Optimising quality of platelet transfusions
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Chapter 2

Low leukocyte contamination without filtration by preparation of platelet concentrates in cylindrical bags with the buffy-coat method

_Vox Sanguinis in press_
LOW LEUKOCYTE CONTAMINATION WITHOUT FILTRATION BY PREPARATION OF PLATELET CONCENTRATES IN CYLINDRICAL BAGS WITH THE BUFFY-COAT METHOD

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Abstract

BACKGROUND AND OBJECTIVES: The buffy-coat (BC) method for platelet concentrate (PC) preparation was modified in order to obtain leukocyte-depleted PCs from single BCs without filtration.

MATERIALS AND METHODS: BCs were centrifuged in cylindrically shaped BC bags, and the optimal centrifugation conditions and optimal haematocrit were determined.

RESULTS: With optimal conditions, a tenfold lower leukocyte contamination was obtained compared to the conventionally shaped, wide BC bag (0.3 ± 0.19 versus 3.0 ± 1.71 x 10^6 leukocytes per PC; 85-ml BCs). The platelet yield obtained with the cylindrical bag did not differ significantly from the yield obtained with the conventional bag (56 ± 16.4 versus 61 ± 15 x 10^9 platelets per PC). Furthermore, when PCs were prepared from 100-ml BCs in cylindrical bags, a leukocyte contamination of 0.2 ± 0.11 x 10^6 and a platelet number of 61 ± 13.5 x 10^9 per PC were obtained.

CONCLUSION: The use of cylindrical BC-bags reduced the leukocyte contamination in PCs to a level required for leukocyte-depletion without affecting the platelet recovery.
INTRODUCTION

The preparation of platelet concentrates (PCs) from buffy coats (BCs) is based on the separation of the platelets from other blood cells by velocity sedimentation [83]. Since the introduction of the Compat [84] it is possible to separate BCs accurately from red cells and plasma. These BCs contain >90% of the platelets, >75% of the leukocytes and only 5% of the red cells [31] and can be used to prepare single PCs or can be combined to prepare PC from pooled BCs. However, during preparation of single PCs in the currently available (wide and short) BC bags, the interface containing leukocytes, is mixed rather easily with the platelet-rich supernatant. This is due to the shape of the BC-bags, because for optimal separation this shape requires a centrifuge rotational speed near the resonance frequency of blood bag centrifuges. Moreover, the width of the BC-bag itself (120 mm) renders the interface between supernatant and sediment rather unstable, in particular during the release of the BC bag from the centrifuge cup and when mounting the bag on the separation device. Remodelling the shape of the BC bag might increase the length of the path available for separation (currently 60 mm), simultaneously decrease the bag's diameter, and thus prevent disturbance by resonance and reduce the width of the interface.

We designed cylindrical bags with a similar nominal volume (100 and 120 ml) as the current wide BC bags, with fixed diameters (50 mm) and variable lengths (115 or 160 mm). The length of the 120-ml cylindrical BC bag was comparable with that of a 500-ml blood collection bag (170 mm). Adapted bag holders (tightly fitting cylinders) were required to keep these bags upright and prevent wrinkling during centrifugation. With these bags we studied the optimal conditions for the production of PCs with a volume of 65 ml, an acceptable yield of platelets [85;86] and a negligible contamination with leukocytes.

MATERIALS AND METHODS

Blood collection and buffy-coat preparation

500 ± 50 ml of whole blood was collected in 70 ml of citrate-phosphate-dextrose (CPD-blood) in PVC/DEHP quadruple bag systems (NPBI, Emmer Compascuüm, The Netherlands). After storage at 20 ± 2°C for 12-16 hours, this CPD-blood was the source material for processing [30].

The CPD blood was centrifuged to buoyant density equilibrium (8 min, 2700 rpm, radius 280 mm measured till bottom of cup, acceleration time 70 s, deceleration time 270 s; 20°C; Hettich Roto Silenta R/P, Dépex, De Bilt, The Netherlands).

