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Preliminary Procedure for the Optimisation of Recovered Pooled Platelets

Galea St John AP[1] and Zammit V[1,2]*

[1]Department of Health Sciences, University of Malta, Malta

[2]National Blood Transfusion Service, Malta

***Corresponding author:** Vanessa Zammit, National Blood Transfusion Service, Telghet Gwardamangia, Pieta, PTA010, Malta

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Abstract

Today's research has opened the door to the development of various new therapies in all therapeutic fields. These new therapies have caused the demand for blood and its components to increase drastically. Due to the short shelf life of platelet units, blood establishments worldwide are constantly being challenged to meet such a demand. The aim of this study is to achieve the optimal platelet count when pooling four BCs instead of the current five. Four new protocols have been drafted and will be evaluated in terms of platelet yield. Two of these protocols involved the direct pooling of four BCs, with one method using a deconstructed pooling kit. The other two methods consisted of using eight and six BCs respectively and aliquoting these to produce two platelet units from each. The results obtained have demonstrated that Method I and Method III, were the most successful protocols with regards to performance and efficiency. Moreover, Method I was the favored technique in terms of practicality and feasibility when compared to both Method III and the conventional method. However, further studies are necessary to confirm this statement.

Keywords: Platelets; Pooling of buffy coats; Blood transfusion

Abbreviations

BC: Buffy Coat; RPs: Recovered Platelets; NBTS: National Blood Transfusion Service; PAS: Platelet Additive Solution; CBC: Cell Blood Count; ANOVA; OneWay Analysis of Variance; TTIs: Transfusion Transmitted Infections

Introduction

Whole blood is one of the most significant body fluids, which via a transfusion is medically utilized to save lives [1]. Blood components are therapeutic constituents of blood that can be prepared by processing whole blood by means of centrifugation, filtration and freezing using conventional blood banking methodology. Once processed the whole blood will yield three different products, which are namely the red cell concentrate, plasma and Buffy Coat (BC). The BCs undergo a further processing step to produce what is known as Recovered Platelets (RPs).

World Health Organization has supported the use of stringent regulations and controls when selecting blood donors. This strict management, together with the improvement in quality of the blood products led to a considerable reduction in the risk of transfusion-transmitted infections [2]. However, with this rigorous selection of donors, and factors such as lack of time and apprehensiveness, have all led to a sudden increase in demand for blood. This has become a fundamental problem in most countries, and numerous strategies are being considered to help recruit more donors. Another way on how to manage this increase in demand is by optimizing the techniques used to prepare and obtain these blood products. Lowering the number of required BCs to pool platelets would help meet this increase in demand and lessen BC wastage *per se* [3]. In this preliminary study, this approach is being evaluated by testing protocols, which have been drafted based on (i) fewer BCs and (ii) increasing the number of BCs and aliquoting the final product thus obtaining two yield RP units. At this stage, the parameter of interest is the platelet.

Methods

Since the scope of this study was to achieve an adequate platelet concentration using different pooling methods, to achieve the number of units required, RPs where pooled as non-ABO specific using BCs that where less than 27 hours old. The integrity of a closed system was maintained by employing the use of a sterile connecting device.

Current pooling method

Presently, at the National Blood Transfusion Service (NBTS) - Malta, RPs are produced by pooling five ABO-specific BCs that have been screened negative for the infectious markers and are less than 24-hours old. A volume of 200ml of Platelet Additive Solution (PAS) is pooled together with the selected BCs in a platelet pooling kit. During this pooling process the integrity of a closed system is maintained. Once the BCs are pooled and washed in PAS, the pooling bag is centrifuged using a soft-spin, after which the platelets are separated and collected. Before collection, the platelets are passed through an in-line filter to remove any leucocytes entering the final bag, producing a leucodepleted pooled platelet product.

