International Blood Research & Reviews

Volume 14, Issue 4, Page 57-69, 2023; Article no.IBRR.104462
ISSN: 2321–7219

Platelet Storage: Time to Rethink the Cold

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/IBRR/2023/v14i4318

Open Peer Review History:
This journal follows the Advanced Open Peer Review policy. Identity of the Reviewers, Editor(s) and additional Reviewers, peer review comments, different versions of the manuscript, comments of the editors, etc are available here: https://www.sdiarticle5.com/review-history/104462

Original Research Article

Received: 12/06/2023
Accepted: 16/08/2023
Published: 24/08/2023

ABSTRACT

Platelet concentrates (PCs) are widely used in transfusion medicine for therapeutic purposes and their demand is constantly on the rise. Current storage regulations make this product highly susceptible to bacterial contaminations and platelet storage lesions (PSL) causing the need for alternative storage methods to be considered. The implementation of cold storage not only reduces unnecessary wastage of valuable donations and overall costs but also decreases both the risk of bacterial contamination and the occurrence of PSL. The current study aimed at determining how a prolonged cold storage may affect PCs. This was accomplished by investigating two different PC cohorts of 10 units each. One of the cohorts, labelled as 'Room Temperature', was stored at 22℃±2℃ for 5 days and then transferred to a temperature of 4℃±2℃. The other cohort, labelled as 'Cold', was stored directly at 4℃±2℃. Both cohorts were stored for a total of 21 days and platelet indices, platelet counts, pH, and platelet factor IV (PF4) were measured at different time intervals. Sterility was performed on Day 21. The key findings showed no significant difference in mean platelet count, platelet distribution width (PDW), mean platelet volume (MPV), platelet-large cell ratio (P-LCR), and plateletcrit (PCT) between the two cohorts. On the other hand, a significant difference in mean pH and PF4 resulted between the two cohorts. Moreover, no significant

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difference in mean platelet count, and PCT was found between Days 1, 5, or 10, and Day 21 in both cohorts. However, an overall significant difference in mean PDW, MPV, P-LCR and pH was discovered between Days 1 and 21, Days 5 and 21, and Days 10 and 21 in both cohorts. Regards PF4, a significant difference was detected between Days 1 and 21, and Days 10 and 21; however, no significant difference was found between Days 5 and 21 in both cohorts. *Corynebacterium freneyi* and *Microbacterium liquefaciens*, were cultured from 1 unit of the room temperature cohort after it was flagged positive during the sterility testing. In conclusion, through the implementation of a delayed cold storage system, PCs can be safely administered to the patient.

Keywords: Platelet concentrates; cold storage; prolonged shelf-life; quality; safety.

1. INTRODUCTION

Platelet concentrate (PC) units are currently stored under continual agitation, inside sterile gas- permeable bags and surfaces at a temperature of 22℃±2℃, in accordance with the European Directorate for the Quality of Medicines & HealthCare (EDQM) guidelines[1]. Under the specified storing conditions units must be stored for a maximum shelf-life of 5 days; although this may be extended to 7 days if adequate pathogen detection techniques are implemented. Such restricted shelf-life could be attributed to the fact that conventional platelet storage presents with two major drawbacks; an elevated risk of pathogen contamination and the occurrence of platelet storage lesions (PSL). Such limitations will consequently lead to an increased likelihood of life-threatening infections, as well as the delivery of an inadequate therapeutic effect *in vivo* [2]. Furthermore, a short shelf-life will bring about an array of issues, which comprise of an increased rate of unnecessary wastage of units, a decrease in availability, an intensified sense of pressure to keep up with high demands and enhanced financial losses. In hopes of prolonging the PC's shelf-life, multiple studies have recognized cold storage as an alternative approach, whereby units are stored at a temperature of 4℃±2℃. Cold storage was first introduced in 1970 and although this method of storing platelets was proven to be beneficial, it was discontinued as upon transfusion, platelets were cleared faster *in vivo* when compared to room temperature platelets, thus limiting the therapeutic effect of the platelets in the recipient[3]. The advances made over the years in the field of transfusion science have opened the possibility of reconsidering this type of storage. Since both the risk of pathogen contamination and the occurrence of PSL are diminished at low temperatures, cold storage could potentially be beneficial in the extension of PC shelf-life; hence, preventing the acknowledged related issues of a limited storage life. In fact, cold-stored platelets are known to be viable for up to 21 days, and bacterial proliferation is limited at such low temperatures [4]. Furthermore, metabolism is greatly slowed down in cold platelets [5]. The main disadvantage of such alternative approach to platelet storage still remains a short life-span post- transfusion. In view of this quality assessments are being conducted to better examine the *In vitro* quality of the platelets prior to transfusion.