BCs (containing >90% of the platelets, >75% of the leukocytes and 5% red cells) were separated from plasma and red cells by use of a Compat [84](NPBI; version G4 used) with an optimal separation programme giving minimal red cell
loss combined with maximal platelet and leukocyte removal [31]. For preparation of BCs with a higher than usual (35% instead of about 12%) Ht, the amount of plasma added to the BC was decreased, by adapting the Compomat programme.

BCs with a standardised volume, a standard amount of platelets and leukocytes, but a variable Ht were prepared to study the influence of Ht on the quality of PC separation. Pools of 6 BCs with a high Ht derived from CPD-blood of the same AB0- and RhD- blood groups were mixed in transfer bags (NPBI). From these pools, standard amounts of BC were filled up to 100 ml with cell-free plasma (obtained after centrifugation at 5000 x g, 10 min, Hettich, Roto Silenta), and leukocyte-depleted, AB0, RhD compatible red cells to obtain the required Ht. Sterile syringes (50 ml, Becton Dickinson, San Jose, CA, USA), cylindrical BC bags (120 ml, NPBI), and aseptic techniques were used. In this way, comparable BCs with 5 different Ht values could be made from one pool of six BCs.

The 4 bag systems (NPBI) were adapted to the required configuration by replacing the standard (Wide) BC bags by sterile cylindrical bags (100 or 120 ml) with the aid of a sterile connection device (SCD, Haemonetics, Braintree, MA, USA) after the blood collection and prior to the BC separation.

**Preparation of PCs**

For PC preparation in cylindrical bags, BCs were centrifuged in cups provided with tightly fitting (cylindrical) holders (radius 280 mm, measured till bottom of cup). The wide BC- bags were centrifuged in the same cups without special holders at 800 to 1000 rpm, for 5 min, brake 3, at 20 °C (200-313xg)(Hettich Roto Silenta).

BCs (with fixed volume and different Ht) made from a BC pool, in cylindrical bags, were centrifuged at 1700 to 2700 rpm (905-2282xg) for 5 min, brake 3, at 20°C (Hettich Roto Silenta).

After centrifugation, PCs were transferred to a transfer bag either manually or by using an automated separation device (Compomat-G4, NPBI) with an adapted (Compomaster) programme for cylindrical and conventional BC bags.

**Platelet and leukocyte counts**

Platelets were counted with an electronic counter. Either a Cell-Dyn (Sequoia-Turner Co, Mountain View, CA, USA) or an AcT10 (Coulter Electronics Ltd., Dunstable, GB) was used.

Leukocytes were counted in PCs after staining of the leukocytes with a fluorescent dye. The PCs were 5 times diluted with either Syto11 staining solution to a final concentration of 5 (M (Molecular Probes, Leiden, The Netherlands) or an acridine orange solution (final concentration 2.5 mg/l Phosphate-buffered-salt-solution). A Nageotte bright-line counting-chamber (Superior, Bad Mergentheim,
Germany) and a fluorescence microscope (Leitz, Wetzlar, Germany) were used for counting the leukocytes.

**Haematocrit**

The Ht of BC samples was measured in micro Ht tubes (Vitrex, Modulohm A/S, Denmark), which were centrifuged for 5 minutes at 10,000-20,000xg [15,000 rpm] (Haematokrit, Hettich, Germany).

**Statistical analysis**

Statistical comparisons (Student-t, two-sided) were made with the computer programme Instat2 (GraphPad Software, San Diego, CA, USA). p-Values <0.05 were considered significant and corrected for multiple comparison analysis (Tukey, Bonferroni). Values are given as mean ± SD.

**RESULTS**

**Optimal platelet yield with minimal leukocyte contamination in wide BC-bags**

Low volume BCs (84 ± 5.0 ml) with a low Ht (12%) were obtained by applying the method described by de Korte et al. [31]. It was expected that low Ht of the BC might improve the PC separation, as it increases the available path length of separation. Therefore, the centrifugation conditions for PC preparation from these BCs, in wide BC-bags, were re-evaluated.