Method I (Pooling with four Buffy Coats)

In this method (Figure 1), four BCs were pooled in a volume of 300ml PAS. At this stage, an initial Cell Blood Count (CBC) was performed to obtain the initial platelet count, which would later be used to measure the platelet recovery rate. Since the overall density was less than that of a traditional pool, a support (Figure 2) was added to the centrifugation bucket to prevent the platelets from seeping back into the red cell layer. Pools were centrifuged using a soft spin. Once centrifuged separation was performed using an automated compressor. Another CBC was taken from the final unit to confirm

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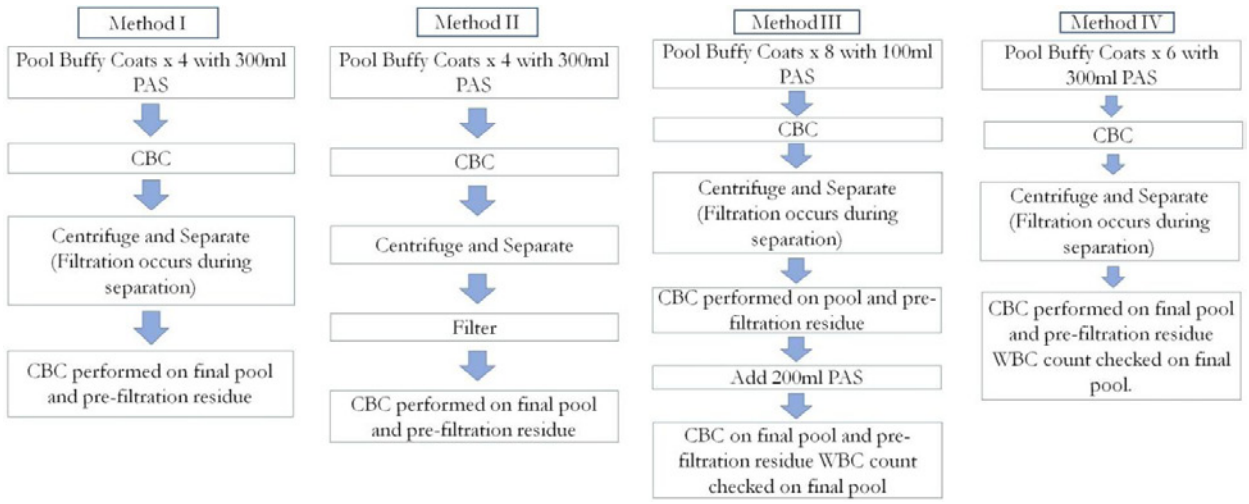

Figure 1: Protocol Flow Chart.
This flow diagram depicts the basic steps required to perform the drafted protocol for the new methods.
Legend: PAS: Platelet Additive Solution; CBC: Complete Blood Count.

the platelet yield.

Method II (Pooling with four Buffy Coats using the deconstructed pooling kit)

This method (Figure 1) is a modification of Method I. The difference is the employment of a deconstructed pooling kit. A deconstructed pooling kit is a pooling kit whose in-line filter has been removed (Figure 3). Once the RP was produced the filter was re-attached, and the product filtered into a new pooling bag. CBCs where again performed on the pre-centrifuged pool and on the final unit.

Method III (Pooling with eight Buffy Coats)

A total of eight BCs were pooled in 100ml PAS centrifuged using a low soft spin and separated (Figure 1). Another volume of 200ml PAS was added. This enabled the final pool to be aliquoted. CBCs was performed before centrifugation and prior to aliquoting. Due to the number of BCs used and consequently the increase in number of leucocytes present, to ensure the integrity of the in-line filter the final unit was tested for leucocytes via flow cytometry.

Method IV (Pooling with six Buffy Coats)

Based on the same principle as Method III, this method employed the use of six BCs (Figure 1). As previously done BC were pooled in 300ml PAS, centrifuged using a soft spin, separated and aliquoted. CBCs was performed before centrifugation and prior aliquoting. Leucocyte testing was also carried out.

