Original article

Assessment of platelet product quality by Wright's stain platelet smear

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Abstract:

Introduction: *Platelet has a discoid shape and changes its shape upon stimulation and activation. In the past, platelet shape can be visualized by the electron microscope or phase-contrast microscope. Currently, the image from a light microscope can be further magnified by a digital camera. We explored the utility of Wright's stain platelet smear examined under a light microscope to evaluate the extent of shape change and compared it with pH and swirling which are the current platelet product QC tools.* ***Materials and Methods:*** *We evaluated 72 platelet-rich plasma platelet (PRP-PC) products, 72 buffy coat derived platelets (BC-PC) products, and 72 apheresis platelet (AP-PC) products at day 3 of storage. We evaluated the proportion of discoid platelet from the Wright's stain platelet smear and compared it with pH and swirling score.* ***Results:*** *Wright's stain platelet smear enables visualization of platelet shape under a light microscope and can discriminate discoid-shaped platelets from other activated shapes. BC-PC had a higher proportion of discoid platelet compared with PRP-PC and AP-PC ($p < 0.05$). The proportion of discoid platelets and swirling score were significantly different between platelet products of different ranges of pH ($p < 0.05$). The reliability of the proportion of discoid platelet is good for both intra-observer ($r = 0.98$) and inter-observer ($r = 0.99$).* ***Conclusion:*** *Wright's stain platelet smear visualized under a light microscope is simple, affordable, and requires limited resources to visualize platelet shape change. The proportion of discoid platelets was correlated with the current platelet QC in blood bank. This method is an alternate tool for in-process monitoring, or the development of a better method to produce platelets.*

Keywords : ● *Platelet product* ● *Wright's stain* ● *Discoid platelet* ● *Quality assessment*

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นิพนธ์ต้นฉบับ

การประเมินคุณภาพผลิตภัณฑ์เกล็ดเลือดโดยการย้อมสีเกล็ดเลือดด้วยสีไรท์

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บทคัดย่อ

บทนำ *เกล็ดเลือดซึ่งในภาวะปกติมีรูปร่างเป็น discoid จะมีการเปลี่ยนแปลงรูปร่างเมื่อมีแรงกระแทกหรือมีการกระตุ้นการทำงานของเกล็ดเลือด ในอดีตการสังเกตรูปร่างเกล็ดเลือดต้องใช้กล้องจุลทรรศน์อิเล็กตรอนหรือกล้องจุลทรรศน์เฟสคอนทราสต์ ในปัจจุบันมีการพัฒนาการใช้กล้องจุลทรรศน์แบบใช้แสงที่สามารถขยายวัตถุได้มากขึ้นเพราะมีการใช้กล้องดิจิตอลมาประกอบ การศึกษานี้ได้ทดลองใช้การย้อมสีไรท์และสังเกตรูปร่างเกล็ดเลือดที่เก็บรักษาไว้ในวันที่สามนำมาเปรียบเทียบกับวิธีการตรวจสอบคุณภาพเกล็ดเลือดที่ใช้ในงานบริการ* ***วัสดุและวิธีการ*** *ผู้ทำการศึกษาทำการประเมินคุณภาพเกล็ดเลือดที่ผลิตจากวิธี platelet rich plasma (PRP-PC), buffy coat method (BC-PC) และเกล็ดเลือดจากผู้บริจาครายเดียว (apheresis platelet: AP-PC) ชนิดละ 72 ถุง ณ วันที่สามของการเก็บรักษาโดยการนำสเมียร์เกล็ดเลือดที่ย้อมสีไรท์ ตรวจสอบสัดส่วนเกล็ดเลือดที่มีรูปร่างเป็น discoid เทียบกับวิธีการตรวจสอบคุณภาพเกล็ดเลือดที่ใช้ในงานบริการ คือ การตรวจสอบการเคลื่อนไหวของเกล็ดเลือดในถุงหรือ swirling และการวัด pH* ***ผลการศึกษา*** *การตรวจดูสเมียร์เกล็ดเลือดที่ย้อมสีไรท์โดยกล้องจุลทรรศน์ สามารถสังเกตเห็นรูปร่างของเกล็ดเลือดได้ว่ามีรูปร่างเป็น discoid หรือมีการเปลี่ยนรูปร่างมีแขนงยื่นออกไป เกล็ดเลือดชนิด BC-PC มีสัดส่วนเกล็ดเลือดที่ยังมีรูปร่างเป็น discoid สูงกว่า PRP-PC และ AP-PC อย่างมีนัยสำคัญ ($p < 0.05$) และสัดส่วนเกล็ดเลือดรูปร่างเป็น discoid และ swirling score ที่มีค่าสูง มีความสัมพันธ์กับผลิตภัณฑ์เกล็ดเลือดที่มี pH ระดับสูง อย่างมีนัยสำคัญ ($p < 0.05$) การตรวจสอบรูปร่างเกล็ดเลือดโดยการย้อมสีไรท์ มีความแปรปรวนต่ำ เมื่อตรวจโดยบุคคลเดียว ($r = 0.98$) และระหว่างบุคคล ($r = 0.99$)* ***สรุป*** *การตรวจดูสเมียร์เกล็ดเลือดที่ย้อมสีไรท์โดยใช้กล้องจุลทรรศน์เป็นวิธีการที่ง่าย สามารถตรวจสอบสัดส่วนการเปลี่ยนรูปร่างของเกล็ดเลือดได้มีความน่าเชื่อถือ อาจเป็นทางเลือกสำหรับใช้ตรวจสอบคุณภาพเกล็ดเลือดในการเลือกวิธีการผลิตหรือการพัฒนาระบบการผลิตได้*