2. MATERIALS AND METHODS

The aim of our study was to establish whether cold storage preserves, weakens, or enhances platelet morphology and function when compared to conventional storage. Furthermore, the objective was to determine how platelets are influenced under lower temperatures over a 21-day storage period. The research question was addressed through the collection and monitoring of 20 PC units, which were divided into two cohorts consisting of 10 PC units each. The Group 1 cohort which consisted of 10 PC units (Room Temperature) were stored inside a platelet incubator/ agitator (KW Apparecchi Scentifici, Italy) at a temperature of 22 ± 2℃ for the initial 5 days. After five days, the units were transferred to a fridge at a temperature of 4 ± 2 ℃ (Fiocchetti The Cold Manufacturer, Italy), for the remaining 16 days. The second cohort (Group 2) also consisting of 10 PC units were stored directly at a temperature of 4 ± 2 ℃ by placing the units inside a fridge, for a total of 21 days. As per EDQM guidelines, both cohorts were stored inside sterile gas-permeable bags under constant agitation (EDQM, 2020). Each cohort was tested on Day 1, Day 5, Day 10, and Day 21 for the following platelet parameters: the platelet count, platelet indices, pH level and platelet factor 4 (PF4) concentration. On Day 21 sterility testing was also performed on all units. To note that Day 1 is considered to be the day of pooling.

2.1 Preparation of Pooled Platelets

Therapeutic doses of platelets were prepared by compiling five ABO-specific buffy coats (BC) together with SSP+ platelet additive solution (PAS) (Macopharma, ATC Code: V07AC). Pooling was performed through the utilization of a Terumo sterile connecting device (TSCD) sterile tubing welder (Terumo, Tokyo, Japan). Next, the prepared platelet units were centrifuged (Andreas Hettich GmbH & Co. KG, Germany) using a soft spin, allowing the separation of platelets from erythrocytes and leukocytes. Separation ensued through the utilization of a Compomat G5 Plus automatic separator machine (Fresenius Kabi, Bad Homburg). The end product was left standing for one hour before being placed on an agitator at 22 ±2℃ or refrigerated at 4 ±2℃; depending on the unit's assigned cohort.

2.2 Sampling for Analysis

Cold-stored PC units were left on an agitator at a temperature of 22℃ for 1 hour prior to testing in order to prevent cell rupture upon handling and ensure that any formed aggregates were dissociated. As to preclude contamination of the main mother bag, PC units were sampled using sample transfer bags (Fresenius Kabi Cat No.: R6R2023) which were connected to the mother bag through the utilization of the TSCD Sterile Tubing Welder (Terumo, Tokyo, Japan). Each unit was mixed for a total of thirty times by gently rotating and inverting the bag back and forth, as to ensure homogeneity. Approximately 8-10 millilitres of the PC was allowed to flow into the sample transfer bag. The sample transfer bag was finally detached from the mother bag through the utilization of a sealing device (Delcon, Italy), and the created seal was carefully cut using a pair of scissors. The PC unit was weighed on an electronic balance scale (Precisa Gravimetrics AG, Switzerland), and stored back at its corresponding temperature.

2.3 pH Level

The pH level of the PC unit was measured on Days 1, 5, 10 and 21 using a blood gas analyser (Radiometer, Copenhagen, Denmark).

2.4 Platelet Count and Platelet Indices

The platelet count and platelet indices measurements were carried out on Days 1, 5, 10 and 21 through the utilization of the Sysmex XN-550 automated analyser (Sysmex Corporation, Kobe, Japan). Measured platelet indices comprised of the platelet distribution width (PDW), mean platelet volume (MPV), platelet large cell ratio (P-LCR) and plateletcrit (PCT).

2.5 PF4 Concentration

Platelet activity was determined by measuring the PC's PF4 concentrations. Analysis was carried out through the use of a sandwich enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Minneapolis, Cat No: DY795). Reagents and standards were prepared according to the provided Certificate of Analysis (CoA), and all prepared reagents were brought to room temperature before use. The plate preparation and ELISA procedure was carried out as per manufacturer's instructions. Optical densities (ODs) obtained were measured using a microplate reader (Berthold Technologies, Mithras LB 940). A calibration curve was created using the OD values obtained for the standards and the PC's PF4 concentration was found through extrapolation.

2.6 Sterility Testing

On Day 21, a pair of aerobic BacT/ ALERT BPA (BioMérieux, Cat No.: 279044) and anaerobic BacT/ ALERT BPN (BioMérieux, Cat No.:279045) were inoculated for each PC unit. The inoculation process was conducted under strictly aseptic conditions to prevent contamination of the PC units and false positive results. Aseptic conditions were ensured through the usage of N95 medical face masks (Bi-Jie, Cat No., EN14683: 2019) and nitrile gloves which were disinfected with 70% isopropyl alcohol. Sterility was further ensured as inoculation took place inside an alcohol-wiped Microflow Horizontal Laminar Flow Cabinet (Bioquell, UK).

Culture bottles which were flagged as positive were followed up through subculturing on a pair of Columbia agar (BioMérieux, Cat No.: 43050) with the addition of 5% horse blood (COH) plates; one incubated aerobically and the other anaerobically at a temperature of 30-35℃. Once a pure colony growth was obtained from the subculture, the plate was sealed and sent to an external laboratory for identification.