Results

The Platelet Count per unit ($x10^{11}$/L) and Volume per $60x10^{9}$ platelets (ml) of the final RP obtained from the four different methods, as well as the conventional pooling of platelets (using five BCs) performed at the NBTS, were statistically analyzed by the OneWay Analysis of Variance (ANOVA) test using the IBM SPSS Statistics 20 Software. Raw data has been summarized in Descriptive Data Tables 1 and 2. Tukey Post Hoc test was used to compare mean platelet counts and mean volume between several methods,

Figure 2: The support.
A support (indicated by the arrow) was added to the centrifuge insert. This enabled the unit and its components to keep steady throughout the centrifugation process.

comparing these two at a time. Results may be viewed at Table 3 and 4. The null hypothesis specifies that the mean platelet count and mean volume varies marginally between the two methods and is accepted if the p-value (probability value) exceeds the 0.05 level of significance. The alternative hypothesis specifies that the mean platelet count and mean volume varies significantly between the two methods and is accepted if the p-value is less than the 0.05 criterion.

When describing the Platelet Count per unit of the pooling method using five BCs, the mean platelet count per unit of all the samples from this method was that of $2.57x10^{11}$/L. On the other hand, this method suffered from the highest standard deviation with a value of 0.431 and has a standard error of 0.035. Since the sample size of the conventional method is relatively large, and the RP prepared contained a platelet count per unit that varied significantly from the average, this method generated the largest standard deviation. All the new protocols carried out had a sample size of 5. The "Pooling with four Buffy Coats" mean platelet count

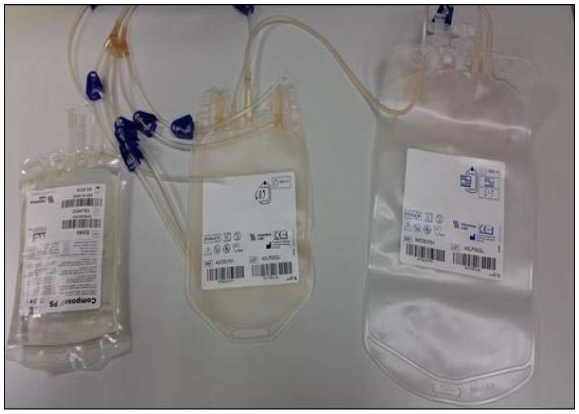

Figure 3: Deconstructed pooling kit.
An example of the deconstructed pooling kit utilized for Method II. The bag in the middle is the mother bag and is directly attached to the final pooling bag (on the right-hand side) without the filter in-between.

Table 1: The Descriptive Table for the Platelet Count per unit.

Platelet Count per unit ($x10^{11}$/L)	Sample size	Mean	Std. Deviation	Std. Error
Conventional Method	150	2.57	0.431	0.035
Method I	5	2.35	0.249	0.111
Method II	5	2.27	0.336	0.150
Method III	5	2.72	0.142	0.063
Method IV	5	2.01	0.008	0.004

Table 2: The descriptive table for the Volume per $60x10^9$ platelets.

Volume per $60x10^9$ platelets (ml)	Sample size	Mean	Std. Deviation	Std. Error
Conventional Method	150	70.78	12.577	1.027
Method I	5	95.47	11.085	4.957
Method II	5	91.90	15.682	7.013
Method III	5	53.83	2.713	1.213
Method IV	5	55.95	3.296	1.474

is $2.35x10^{11}$/L. The standard deviation of this mean is 0.249 and the standard error is 0.111. In Method II, "Pooling with four Buffy Coats using deconstructed pooling kit", the mean count is less than the previous method ($2.27x10^{11}$/L). However, the standard deviation is greater than Method I (0.336) and has the largest standard error (0.150). The platelet count average of the "Pooling with eight Buffy Coats" method is $2.72x10^{11}$/L. The standard deviation of this sample mean is 0.142 and the standard error is 0.063. "Pooling with six Buffy Coats" produced the lowest platelet count mean; that of $2.01x10^{11}$/L. The standard deviation and standard error are also the lowest (0.008 and 0.004 respectively). Using the Tukey Post Hoc test, the mean platelet count varied significantly when comparing the "Pooling of platelets with five Buffy Coats" method with the "Pooling with six Buffy coats" method. The p-value obtained was 0.026; thus, the null hypothesis was rejected. The platelet counts of all the other pooling methods performed were not significantly different from each other as they obtained a p-value of more than 0.05. This means that the null hypothesis (stating that the mean platelet count varies marginally between the two methods) was accepted for the rest of the methods.