คำสำคัญ : ● *ผลิตภัณฑ์เกล็ดเลือด* ● *ย้อมสีไรท์* ● *รูปร่างของเกล็ดเลือด* ● *การประเมินคุณภาพ*
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Introduction

A platelet is a delicate megakaryocyte fragment that functions to initiate hemostasis. A platelet product is needed to treat bleeding in thrombocytopenic patients or prophylaxis against serious bleeding in thrombocytopenic hematologic and oncologic patients, thus a high-quality platelet product is required. Monitoring platelet quality is an essential tool to assure the quality of platelet products.

Blood banks implement several methods for harvesting platelets and storage under recommended conditions before transfusion. The two widely used methods to separate platelet from donated whole blood are platelet-rich plasma and buffy-coat derived platelet which have different manipulation of platelets. Platelet products can be produced by passing donor blood through an automated blood cell separator and selectively collecting only platelet from this single donation. Thus, there are several methods to prepare platelet products that have different platelet manipulation but the final product is used for the same therapeutic purpose. There are methods to monitoring platelet product quality, the current widely used methods are swirling score and measurement of pH of platelet product.

The resting platelet has a discoid shape and changes its' shape when stimulated and activated[1]. The platelet shape change is an early morphological manifestation of platelet activation. Therefore, the ability to visualize and estimate the proportion of platelets in the resting stage, a discoid shape, would be useful to quantitatively measure platelet quality. The scanning electron microscope (EM)[2] and a phase-contrast microscope[3,4] are the most common tools used to visualize platelet shape, but these tools are rarely available in blood banks in developing countries. Wright's stain has been used to visualize blood cells with a light microscope for a century[5] but it is never applied to assess the platelet shape in platelet product. Currently, the visualized technique by light microscopy is much improved, the image from light microscopy can be further magnified using a digital camera and thus the tiny platelet shape can be evaluated by a light microscope.

Here, we explore the utility of Wright's stain platelet smear visualize platelet shape under light microscopy for evaluation proportion of discoid platelets from three different platelet products on the third day of storage, and compare the results with the swirling score and pH which are current QC methods for platelet products.

Materials and Methods

This study was conducted according to the requirements of the Siriraj Hospital Institutional Review Board No. 392/2558.