2.7 Data Analysis and Statistical Tests

Data generated was analysed using the IBM SPSS Statistics 27.0 statistical software (IBM:

New York). A two-way ANOVA regression model was computed for each analysed platelet parameter and plot graphs were generated.

3. RESULTS

A two-way ANOVA was used to establish how the average of a quantitative variable differs along two qualitative variables, or predictors. In this study, the two predictors comprised of the sample cohort, as either 'cold' or 'room temperature', and the day on which the data was collected; as either 'Day 1', 'Day 5', 'Day 10' or 'Day 21'.

3.1 Platelet Count

It was established that there was no significant difference (*p-value* >0.05) in the mean platelet count between the cold and room temperature cohort, as well as no significant difference (*p-value* >0.05) in the mean platelet count between Days 1 and 21, Days 5 and 21, and Days 10 and 21. Moreover, an increase in mean platelet count was detected upon transferring room temperature-stored PCs from a temperature of 22± 2℃ to that of 4±2℃ on Day 5. A gradual decrease in mean platelet count was observed after Day 10, for both cohorts (Fig. 1).

3.2 Platelet Indices

Although no significant difference (*p-value* >0.05) in mean PCT was discovered between Days 1 and 21, Days 5 and 21, and Days 10 and 21, an overall significant increase (*p-value <0.05)* in mean PDW, MPV and P-LCR was seen along the 21-day storage (Figs. 2–5). Furthermore, although for most days the cold cohort presented with a mean PDW, MPV and PLCR values higher than that for the room temperature cohort, no significant difference (*p- value* >0.05) in mean platelet indices was found between the two cohorts.

3.3 pH Level

Although both cohorts followed a similar trend (Fig. 6), cold-stored platelets acquired a significantly lower (*p-value <0.05*) mean pH level than that of room temperature platelets. Nonetheless, all stored PCs possessed a pH value which lay within the recommended range, established by the EDQM guidelines, of greater than 6.4. Moreover, an overall significant difference (*p-value <0.05*) in mean pH was seen along the 21-day storage period.

3.4 PF4 Concentration

Although both cohorts exhibited a similar trend (Fig. 7), cold-stored platelets possessed a significantly lower (*p-value <0.05*) mean PF4 concentration than that for room temperature stored platelets. Furthermore, for both cohorts a significant difference (*p-value <0.05*) in mean PF4 concentration was detected between Days 1 and 21, and Days 10 and 21; however, no significant difference (*p-value* >0.05) was found between Days 5 and 21.

3.5 Sterility Testing

Sterility testing revealed that all stored PC units resulted to be negative after 21 days of storage, apart from one sample. The BPN culture bottle inoculated with Sample 10 from the room temperature cohort was flagged as positive after approximately 4 days of incubation. The corresponding BPA culture bottle, however resulted to be negative. Upon further sub-culturing of the positive sample on a pair of COH plates stored either under aerobic or anaerobic conditions, identification revealed the isolation of *Corynebacterium freneyi* and *Microbacterium liquefaciens.*

The cold cohort possessed an overall higher mean platelet count, as demonstrated in Fig. 1, however, no significant difference was detected between the two cohorts. An increase in mean platelet count was observed upon transferring room temperature- stored PCs from a temperature of 22± 2℃ to that of 4± 2℃ on Day 5. A gradual decrease in mean platelet count was observed after Day 10, for both cohorts.

Although for most days the cold cohort presented with a mean PDW value higher than that for the room temperature cohort, as shown in Fig. 2, no significant difference was detected between the two cohorts. For both cohorts, a gradual increase in mean PDW was seen throughout the 21- day storage, which was found to be significant.

Although for most days the cold cohort presented with a mean MPV value higher than that for the room temperature cohort, as shown in Fig. 3, no significant difference was detected between the two cohorts. For both cohorts, a gradual increase in mean MPV was seen throughout the 21- day storage, which was found to be significant.

Although for most days the cold cohort presented with a mean PLCR value higher than that for the room temperature cohort, as shown in Fig. 4, no significant difference was detected between the two cohorts. For both cohorts, a gradual increase in mean PLCR was seen throughout the 21- day storage, whichwas found to be significant.

The cold cohort possessed an overall higher mean PCT, as demonstrated in Fig. 5, however, no significant difference was detected between the two cohorts. Moreover, some variation in PCT values was observed throughout the 21-day storage for both cohorts, which was deemed as insignificant.

Both cohorts followed a similar trend as the average pH demonstrated a slight increase from Day 1 till Day 5, followed by a slight decline from Day 5 till Day 10, and a steep decline from Day 10 till Day 21. Nonetheless, a significant difference in mean pH was found between the two cohorts. Furthermore, the variation in pH throughout the 21- day storage was deemed as significant.

Both cohorts exhibited a similar trend as the average PF4 concentration demonstrated an increase from Day 1 till Day 5, followed by a decline from Day 5 till Day 10, and another increase from Day 10 till Day 21. Nonetheless, the cold cohort possessed a significantly lower mean PF4 concentration than that acquired by the room temperature cohort.