Table 3: Tukey Post Hoc Test for the platelet count per unit.

Method	Compared with Method	Mean Difference	Std. Error	P-value
Conventional Method	Method I	0.218	0.189	0.778
	Method II	0.302	0.189	0.501
	Method III	-0.150	0.189	0.932
	Method IV	0.565	0.189	**0.026**
Method I	Conventional Method	-0.218	0.189	0.778
	Method II	0.084	0.263	0.998
	Method III	-0.368	0.263	0.629
	Method IV	0.347	0.263	0.678
Method II	Conventional Method	-0.302	0.189	0.501
	Method I	-0.084	0.263	0.998
	Method III	-0.451	0.263	0.426
	Method IV	0.263	0.263	0.854
Method III	Conventional Method	0.150	0.189	0.932
	Method I	0.368	0.263	0.629
	Method II	0.451	0.263	0.426
	Method IV	0.715	0.263	0.055
Method IV	Conventional Method	-0.565	0.189	**0.026**
	Method I	-0.347	0.263	0.678
	Method II	-0.263	0.263	0.854
	Method III	-0.715	0.263	0.055

Table 4: Tukey Post Hoc Test for the Volume per $60x10^9$ of platelets.

Method	Compared with Method	Mean Difference	Std. Error	P-value
Conventional Method	Method I	-24.689	5.609	**0.000**
	Method II	-21.127	5.609	**0.002**
	Method III	16.943	5.609	**0.024**
	Method IV	14.827	5.609	0.067
Method I	Conventional Method	24.689	5.609	**0.000**
	Method II	3.562	7.803	0.991
	Method III	41.632	7.803	**0.000**
	Method IV	39.516	7.803	**0.000**
Method II	Conventional Method	21.127	5.609	**0.002**
	Method I	-3.562	7.803	0.991
	Method III	38.070	7.803	**0.000**
	Method IV	35.954	7.803	**0.000**
Method III	Conventional Method	-16.943	5.609	**0.024**
	Method I	-41.632	7.803	**0.000**
	Method II	-38.070	7.803	**0.000**
	Method IV	-2.116	7.8031	0.999
Method IV	Conventional Method	-14.827	5.609	0.067
	Method I	-39.516	7.803	**0.000**
	Method II	-35.954	7.803	**0.000**
	Method III	2.116	7.803	0.999

Table 5: Rate of Platelet Recovery (%) for the new protocols.

Trial number	Method I	Method II	Method III	Method IV
1	67	91	71	60
2	58	39	75	78
3	61	52	68	71
4	62	62	70	88
5	66	60	81	79
Average (%)	62.8	60.8	73	75.2

Table 6: Leucocyte count (x10^6/unit).

Trial number	Method III	Method IV
1	0.00	0.00
2	0.009	0.00
3	0.00	0.00
4	0.01	0.00
5	0.00	0.00

The Volume per 60x10^9 of platelets is another essential parameter of RP used to determine whether the sample is fit for therapeutic use. The mean volume of the conventional pooling method is 70.78 per 60x10^9 of platelets, and the standard deviation is 12.577. The standard error of the conventional method is the lowest of all techniques (1.027). Method I, "Pooling with four Buffy Coats", has the greatest mean (95.47 per 60x10^9 of platelets). The standard deviation and standard error of this method are 11.085 and 4.957 respectively. The mean of Method II is that of 91.90 per 60 x10^9 of platelets. The standard deviation and standard error of this method are the largest (15.682 and 7.013 respectively). The method of "Pooling with eight Buffy Coats" obtained the lowest mean (53.83 per 60 x10^9 of platelets). The standard deviation is also the lowest (2.713) and has a standard error of 1.213. The final method, "Pooling with six Buffy Coats", produced an average of 55.95 per 60x10^9 of platelets. The standard deviation is 3.296, while the standard error of Method IV is 1.474. The final volume of the RP per 60x10^9 of platelets was also compared between each method. When comparing the mean volume of the conventional method with Method I, the mean value varied significantly (p-value of 0.000). The probability value of the conventional method, when comparing its mean final volume with that of Method II and Method III, was also significant (p-value of 0.002 and 0.024 respectively). Furthermore, when comparing Method I with Method III and Method IV, the mean volume also varied significantly (both p-values obtained are 0.000). The mean volume obtained from Method II, varied significantly when compared Method III and Method IV (again, both obtained a p-value of 0.000). When comparing the mean volume of the rest of the methods to each other, the null hypothesis (that states that the mean volume varies marginally between the two methods tested), was accepted as the 0.05 level of significance was exceeded. However, when comparing the mean volume of the conventional method with Method IV, the p-value obtained was 0.067; this is considered borderline in statistic terminology.