PCs were prepared by centrifugation of the wide BC bags for 5 min at a variety of rotational speeds (800-1000 rpm). The yield of platelets and the contamination with leukocytes was measured and the means and SDs were plotted as bars (Y-axis) against the rotational speeds (X-axis, fig.2.1).
Fig. 2.1 - Platelet (solid bars) and leukocyte (shaded bars) numbers in PCs derived from single BCs, centrifuged for 5 minutes in conventional submarine-shaped BC bags at different speeds (rpm, X-axis).

There was no significant difference between platelet yields and between leukocyte contaminations for the different centrifugation speeds. At 900 rpm (250xg), PCs (n=22) contained 61 ± 15 x 10⁹ platelets and 3.0 ± 1.7 x 10⁶ leukocytes. This was comparable with results obtained before [87].

It was concluded that for wide BC-bags variations in volume and Ht do not result in significant effects on the final composition of the PC.

Effects of cylindrical shape of BC-bags on PC-preparation from low volume BCs

BCs with a low volume (86 ± 2.6 ml) and Ht (12%) were now prepared in cylindrical bags (nominal volume 100 ml) which replaced the wide BC bags in the 4-bag systems. Platelets were concentrated from these BCs by a variety of speeds during 5 min of centrifugation and were separated from the cell sediment with a Compomat-G4 (adapted Compomaster programme). Platelet yields and leukocyte contaminations of the PCs were measured, and the means and SDs were plotted as bars (Y-axis) against the rotational speeds (X-axis, fig. 2.2).
At 1700 rpm the platelet yield was $56 \pm 16.4 \times 10^9$ platelets with a contamination of $0.3 \pm 0.19 \times 10^6$ leukocytes, in a volume of $64 \pm 4.5$ ml PC (n=17). At 1800 rpm a yield of $56 \pm 12.5 \times 10^9$ platelets with a contamination of $0.2 \pm 0.20 \times 10^6$ leukocytes per PC of $63 \pm 3.2$ ml was obtained (n=11). At 1600 rpm the yield of platelets ($62.1 \pm 12.8 \times 10^9$) rose, but this rise was accompanied by a slight increase of the contamination to $0.5 \pm 0.44 \times 10^6$ leukocytes per PC (not significant, small differences). The volume of the PCs was $64 \pm 3.3$ ml (n=12). These results indicated that in cylindrical bags the effects of the path length of separation came to expression, because the separation of platelets and leukocytes was greatly (almost tenfold) improved as compared to the wide BC-bags. However, further study on any particular effect of the Ht was needed, as the mere change of the shape of the bag itself might have provided already the optimal path length of separation.

![Fig. 2.2 - Platelet (solid bars) and leukocyte (shaded bars) numbers in PCs derived from single BCs (Ht 12%, volume 86 ± 2.6 ml, n = 69), centrifuged for 5 minutes in 100-ml cylindrical BC bags at different speeds (rpm, X-axis).](image-url)
Chapter 2

Use of BC pools for determination of optimal Ht and volume for PC preparation

A proper study on the influence of the Ht of the BC on the platelet yield and leukocyte contamination of PCs during their preparation required the elimination of all biological variations in the composition of the source material.

Therefore BCs made from pooled BCs, cell-free plasma and leukocyte-depleted red cells to 5 different Ht values were used. The volume of the composed BCs had to be increased (99±1.2 ml, mean±SD, n=20) to allow the same content of leukocytes and platelets per BC at different Ht values. The increase of the volume of the BCs itself had an additional advantage, as it provided a better filling of the cylindrical bags. After centrifugation of the BCs for 5 minutes at 2300 rpm, PCs were made. The Compomaster programme did only allow preparation of PCs from BCs with a fixed Ht. Therefore, automated PC preparation with the Compomat G4 was rather unsuitable for this kind of experiments, and the PCs had to be made manually.