Sample collection

Our study included randomly selected platelet products from the Department of Transfusion Medicine, Faculty of Medicine Siriraj Hospital, Mahidol University from April to October 2016. To sample platelet for quality assessment, the platelet product was mixed thoroughly in the storage bag before being transferred to an integrated segment of a platelet bag.

Preparation of platelet products

Centrifugation of whole blood-derived platelets was performed in a refrigerated blood bank centrifuge (Sorvall™, Thermo Fisher Scientific, USA). PRP was separated from 450 ml of donated whole blood by the platelet-rich plasma method described elsewhere[6] using a triple bag system (TERUMO PENPOL, Ltd. Puliyarakonam, Trivandrum, India). The platelet concentrate (PRP-PC) was suspended in 50-70 mL of plasma. BC-PC was prepared from four units of buffy coat and one unit of plasma. Each buffy coat containing platelets was separated from 450 ml of donated whole blood in a top-bottom bag system (TERUMO PENPOL, Ltd., Puliyarakonam, Trivandrum, India or JMS, Singapore PTE, LTD, Singapore) following the manufacturer's instruction. Separation was done using a semiautomated presser (T-ACE, Terumo BCT Inc., USA). All tubing connection was done with a sterile connecting device (TSCD-II, Terumo Corporation, Tokyo, Japan) and the final BC-PC product was stored in

a 1,300 mL bag (Compoflex, Fresenius Kabi, Germany). Apheresis platelets (AP-PC) were prepared from a qualified blood donor using the Trima Accel automated apheresis system (Terumo BCT Inc., USA) with ACD-A as an anticoagulant. All production was done following the manufacturer's protocol. All platelet products were stored in a standard platelet storage incubator with continuous agitation (Helmer Inc., USA) and monitored by a temperature monitoring system (Labguard, Affinitech Co., LTD, France).

Examination of platelet morphology by Wright's stain

Five milliliters of a platelet suspension were collected from the cut segment. The platelets were smeared on a glass slide using a circular motion to two centimeters wide, then allowed to air dry for 15 minutes. The slide was flooded with Wright's stain (Merck KGaA, Germany) before adding an equal volume of distilled water, mixed gently with air from a rubber ball, and left for two minutes before rinsing gently with water to clean the back of the slide and then allowed to dry. We examined the platelet smear with a 100 x. oil immersion objective lens under a light microscope (Axiostar Plus, Zeiss, Gottingen, Germany). The image was taken by a digital camera (Canon EOS1100D, Canon Inc, Japan) and all evaluation was done with the image from a digital camera using the computer.

Evaluation of quality of stored platelets

A total of 216 platelet products on day 3 of storage were studied, including 72 samples of each platelet concentrate produced by 1) the platelet-rich plasma method (PRP-PC), 2) buffy coat derived pooled platelets (BC-PC), and 3) apheresis platelet (AP) at the Department of Transfusion Medicine, Faculty of Medicine Siriraj Hospital, Mahidol University from April 2016 to October 2016. These platelet products were evaluated by the swirling score, pH, and proportion of discoid platelet from the Wright's stain platelet smear.

The swirling score

The swirling score was evaluated by observing the movement of fluid in the platelet bag when gently rotating the bag against the light source at a distance of about 30 cm. Swirling was graded by experienced blood bank staff using a score of 3 (excellent), 2 (good), 1 (poor), and zero (no swirling)[7].

pH Measurement

The pH of each platelet product was measured by a pH meter (Orion 2 star, Thermo Scientific, USA) within one hour after sampling time.

The proportion of discoid platelets

We examined 500 platelets from the Wright's stain platelet smear and recorded their shape as discoid, non-discoid, or barbell (proplatelets) before calculating the proportion of discoid-shaped platelets in each sample.

Reproducibility of the proportion of discoid platelets

To study the inter-observer variation, two technicians examined the same set of 20 Wright's stain platelet smears and the proportion of discoid platelets. For intra-observer variation, one technician examined a single set of 20 platelet smears to determine the proportion of discoid platelets two times.