The CBCs taken as part of an internal quality control check helped ensure whether platelets were being lost during the procedures. A percentage recovery rate (Table 5) for the platelet yield was calculated for all four alternative methods.

Leucocyte testing (Table 6) demonstrated that the integrity of the filter was maintained when filtering pools produced from eight and six BC.

Discussion

Platelet transfusions are required to treat or to prevent bleeding in patients who are severely thrombocytopenic or have a qualitative platelet disorder [4]. At NBTS, RP are prepared by pooling together five BCs obtained from the processing of whole-blood units. A major problem that all Blood Establishments encounter are the days when there is a low blood donation turnout. This puts a strain on meeting the Hospitals' blood component requests. A reason for this low blood donor turnout is the rigorous protocol required for the RP preparation; this further affects their production rate. Currently, five regular donors of the same ABO blood group, who have donated within a maximum of 24 hours and are screened negative for viral markers, are required to produce one RP.

In this study, the issue of not having enough BCs to produce RP was tackled by modifying the pooling protocol. Essentially, the aim of this project was to provide the basis for the validation of a new, improved and efficient procedure for producing RPs that are fit for therapy.

In this project, four different pooling methods were designed. Two of these techniques consisted of pooling four BCs, another method used eight BCs, and the final method consisted of pooling six BCs. Optimizing the pooling methods required various modifications and adjustments. One of the hurdles faced during this study was attaining a platelet count above the necessary threshold. The first few trials of pooling with four BCs did not yield satisfactory results by the final platelet count per unit falling below the European Directorate for the Quality of Medicines & HealthCare [5] requirements. Appropriate measures were then taken to increase the platelet yield. One of the adjustments made was to increase the PAS volume to 300ml instead of using the standard 200ml. This was done to compensate for the lack of volume resulting from the reduced BC number. The added volume allowed the platelets to be pushed past the filter during the separation stage. Another alteration that was made during optimizations was the way the pooled units were packed in the centrifuge sleeve. Since less BCs were used and more PAS was added, the unit's density varied from that of a conventional pool. When initially centrifuged, a high number of platelets became entrapped within the red cell layer. Since the separator automatically seals the tubing when red cells are detected, most of the platelets were not being collected, resulting in a low platelet count. This was solved via a simple addition of a support (Figure 2) to the insert prior to centrifugation, to maintain a steady position for the unit during the spinning process creating a discrete zone of platelets. This adjustment was introduced for Methods I and II i.e. where four BCs were used. Statistical analysis was carried out to assess which of the four methods executed in this study were the most successful and whether any of the techniques exceed the conventional pooling method in terms of performance and efficiency. The platelet count (x10^{11}/L) and the volume per 60x10^9 of platelets (ml) were the two essential quality parameters assessed in this study These two fundamental in vitro quality parameters are evaluated to ensure a unit is fit for use. In the Method III, 100ml of PAS was initially added; this was done as the maximum volume the pooling bag can take is 600ml.

