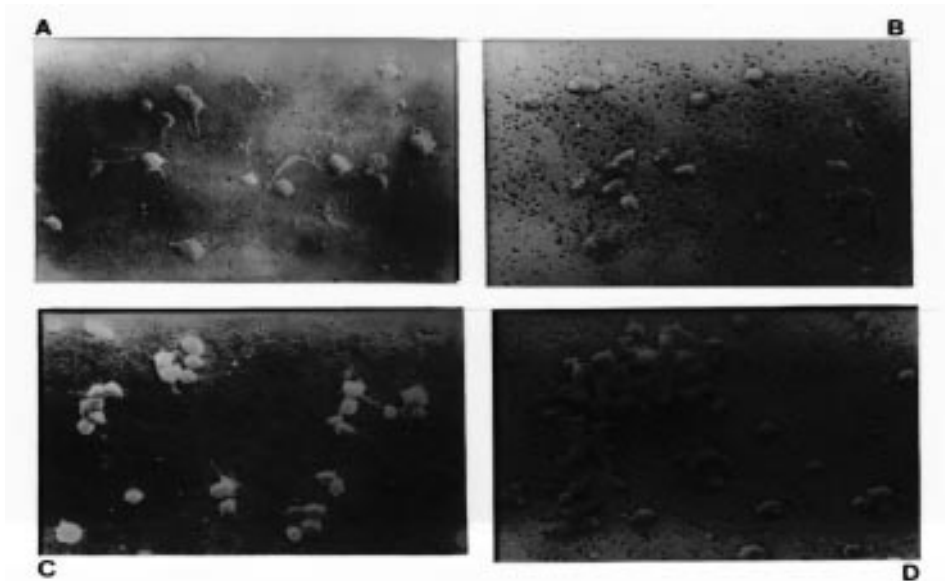


Figure 4. Scanning electron microscopy preparations of pelleted and buffy coat platelets stored at room temperature. A: day 1, buffy coat, **B:** day 5, buffy coat, **C:** day 1, pellet, **D:** day 5, pellet. At day 1, an abundance of discoid platelets with no visible aggregates is observed in buffy coat preparations (**A**), while the majority of platelet forms observed in pelleted platelet preparations were spheroidal forms with small platelet aggregates (**C**). At day 5 of storage, discoid and spheroidal forms are observed in buffy coat preparations (**B**), while pelleted preparations demonstrated an abundance of spheroid and dendritic forms with platelet aggregates present (**D**). Arrow indicates the presence of a bipolar cell. (x2500)

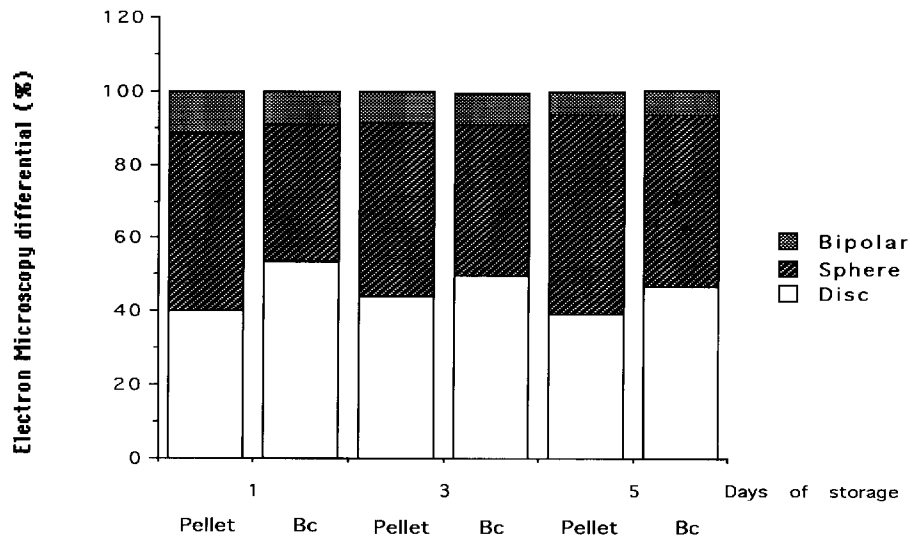


Figure 5. Comparison of pelleted and buffy coat differential during storage at room temperature using scanning electron microscopy. At day 1, the proportion of spheres in pelleted platelet preparations was significantly increased ($p < 0.05$) in comparison to buffy coat preparations. At day 5, pelleted platelet preparations demonstrated a significantly higher proportion of spheres ($p < 0.023$) in comparison to buffy coat preparations.