Statistical analysis

Using Microsoft Excel software 2016, the Pearson correlation coefficient was calculated between the percent discoid platelet and pH or swirling score, between the pH and swirling score, and between the percent discoid from inter-observer variation and intra-observer variation. The difference between the percent discoid in the three platelet products and different pH group were calculated using one way ANOVA test. A *p-value* less than 0.05 is required for statistically significant.

Results

Figure 1 showed the platelet morphology visualized under the light microscope. We could differentiate platelet shape between discoid, dendritic, balloon, and barbell-shaped platelets from the Wright's stain platelet smear under a light microscope. Figure 2 showed the Wright's stain platelet smear from the sample of 3 different platelet products on day 3 of storage. At a glance, the BC-PC had more proportion of discoid platelets than AP-PC and PRP-PC.

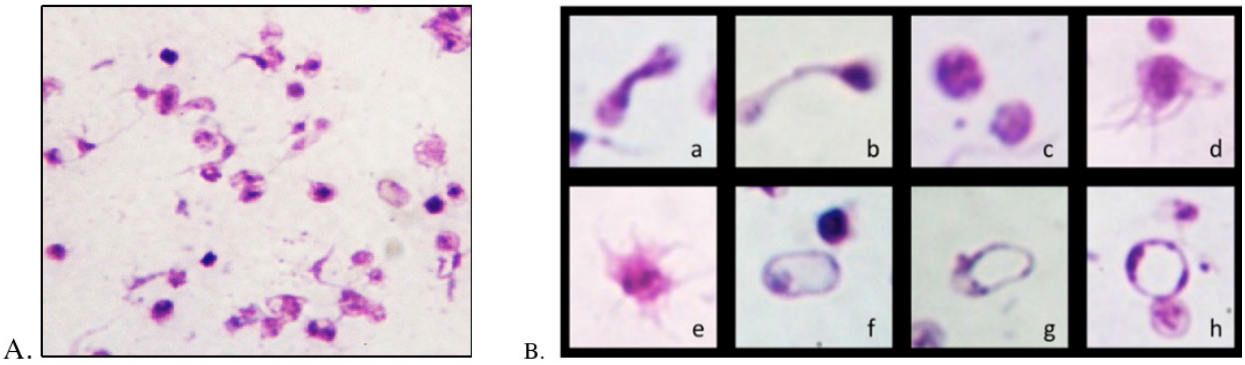

Figure 1 (A) Morphology of platelets visualized by a light microscope under 100x oil immersion objective lens. (B) Different platelet shapes: pro-platelet barbell shape (a-b), discoid (c) dendritic (d-e), procoagulant platelet process; balloon shape (f-g) balloon with cap (h)

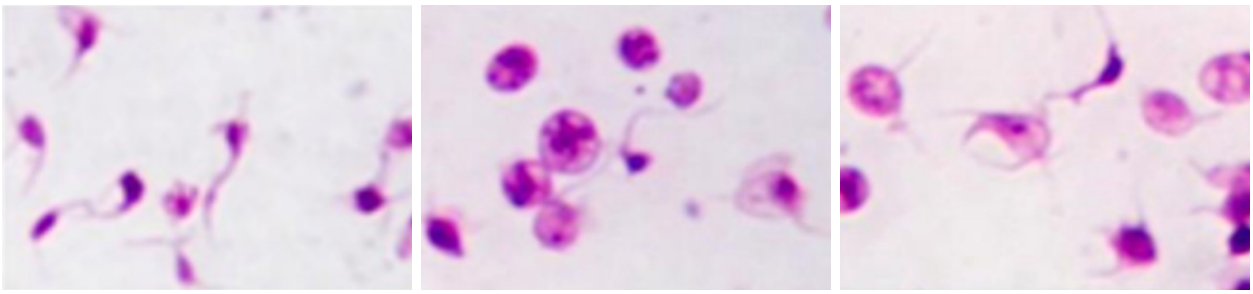

Figure 2 Wright's stain platelet smear from three different platelet preparations at day 3 of storage: (**A**) PRP-PC, (**B**) BC-PC, and (**C**) AP-PC