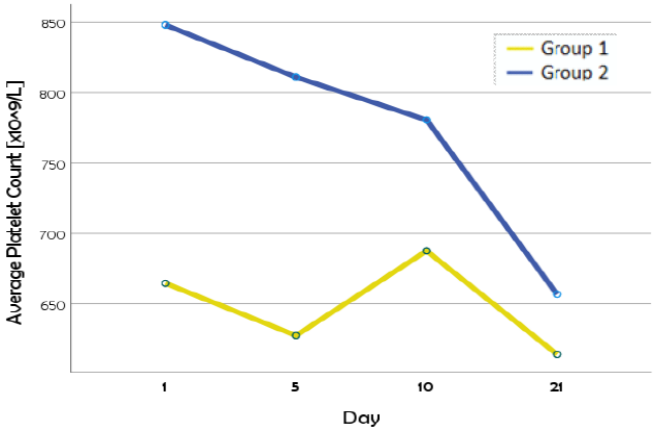

Fig. 1. A plot graph of average platelet count [x109/L] against day

The cold cohort possessed an overall higher mean platelet count, as demonstrated in figure 1, however, no significant difference was detected between the two cohorts. An increase in mean platelet count was observed upon transferring room temperature- stored PCs from a temperature of 22± 2℃ to that of 4± 2℃ on Day 5. A gradual decrease in mean platelet count was observed after Day 10, for both cohorts

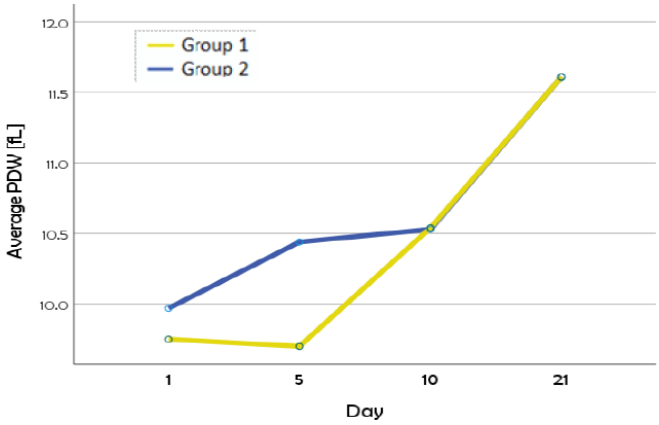

Fig. 2. A plot graph of average MPV [fL] against day

Although for most days the cold cohort presented with a mean PDW value higher than that for the room temperature cohort, as shown in figure 2, no significant difference was detected between the two cohorts. For both cohorts, a gradual increase in mean PDW was seen throughout the 21- day storage, which was found to be significant

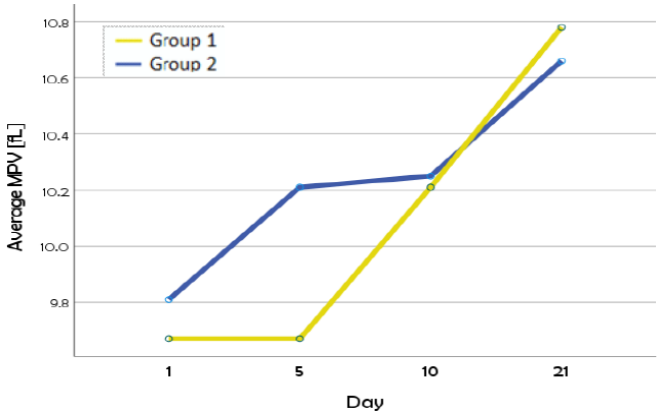

Fig. 3. A plot graph of average MPV [fL] against day

Although for most days the cold cohort presented with a mean MPV value higher than that for the room temperature cohort, as shown in figure 3, no significant difference was detected between the two cohorts. For both cohorts, a gradual increase in mean MPV was seen throughout the 21- day storage, which was found to be significant.

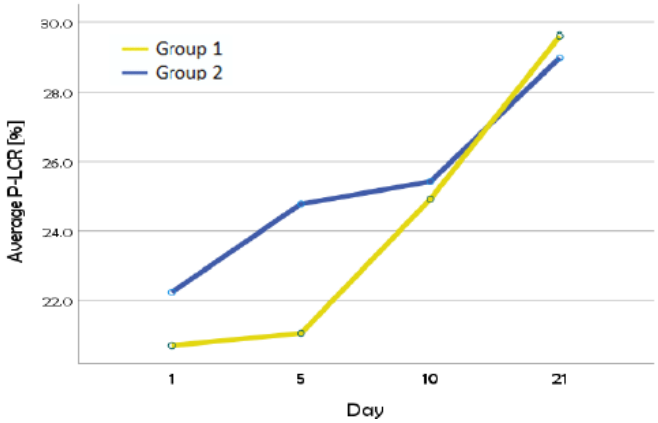

Fig. 4. A plot graph of average PLCR [%] against day

Although for most days the cold cohort presented with a mean PLCR value higher than that for the room temperature cohort, as shown in figure 4, no significant difference was detected between the two cohorts. For both cohorts, a gradual increase in mean PLCR was seen throughout the 21- day storage, which was found to be significant.