Figure 5 depicts the percentages of morphologic platelet forms, as assessed by SEM, of buffy coat and pelleted platelet concentrates during 5 days of storage. A 24% increase in spheroidal forms was observed in day 1 pelleted platelet preparations in comparison to buffy coat preparations ($p < 0.05$). Similarly, a 17% increase in spheroidal forms was noted in pelleted platelet preparations in comparison to buffy coat preparations at day 5 of storage ($p < 0.023$).

In order to confirm these results, expression of GMP-140 was assayed by flow cytometry. Platelet concentrates from pelleted platelet preparations demonstrated a 75% ($p < 0.017$) increase in GMP-140-positive cells in comparison to platelet concentrates of buffy coat preparations on day 1 of storage. Similarly, on day 3 and 5 of storage, platelets of pelleted preparations showed a 54% ($p < 0.031$) and 25% ($p < 0.05$) increase, respectively, in GMP-140-positive cells in comparison to platelets derived from buffy coat.

DISCUSSION

Despite technological advances in platelet

storage conditions, functional platelet defects (16) and aberrations in membrane composition (17) occur during the storage period. As a consequence, platelet recovery after transfusion remains inferior to that of fresh platelets (18). Platelet injury occurs early in the process of platelet preparation and it has been suggested that platelet damage results in platelet activation which ultimately leads to platelet refractoriness (19). Thus, conditions which minimize platelet activation during platelet procurement and platelet processing, as well as during platelet storage, should substantially improve the quality of platelet preparations utilized for transfusion.

Studies by others have found that platelet concentrates prepared from pelleting by high speed centrifugation showed abnormal aggregation and an enhanced secretory response as compared to preparations of fresh platelet rich plasma (1,19-21). Although platelet concentrates processed by the buffy coat technique are centrifuged twice, these platelets are not subjected to high speed centrifugation, hence avoiding pelleting. The superior *in vitro* functional quality of these preparations has been confirmed herein

and by others by assaying ADP-induced platelet aggregation (19). Further, in this report, values of O_2 consumption of platelet concentrates prepared from buffy coat preparations stored for 1 day were significantly increased in comparison to platelet concentrates of pelleted platelets, as indicated by the lower O_2 partial pressure in the storage container. In addition, our electron and light microscopic morphological studies demonstrated a significantly reduced proportion of activated platelets in buffy coat preparations in comparison to pelleted preparations. These morphologic findings have been demonstrated to correlate with enhanced viability of platelets *in vitro* and *in vivo* after transfusion (1,12, 22). Lastly, a significantly reduced population of activated platelets in buffy coat preparations was confirmed by GMP-140 expression.

Although others have utilized the buffy coat technique for platelet concentrate preparation, the previous technique involves the use of a bucket consisting of inflexible compartments for the upright placement of storage bags (9). As a result, such a device has the potential for inferior cellular separation resulting in excessive contamination of the platelet rich plasma with leukocytes.

The bucket design presented herein, allows for free suspension of the storage bag during centrifugation, not only resulting in superior cellular separation, but enabling a bag of any manufacturer to be utilized in this centrifugation system.

A significant factor contributing to morbidity associated with platelet concentrate transfusions is the problem of leukocyte contamination. Recent reports have indicated that levels of leukocyte contamination of platelet concentrates correlated with the secretion of pro-inflammatory cytokines,

IL-8, IL-1 β and IL-6 (23) the latter, having been demonstrated to activate platelets *in vitro* (24) and *in vivo* (25). As such, a technology which reduces leukocytes in platelet concentrates should not only reduce the risk of febrile transfusion reactions, but should prevent platelet activation resulting from pro-inflammatory

cytokine secretion.

Prior reports investigating the functional capacity of platelet concentrates prepared by the conventional buffy coat technique, have reported considerable leukocyte contamination. Using the previous methodology, Fijnheer et al (1), Eriksson et al (26) and Pietersz et al (27) reported total leukocyte contamination of 6×10^6 , 110×10^6 , and 14×10^6 per unit of platelet concentrate, respectively. Such values represent 19-fold, 365-fold and 45-fold increases in leukocyte contamination in comparison to our suspended bag system. Further, Washitani et al (19), also utilizing the previous buffy coat methodology, reported platelet maximum aggregation values 47% and 85% below those of the present study on day 1 and day 3, respectively. As such, the suspended storage bag technology not only lowers leukocyte contamination, but produces platelets with an improved functional response to agonists.

In summary, our results demonstrate that platelet concentrates prepared by a buffy coat technique, utilizing a methodology which allows for the free suspension of platelet storage bags while undergoing centrifugation, results in reduced leukocyte contamination of platelet concentrates, enhanced functional capacity of platelets, and reduced platelet activation. Furthermore, this technology could be applied to any bucket design, irrespective of the manufacturer. The potential *in vivo* beneficial effects of platelets prepared with this technique remain to be investigated.

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