Only one rotational speed (2300 rpm) was chosen based on results of preliminary experiments with 100-ml BCs in cylindrical bags. The PC-preparations were repeated 4 times with 4 different series of composed BCs. The yield of platelets and the contamination with leukocytes were measured and the means and SDs were plotted as bars (Y-axis) against the Ht (X-axis, fig.2.3).

![Fig. 2.3 - Platelet (solid bars) and leukocyte (shaded bars) numbers in PCs derived from BCs (volume 99 ± 1.2 ml) with different Ht values (HtBC, X-axis) centrifuged at 2300 rpm for 5 minutes. The optimal yield of platelets 53 ± 0.8 x 10⁹ was found at an Ht of 19 ± 1.1%. The leukocyte contamination was 5 ± 3.6 x 10⁶ at an Ht of 10 ± 0.6%, decreased to 0.9 ± 0.45% at an Ht of 19 ± 1.1%, but showed a tendency to increase again at higher Ht values (no significant differences).](image-url)
Optimal centrifugation speed for PC-preparation from high volume BCs with 20% Ht in cylindrical BC bags

High volume BCs (113 ± 2.8 ml) with a standard Ht (21 ± 0.6%) were composed from a BC-pool, cell-free plasma and leukocyte-depleted red cells as described previously. These BCs were centrifuged at a variety of speeds (1900 to 2700 rpm), for 5 minutes in cylindrical bags with a nominal volume of 120 ml. PCs were prepared manually.

The yield of platelets and the leukocyte contamination were measured and plotted as bars (Y-axis) against the rotational speeds (fig. 2.4). At a rotational speed of 2400 and 2500 rpm the platelet yield was similar (71 ± 6.4 x 10⁹ platelets) and the leukocyte contamination was decreased to a minimum (0.3 ± 0.13 x 10⁶ leukocytes). Due to the correction of the p-values for multivariate analysis, no significant differences were measured, but there was a significant linear trend toward lower leukocyte contamination at higher speeds. The platelet recovery with respect to the BCs was 65 ± 5.4% and the PC volume was 80 ± 2.6 ml. Above 2500 rpm, the platelet recovery showed a decrease and then no further reduction of the leukocyte contamination was obtained. These results confirmed that in cylindrical bags, each combination of volume and Ht of the BCs needed optimisation of the centrifugation conditions to prepare an optimal PC.

Optimisation of centrifugation conditions for PC preparation from random single BCs with low Ht by adapting volume.

Single BCs were prepared automatically from 500-ml units of CPD blood with a minimum loss of haemoglobin as described previously [31]. The cylindrical BC bags (nominal volume 120 ml) were part of the 4-bag system during preparation. The volumes of the BCs were now adapted to 99 ± 12.7 ml by addition of extra plasma during the BC preparation with the Compomat, resulting in an Ht of 17 ± 5.3% (n=56). Deformation of the BC-bags during centrifugation was avoided, as they were now completely filled. Therefore the variation of the rotational speed was the only parameter during optimisation of the conditions for platelet preparation (fig. 2.2).

PCs were prepared manually from these BCs, after centrifugation for 5 min, at different speeds. The yield of platelets and the leukocyte contamination were measured and plotted as bars (Y-axis) against the rotational speeds (X-axis, fig. 2.5). At 2300 rpm PCs of the favourite specifications were obtained. These PCs contained 61 ± 13.5 x 10⁹ platelets and 0.2 ± 0.11 x 10⁶ leukocytes (significantly (p < 0.01) lower than at 2100 rpm) in a volume of 68 ± 2.8 ml (n = 10). At higher speeds of centrifugation almost similar high platelet yields and low leukocyte contaminations were found. However, at these speeds (2400-2500 rpm), the PCs had a volume of 80 ± 2.9 ml, which is above the preferred volume for clinical use [88].
Fig. 2.4 - Platelet (solid bars) and leukocyte (shaded bars) numbers in PCs derived from single high volume BCs (Ht 21 ± 0.6%, volume 113 ± 2.8 ml), centrifuged in 120-ml cylindrical BC bags at different speeds (rpm \ n-value; X-axis) for 5 minutes.