Following centrifugation and separation of the platelets into the final bag, a further 200ml of PAS was added. If this step was avoided, the volume of the final pool would be insufficient (that is, below 40ml per $60x10^9$ of platelets). With the addition of the 200ml of PAS, the platelet count soared above the 40ml per $60x10^9$ of platelets threshold required. Also, the final pool contains a total PAS volume of 300ml. This was vital in keeping the volume constant between the different methods to improve comparability. Past records at NBTS showed that the leucocyte filter is capable of filtering leucocytes derived from a five-BC pool, so the white cell count was investigated for Methods III and IV where the total BC number in a single unit exceeded the conventional five BCs. The mean platelet count of the methods were compared via the statistical Tukey Post Hoc test. According to the obtained results, the only two techniques that were significantly different with regards to the mean platelet count, were the conventional method and Method IV. This indicates that the conventional method generates a statistically superior platelet count than the other method. When the mean platelet count of all the other methods were compared together, they all achieved a p-value greater than the 0.05 criterion, thus accepting the null hypothesis that states that the mean platelet count varies marginally between the two methods. However, when comparing the mean platelet count of Method III with Method IV, the p-value obtained was 0.055. Despite this value being above the required threshold, it is still considered borderline. This indicates that the mean platelet count of Method III is larger than that of Method IV, but the difference is not yet significant. For this to be resolved and to obtain a more unambiguous result, a larger sample size is required [6]. This is because the One-Way ANOVA test considers the sample size. Therefore, other factors must be analysed to determine which of the techniques mentioned are the most favorable. The mean volume of the platelet concentrates prepared from the conventional method, Method I, Method II, Method III and Method IV were also compared together using the Tukey Post Hoc test. From the results obtained, all the platelet concentrates prepared using the different methods produced a volume of more than 40ml per $60x10^9$ platelets. Based on the p-value obtained, the null hypothesis, that states that the mean volume varies marginally between the two methods if the p-value exceeds the 0.05 level of significance, was accepted when comparing the conventional method with Method IV. However, a significant difference in the mean volume was expected when comparing the two. This is because products produced from the conventional method are composed of a total of five BCs, while those produced in the Method IV are ultimately aliquoted into two units, each containing an average of three BCs. The p-value obtained was 0.067, which despite being above the predetermined level of significance, is in fact considered as borderline. The reason for this equivocal result is that the trial was underpowered due to the limited number of pools which could have been produced. As previously mentioned, this can be overcome by increasing the sample size and, thus, the statistical power [6]. Collectively, the alterations to Method I are the use of one less BC, added PAS and a support for centrifugation. Method II differed from Method I due to the use of a deconstructed kit which was solely composed of a mother bag and final pooling bag, i.e. the leucocyte-reducing filter was by-passed. This modification was made to analyse whether there was any significant platelet loss during separation of platelets through the filter into the final bag. The final product was then filtered after separation was complete. The results provided evidence that no significant platelet loss occurred during separation when the filter was still attached to the pooling kit, as a p-value of 0.998 was obtained when comparing the mean platelet count of Method I with Method II. Method I was the preferred technique since the hypothesis that platelets are lost in the filter during separation was rejected; this is preferred since fewer adjustments are required to carry out this method. This is important as resources are utilized more efficiently and the pooled unit is made available in a shorter time.

In the study conducted by Chatterjee et al., the BCs were pooled together in a single unit of plasma via the train method. Certain aspects such as the quality control checks used in their study, may have improved the true value of our study. Chatterjee et al., took samples of the same platelet concentrate on the first, third and final day of storage, to measure the platelet count and observe any changes that occur [7]. This is ideal as it assures the blood establishment whether there was any significant change in the count and, thus, whether the unit is still fit for use. This quality monitoring is especially essential when the platelet counts are initially borderline, where the platelet concentrate may fail the quality control check on the third or fifth day of storage. Other quality control checks that could have been tested on the prepared products are the pH, glucose, and lactate levels. These parameters are assessed to ensure that the viability and metabolic function of the platelets were not compromised during storage [7]. In their project, Chatterjee et al. also investigated bacterial contamination throughout seven days of storage [7]. Despite the precautionary measures taken during donation and processing, platelet concentrates remain at an increased risk of transmitting microbial infections due to the required storage conditions that favor bacterial growth [8].