Table 1 Quality assessment of PRP-PC, BC-PC, and AP-PC at day 3 of storage (n =216)

	All PC (n = 216)	PRP-PC (n = 72)	BC-PC (n = 72)	AP-PC (n = 72)	*p*-value
Platelet conc.(10^6/μL)	1.52±0.33	1.56±0.46	1.42±0.19	1.59±0.28	$p < 0.05$
pH	7.3±0.3	7.3±0.3	7.5±0.1	7.2 ± 0.3	$p < 0.0001$
Swirling score	2.8±0.9	2.8±0.5	3.0±0.2	2.8 ± 0.4	$p = 0.131$
Discoid platelet (%)	78.7±13.0	75.0±13.4	88.0±5.1	73.0±13.1	$p < 0.0001$

Data display mean ± SD

Table 1 showed the results of three platelet product quality assessments by the swirling score, pH, and proportion of discoid platelet from visualized the Wright's stain platelet smear under a light microscope. The comparison of quality parameters between three groups of platelet products were shown in Table 1. pH and proportion of discoid platelets had significant differences between the three groups. BC-PC had the highest proportion of discoid platelets (88.0±5.1%). We also noticed that the mean platelet concentrations between the three groups were different, in our analysis, the platelet concentration was significantly different between PRP-PC and BC-PC ($p = 0.03$), BC and AP-PC ($p = 0.006$) but it was not significantly different between PRP-PC and AP-PC ($p = 0.84$). The swirling score showed no significant difference between different platelet product groups. Table 2 showed the swirling score and pH range of platelet products. The results showed that the higher pH range was correlated with the higher swirling score and proportion of discoid platelets ($p < 0.05$).

Table 2 Platelet product pH range, swirling score and proportion of discoid platelets (n = 216)

pH	N	Swirling score (min, max)	Discoid platelet (%) (min, max)
6.5-6.7	8	2.4±0.5 (2, 3)	59.4±7.4 (47, 71)
6.8-7.0	23	2.5±0.7 (1, 3)	60.4±8.2 (52,81)
7.1-7.3	58	2.8±0.5 (1, 3)	72.3±11.3 (52, 91)
≥ 7.4	127	3.0±0.2 (2, 3)	86.2±7.4 (64, 96)