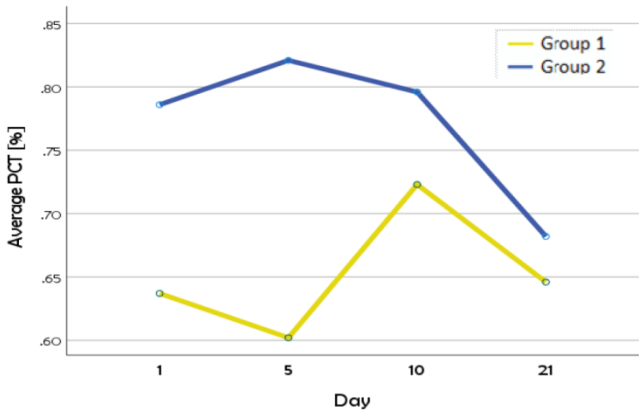

Fig. 5. A plot graph of average PCT [%] against day

The cold cohort possessed an overall higher mean PCT, as demonstrated in figure 5, however, no significant difference was detected between the two cohorts. Moreover, some variation in PCT values was observed throughout the 21- day storage for both cohorts, which was deemed as insignificant.

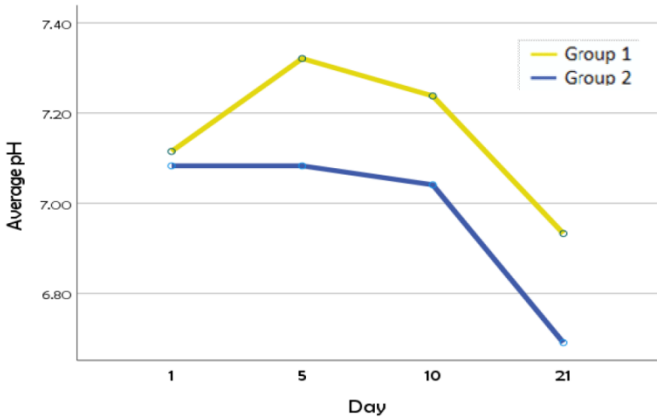

Fig. 6. A plot graph of average pH against day

Both cohorts followed a similar trend as the average pH demonstrated a slight increase from Day 1 till Day 5, followed by a slight decline from Day 5 till Day 10, and a steep decline from Day 10 till Day 21. Nonetheless, a significant difference in mean pH was found between the two cohorts. Furthermore, the variation in pH throughout the 21- day storage was deemed as significant.

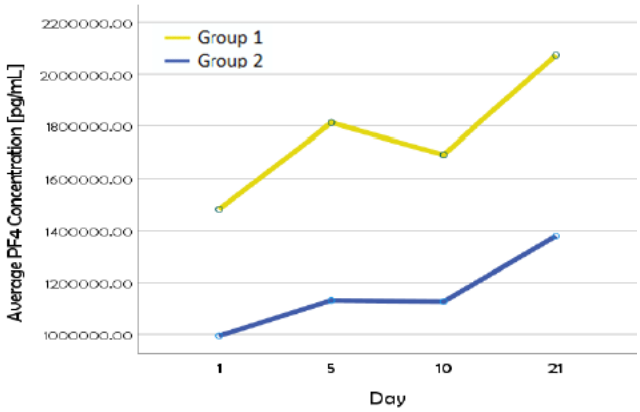

Fig. 7. A plot graph of average PF4 concentration [pg/mL] against day

Both cohorts exhibited a similar trend as the average PF4 concentration demonstrated an increase from Day 1 till Day 5, followed by a decline from Day 5 till Day 10, and another increase from Day 10 till Day 21. Nonetheless, the cold cohort possessed a significantly lower mean PF4 concentration than that acquired by the room temperature cohort.

4. DISCUSSION

Cold storage can serve as an alternative approach allowing for the extension of PC shelf-life since under low temperatures, both the risk of pathogen contamination and the occurrence of PSL are reduced. Extending the unit's shelf-life will, consequently, decrease the rate of unnecessary wastage of units, increase PC availability, allow one to keep up with high demandsand prevent financial losses.