g-values:  1130  1252  1381  1515  1656  1803  1957  2116  2282
Fig. 2.5 - Platelet (solid bars) and leukocyte (shaded bars) numbers in PCs derived from single BCs with low Ht (Ht 17 ± 5.3%, n=56), centrifuged in 120-ml cylindrical BC bags at different speeds (rpm, X-axis) for 5 minutes.
DISCUSSION

For the preparation of PCs, the BCs are centrifuged until the platelets are separated from the other blood cells by velocity sedimentation, according to their differences in cell size. In addition, the concentration of platelets in the supernatant is promoted by the upward displacement of plasma induced by the sedimentation of red cells [89].

PCs prepared from a pool of BCs had lower leukocyte contamination than expected on the basis of the calculated contamination for the same number of single PCs [90;91]. The preparation of PCs from a pool of BCs was performed in a 500-ml transfer bag, whereas PCs from single BCs were made in the 100-ml submarine-shaped, wide BC bag of the 4-bag system. These findings and identical findings of Mrowiec et al. [92] indicated that the shape and stabilisation of the bag in which the PCs are prepared from BCs influence the quality of separation. Therefore, optimal separation is dependent on the path length available for separation and on the centrifugal force that drives the blood components, viz. the height of the BC-bag, the volume ratio of cells and plasma, and the rotational speed during a constant time of centrifugation.

Cylindrical BC bags enabled us to evaluate the effects of the volume of the BCs, of the volume ratio of cells and plasma in the BCs, and of the effects of the centrifugation speed on the concentration and separation of the platelets.

Biological variations in BCs were eliminated, by composing a variety of Ht values through combining standard amounts of pooled compatible BCs with different amounts of cell-free plasma and leukocyte-depleted red cells.

First, we showed that the mere increase of the path length of separation by using cylindrical bags decreased the leukocyte contamination of the PCs more than six-fold (fig. 2.2).

The evaluation of the effects of variation of the Ht on the separation appeared to be rather complicated. Variation of the Ht induced four (sometimes opposing) effects:

1. the lower the Ht the longer the path length of separation
2. the lower the Ht the smaller the draw and duration of the upward plasma displacement induced by the red cell sedimentation
3. the lower the Ht the lower the rotational speed required for optimal separation
4. the lower the Ht the greater the volume of the recovered PC.

Therefore, the optimal platelet yield with the minimal leukocyte contamination that could be reached at a fixed speed of centrifugation (2300 rpm) was found at an Ht of 20% (fig. 2.3). The optimal leukocyte depletion (0.3 ± 0.13 x 10^6 leukocytes per PC) that could be reached at this Ht was at a higher rotational
speed of 2400-2500 rpm (fig. 2.4). However, this resulted in quite high volumes (80 ± 2.6 ml), not longer suitable for practical clinical use [88].

With these results it was now possible to adapt the volume of random BCs and to find the optimal conditions for the preparation of PCs with a leukocyte contamination below the level required for leukocyte-depletion (fig. 2.5). The draw and duration of the upward displacement of plasma is inversely related to the Ht, as these parameters (upward plasma displacement and Ht) are both proportional to the volume of the sedimenting red cells. The combined effects of the Ht variation, therefore, resulted in opposing influences on the movements of the platelets during the separation from the other blood cells.

It was concluded that each combination of Ht and volume needs its own optimisation of the rotational speed for the improvement of the PC separation. After optimisation, the PCs thus obtained had a volume, a platelet concentration and platelet yield in accordance with the most favourite requirements (small volume, high platelet amount [88]) for single donor platelets (fig. 2.5).

We conclude that cylindrical BC bags, with a nominal volume of about 100 ml, made it possible to obtain leukocyte-depleted PCs without filtration, from single donations. Single PCs are very helpful in paediatrics because they are ready to use. Moreover, single PCs can be matched individually with the recipient before they are combined to a pool with the dose of platelets required for adults.