The steps required to carry out the procedures of Method III and Method IV are very similar, and the latter was introduced to compare the two techniques. Both pooling methods provide a final RP that can be aliquoted, thus obtaining two units. However, it is obvious that since Method III utilizes a total of eight BCs, more BCs need to be made available. Therefore, Method IV offers an improved method with less BCs and resources while still producing a double dose. Unfortunately, despite obtaining a final platelet count of more than $2x10^{11}$/unit in all the five trials produced by the latter method, the final counts were all marginal and the mean platelet count was significantly different to the conventional method. Thus, from all the five methods being investigated, Method IV proved to be the least statistically successful technique. The results showed that Methods I and III were the most successful of the alternative techniques. When comparing the two procedures to each other, the mean final platelet count of Method I is $2.35x10^{11}$/L, while the mean of Method III is $2.72x10^{11}$/L. Nevertheless, the null hypothesis was accepted, as the values did not differ significantly from each other (p-value of 0.629). This suggested that other non-statistical aspects needed to be considered to determine which of the two methods is better. The average platelet recovery rate of Method III (73%) is better than that of Method I (62.8%). This indicates that less platelets are lost in Method III. However, when pooling with eight BCs, more effort is required to recruit eight ABO-matched donors in a single setting of 24 hours. Obtaining four ABO-specific BCs is much more feasible. Furthermore, the potential recipient would be exposed to antigens from eight different donors, thus increasing the risk of transfusion

related reactions including, but not limited to, allergic reactions and alloimmunization [9]. Another drawback is that exposure to eight donors doubles the risk of Transfusion Transmitted Infections (TTIs) when compared to exposing a recipient to four donors [4]. One of the main concerns when pooling with eight BCs was the possibility of rupturing the leuco-reduction filter caused by the increased number of leucocytes. The integrity of the filter was examined by measuring the white blood cell count in the final bag using the flow cytometry. The leucocyte count was also checked in Method IV, since a total number of six BCs were pooled together. The results obtained demonstrated that none of the final pool trials contained a leucocyte concentration of more than the $1x10^6$/unit limit. Thus, this provided evidence that the leuco-reduction filter remained intact despite the increased volume of cells. All in all, the disadvantages posed by Method III outweigh the disadvantages of Method I. Therefore, despite the overall superior performance of Method IV, Method I appears to be a more apt and practical technique. Between the four modified methods, Method I was deemed the best pooling method and thus, was compared to the conventional pooling method in which five BCs are used. The mean platelet count per unit obtained when using the conventional method ($2.57x10^{11}$/L) was greater than that obtained from Method I ($2.35x10^{11}$/L); however, statistically, this variation was described as marginal (p-value of 0.778). Hence, other characteristics were taken into consideration to determine whether Method I has a definite edge over the conventional method of five BCs. There are several advantages of using four BCs for pooling, rather than five. The main goal of this study is to cope with the increased demands for RPs. Therefore, this optimized alternative method provides an easier way of obtaining BCs that are of the same ABO blood group within their short 24-hour shelf life period from collection time. Since there is an improved chance of selecting the BCs in such a limited period, it also reduces BC wastage. By reducing the BC number, one is essentially reducing the exposure of antigens present in the final blood product to the potential recipient. This lessens the risk of transfusion-related reactions as well as of TTIs [3,9]. From the economical aspect, Method I has a positive effect on the conservation of resources. However, Method I still necessitates further evaluation to ensure that the platelet count obtained is always above the desired threshold of $2x10^{11}$/unit. Additionally, viability tests need to be performed to ensure that platelets maintain their correct function.

Conclusion

Overall, considering the obstacles and limitations of this project, this study was a successful one with the obtained satisfactory and expected results. Method I, proved to be best of the four methods carried out and is possibly more advantageous than the current pooling procedure. However, more work is required to solidify this statement and to validate a system that allows for pooling of platelets using four BCs. Nevertheless, this pilot study has achieved the desired information, which may eventually lead to a more in-depth study and consequent validation of a four BC pooled platelet product.

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