Platelet morphology and function were investigated through the measurement of platelet indices, pH levels and PF4 concentration, whilst the occurrence of contaminating microorganisms in the PC units was analysed through sterility testing at the end of storage. The key findings gathered from this study are summarized as follows. No significant difference in mean platelet count, PDW, MPV, P-LCR, and PCT was found between the cold and room temperature cohorts. On the other hand, a significant difference in mean pH and PF4 concentration was discovered between the two cohorts. Moreover, for both cohorts, no significant difference in mean platelet count, and PCT was found between Days 1, 5, or 10, and Day 21. On the contrary, for both cohorts, an overall significant difference in mean PDW, MPV, P-LCR and pH was discovered between Days 1 and 21, Days 5 and 21, and Days 10 and 21. In the case of PF4 concentration, a significant difference was detected between Days 1 and 21, and Days 10

and 21; however, no significant difference was found between Days 5 and 21 for both cohorts. Sterility testing established that the anaerobic BacT/ ALERT BPN bottle inoculated with Sample 10 from the room temperature cohort was flagged as positive. Further sub-culturing and identification revealed the isolation of *Corynebacterium freneyi* and *Microbacterium liquefaciens*.

The quantity of thrombocytes in the sample was indicated by the platelet count. The ability of PC units to provide donors with the needed therapeutic effect increases with platelet count. These findings demonstrated that, although not significant, PC units present a slightly enhanced platelet count under lower temperatures. A study conducted by Braathen et al. [6] revealed similar findings since room temperature-stored PC units which had been relocated to lower temperatures after 7 days of storage resulted to have a temporarily stabilized platelet count. Upon further research, however, some contradicting findings were discovered from additional studies. Two studies conducted by Reddoch et al. [3] and Hedge et al. [7] reported that platelet counts were significantly lower in cold-stored PC units as compared to room temperature units. It was suggested that such reduction was attributed to the use of smaller bags [3]. Since smaller bags possess a higher surface-area-to-volume ratio, platelets will acquire a higher chance of sticking onto the inner-surfaces of the bag, hence, leading to the presence of a reduced amount of thrombocytes within the collected sample. Furthermore, in our study, a gradual decrease in platelet count was observed for both cohorts after Day 10, implying that the quantity of thrombocytes within a sample will start to deplete after 10 days of storage, irrespective whether units are stored directly at a temperature of 4℃±2℃ or if they are stored at a temperature of 4℃±2℃ after a 5-day storage at 22℃±2℃.

The size and morphology of the platelets was assessed through PDW, MPV and P-LCR values. PDW determines the degree of variability in platelet size, or platelet anisocytosis, whilst MPV represents the mean size, or volume of the platelets [8]. Both values ultimately indicate platelet function and activation [9]. P-LCR is a parameter which indicates the percentage amount of platelets within the sample which surpass the normal platelet volume of approximately 12 femtoliters (fL) [10]. Values will rise upon platelet activation as thrombocytes undergo swelling and pseudopodia formation [11]. The overall significant increase in mean PDW, MPV and P-LCR values along the 21-day storage implied that the stored platelets were starting to significantly vary and increase in size. Moreover, the fact that no significant difference in mean PDW, MPV and P-LCR was seen between the two cohorts, indicated that values are not significantly different between units which are stored directly at a temperature of 4℃±2℃ and units which are stored at a temperature of 4℃±2℃ after a 5-day storage at 22℃±2℃.

Further studies conducted by Baghdadi et al. [12] discovered that cold-stored platelets at 4℃ were found to have higher PDW and MPV values than room temperature-stored platelets at 22℃. Such an outcome is linked to the occurrence of platelet deformation under low temperatures. It has been reported that cold-stored platelets most often undergo reversible morphological changes during storage whereby their shape is altered from discoid to spherical, characteristic of activated platelets [12–14]. Furthermore, such transformation consequently causes swirling to be imperceptible since light fails to be scattered off spherical platelets as it does off normal discoid platelets [6]. Cold-induced platelet shape distortions mainly occur through a process known as actin polymerization which is characterized by the fragmentation of actin filaments followed by reassembling and an elongation step through the addition of actin sub-units [13,15]. Such rearrangement is thought to influence the platelet's lifespan in the recipient's circulation.

The percentage of blood which is occupied by thrombocytes is denoted by the PCT parameter [10]. In this study it was established that PCT values were relatively stable during storage since no significant variation was detected throughout the 21 days of storage. Moreover, since no significant difference in mean PCT was discovered between the two cohorts, it was determined that values are not significantly different between units which are stored directly at a temperature of 4℃±2℃ and units which are stored at a temperature of 4℃±2℃ after a 5-day storage at 22℃±2℃. Looking at the trend created by the cold cohort, PCT values seemed to increase up till Day 5, followed by a reduction thereafter. This finding was interestingly comparable to a similar outcome gathered by Sidorkevich et al. as such study also revealed that PCT values of platelets stored at 4℃±2℃ increased by the 5th day of storage, followed by a decrease.

PC quality is ensured by measuring the pH level which is an instrumental parameter utilized to determine the unit's acidity or basicity. As indicated by EDQM guidelines, pH levels of PC units should never fall below 6.4, since this may cause platelets to undergo irreversible morphological alterations which would ultimately affect their viability *in vivo* (1). Results obtained illustrated that for the first 5 days of storage, the mean pH level in cold-stored platelets was stable, whilst the mean pH level in room temperature-stored platelets increased (Fig. 6). Such an initial increase in pH was also evident in a study performed by Hornsey et al. [16], however, the mean pH level subsequently started to gradually decrease, as also shown in our study. Furthermore, Hornsey et al. [16] and Reddoch et al. [3] demonstrated how, eventually, glucose levels started to decrease whilst lactate levels started to increase indicating that platelets were undergoing metabolism as glucose was being broken down into pyruvic acid through glycolysis and converted into lactate through lactic acid fermentation. Since lactate is an acidic component, PC units will encounter a decline in pH. Such glycolysis reaction will be enhanced upon an alternation in platelet metabolism, which can be caused by the occurrence of PSL [17].

As also reported by Johnson et al. [18] and Braathen et al. [6], our study unexpectedly discovered that cold-stored platelets possessed an overall lower pH than room temperature platelets along the storing period, suggesting that platelet metabolism was not slowed down under lower temperatures. Such outcome could, however, be linked to the fact that the concentration of bicarbonate, which functions as a buffer to maintain a stable acid-base balance within PC units, was stable in room temperature platelets, and diminished in cold-stored platelets [5]. The suitability of the PAS which was used in this study is only validated for room temperature storage, but not for cold storage.

Contradicting results were discovered by Reddoch et al. [3] since it was found that although both cold-stored and room temperature platelets increased in glucose consumption and lactate production, lactate production in room temperature platelets was much higher than in cold- stored platelets. This, hence, implied that a slowdown in platelet metabolism did indeed occur under lower temperatures. Similarly, Johnson et al. [5] established that in the case of cryopreserved platelets that have been thawed and then stored at room temperature, possessed a significantly lower pH than thawed platelets stored at 2-6℃.

Nonetheless, all stored platelets in this study possessed a pH level which lay within the recommended value of >6.4 [1]. Therefore, pH levels suggest that platelets from either cohort would have been adequate for transfusion under the established guidelines.

It is important to note that pH levels may also vary through the presence of bacterial contamination and volume changes. pH levels are indirectly proportional to the amount of PC volume whereby the lower the volume, the greater the likelihood of pH variation. The fact that in this study an overall significant variation in pH was obtained along the 21 days of storage, could have been linked to the fact that PC volume was gradually decreasing throughout storage upon sampling. Furthermore, pH levels will decline in the event of bacterial contamination, as microorganisms will carry out metabolic reactions which produce CO_2 as by-product, which is acidic in nature. The sterility testing was, thus, used to confirm or deny the presence of bacterialcontamination as a cause of pH decline.

Sterility testing on PC samples indicated the lack of contaminating microorganisms in all PC samples, except for the tenth sample from the room temperature cohort. Whilst the aerobic BacT/ ALERT BPA culture bottle inoculated with such PC sample resulted to be negative, the corresponding anaerobic BacT/ ALERT BPN was flagged as positive, indicating the presence of anaerobic or facultative anaerobic microorganisms. Further sub-culturing on a pair of aerobic and anaerobic COH plates demonstrated the presence of small grey colonies on the anaerobic COH plate, and one big grey colony and one small grey colony on the aerobic COH plate. Further investigations revealed the isolation of two species: *Corynebacterium freneyi* and *Microbacterium liquefaciens*.

Corynebacterium freneyi are Gram-positive facultative anaerobes, which form part of the normal human skin flora, hence, making it a common skin contaminant [19,20]. The presenceof this species in the PC unit, thus, must have been introduced upon venepuncture during blood collection, due to a lack of proper blood donor arm disinfection [21]. *Microbacterium liquefaciens*; also known as *Aureobacterium*

liquefaciens, are aerobic, mesophilic, Gram-positive bacteria. *Microbacterium* species has been frequently found to be a nosocomial pathogen, affecting especially weak, immunocompromised patients [22]. *Microbacterium liquefaciens* colonies appear yellow or ivory in colour [23]. Since, one big ivory-coloured colony was noted on the aerobic COH plate in this study, the presence of such aerobic organism was further confirmed. Unlike *Corynebacterium freneyi*, since *Microbacterium liquefaciens* is not considered to be a skin commensal it is suspected that such contaminant was introduced during inoculation. It is important to note the fact that sterility testing was carried out after 21 days of storage, as well as that positivity was detected after an additional 38.4 hours of incubation under optimal growth conditions. This, hence, indicates that the microbial load was relatively low and that the positive PC unit originally contained very few colonies. It is probable that had sterility testing been performed at an earlier stage during storage, these contaminants would probably have not been detected.

Moreover, it is worth mentioning that no contaminating microorganisms were detected in PC units stored under low temperature. This could have been credited to the fact that most bacterial species and common human pathogens are mesophiles whose optimal growth temperatures range from 20℃ - 45℃ [24]. Therefore, at a temperature of 4℃, optimal growth conditions for mesophile species were no longer available, and hence, the risk of pathogen contaminationwas, in fact, reduced.

Platelet activity was assessed through the measurement of PF4 concentration. PF4 is a chemokine protein which possesses a key role in hemostasis as it is released out from the platelet's alpha granules during activation [25]. Hence, PF4 levels are directly proportional to the platelet's activity. Measured PF4 concentrations indicated that although both cohorts exhibited a similar trend, the cold cohort possessed significantly lower PF4 levels than the room temperature cohort, signifying that *in vitro* platelet activation is significantly lower in cold platelets than in room temperature platelets. Similarly, a study conducted by Winskel- Wood et al. [26] discovered that cold-stored platelets contained a lower concentration of PF4. In addition to this, such study also measured other components which are released upon platelet activation such as Regulated on Activation, Normal T-cell Expressed and Secreted (RANTES) and complement C3a protein. Similarly, to PF4, concentrations of both RANTES and C3a were also found to be significantly lower in cold-stored platelets. The discrepancy in PF4 levels between the two cohorts assessed may be credited to the fact that under conventional storage, PCs will undergo significant platelet activation during the first 5 days of storage [27].

Alongside pH levels and platelet counts, other platelet parameters which determine PC quality were measured including the weight, volume, platelet content per final unit [$x10^{11}$/L] and volume per 60 $x10^9$ of platelets [mL], whose calculations depend on the weight of the PC bag. In accordance with the EDQM guidelines, the volume per 60 $x10^9$ of platelets [mL] must be greater than 40 mL per 60 $x10^9$ of platelets, whilst the platelet content per final unit must be equal to or greater than 2 $x10^{11}$/L [1]. Although efforts were made to prevent major volume discrepancies, the weight of the PC units was constantly declining due to sampling. This decrease in weight would have had an impact on the quality requirements of the pooled platelets. Therefore, to mitigate this issue, the original weight in conjunction with the final platelet indices results were considered. Through this action, the obtained quality parameters in this study fell within the requirements established by the EDQM.

4.1 Limitations & Further Studies

The main limitations acknowledged in this study include the fact that testing was only limited to *in vitro* analysis. Hence, monitoring was not able to capture the broader picture of how the platelets function *in vivo*, and their clearance mechanisms upon transfusion. Furthermore, the sample size of the two cohorts assessed was limited to 10 PC units each – this was mainly due to the availability of the product. Additionally, one can never be completely certain that PC units possess no contamination, and that sterility testing is truly negative since the microbial load in the units may be very low, hence, diminishing the likelihood that a colony is sampled and inoculated. Thus, as a suggestion, even though blood culture bottles are the gold standard when it comes to sterility testing, one should consider other methods of detection for such cases. A particularly highly sensitive and specific method known as Nucleic Acid Test (NAT) includes nucleic acid amplification through real-time polymerase chain reaction (RT-PCR) technology [28].

Further studies should consider increasing sample size for the purposes of improving accuracy and enhancing the validity of the research study. Moreover, additional research may opt to look into more advanced investigations which were not carried out in this study. For instance, platelet function upon activation may be assessed through platelet aggregation studies. Furthermore, the stiffness of the clots formed may be evaluated through oscillatory tests.

Platelet metabolism may be more extensively investigated through the measurement of glucose and lactate concentrations, as well as through mitochondrial function tests. Platelet survival can be also explored through the measurement of apoptotic markers and implementation of *in vivo* studies to further assess clearance rates upon transfusion.

5. CONCLUSION

In conclusion, it was established that under low temperatures platelets undergo reversible morphological alterations which contribute to the lack of swirling and a short platelet lifespan within the recipient's circulation. Moreover, regardless of whether PC units are stored directly at a temperature of 4℃ ±2℃ or if they are stored at a temperature of 4℃ ±2℃ after a 5-day storage at 22℃ ±2℃, PC quality parameters are maintained within the corresponding set ranges as indicated by the EDQM guidelines, for the entirety of the 21-day storage period. Ultimately, conventional platelet storage at 22℃±2℃ will always remain a necessity for chronic thrombocytopenic patients, however, through the implementation of a delayed cold storage system, any unused room temperature-stored PCs which exceed the 5-day maximum shelf-life can be safely used in the case of acutely thrombocytopenic patients. Therefore, the use of cold-stored platelets has proven to be a beneficial alternative for emergency situations which will consequently ease inventory management, reduce wastage, enhance product availability and prevent major financial losses.

CONSENT

It is not applicable.

ETHICAL APPROVAL

University of Malta Faculty of Research Ethics Committee (FREC) approval number FHS- 2022-00051.

ACKNOWLEDGEMENTS

The Authors would like to thank the National Blood Transfusion Service Malta for sample collection and analysis, the Department of Applied Biomedical Science, Faculty of Health Sciences, University of Malta for the funds and the Allied Research Unit, Department of Clinical Pharmacology and Therapeutics, Faculty of Medicine and Surgery, University of Malta for providing access to the laboratories. Special thanks to Professor Liberato Camilleri from the Statistics & Operations Research, Faculty of Science, University of Malta for his help with the statistical analysis.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Peer-review history:
The peer review history for this paper can be accessed here:
https://www.sdiarticle5.com/review-history/104462