* $p < 0.05$ for both swirling score and percent discoid platelet

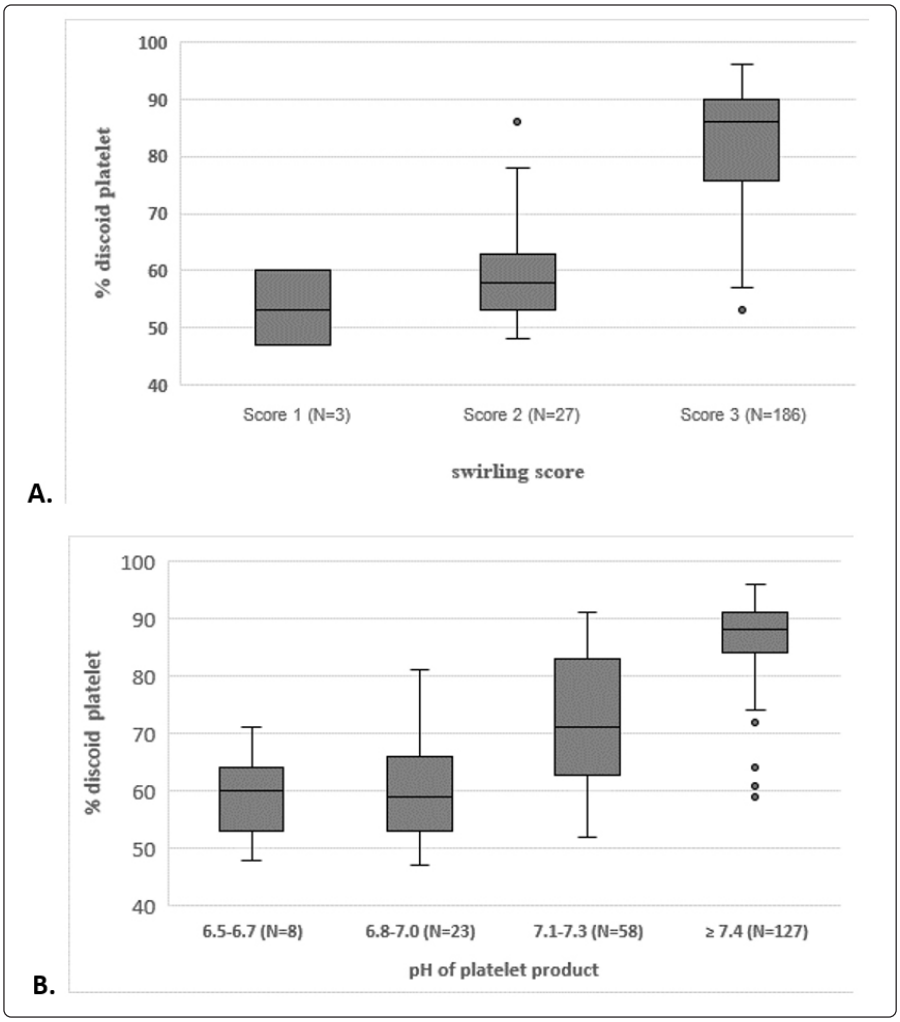

Figure 3 Box plot of quality assessment of platelet products on day 3 of storage (A) swirling score and percentage of discoid platelets (B) pH and percentage of discoid platelets

To evaluate platelet quality, the proportion of discoid platelets showed a strong positive correlation with pH (correlation coefficient 0.77) and a moderate positive correlation with the swirling score (correlation coefficient 0.61). The plot between the proportion of discoid platelets and pH or swirling score were shown in Figure 3.

The intra-observer Pearson correlation coefficient of the proportion of discoid platelets was 0.98 and the inter-observer correlation coefficient was 0.99.

Discussion

From Figure 1, examination of the Wright's stain platelet smear through the light microscope, we could see the various shape of platelets from discoid, dendritic, ballooning, and even barbell-shaped platelet as described in previous studies[8] Our light microscope had a digital camera port and the image can further magnify to see detail. The discoid platelet has a round shape and we could see the purple stain granules, while the dendritic

shape platelet had protruding cytoplasm in every direction. Barbell shapes were also clearly identified entities. The sphere platelet shape which is one of the activated forms of platelet cannot be discriminated from discoid since they have the same round shape and the observation of 2-D picture from platelet smear can see the only round shape. From Maxwell MJ et al. study[9], there were two types of sphere-shaped platelet, a spiny-sphere which transform from discoid which had numerous protrusion, and the smooth sphere which all protrusion was retracted into platelet. To discriminate between the smooth sphere and discoid shape may need the scanning electron microscopy. The spiny sphere may be seen in our Wright's stain platelet smear as dendritic shape. The barbell-shaped platelet was recognized in normal healthy humans at about 3.3±1.6%[10] but there was no data in platelet products. This is an incidental finding of barbell-shaped platelet from stored human platelet products because there were very few direct morphological studies on human platelet products. The barbell-shaped was described as a newly released platelets that transform from a large "pre-platelet" before fission into mature small platelets[10,11]. We found 0.68%, 0.39% and 0.40% barbell-shaped platelets in PRP-PC, BC-PC, and AP-PC, respectively. The different portions of newly released pro-platelets in platelet products may result from the method of separation and collection. The platelet shape can be identified clearly from Wright's stain platelet smear compared with the photo from the previous study using the phase-contrast microscope[3]. We evaluated three different platelet products that were produced by three different methods because there are different platelet manipulation during the collection of whole blood, separation, and storage of platelet products. We selected the third day of storage because platelets are activated during storage[12]. In this study, the third day of storage used the same practice of which US FDA guide for bacterial detection[13] which sampling time on the third day means that the collection date is day zero, and sampling time on day three means that the sampling can occur anytime before midnight. The age of the platelet product may be different within 8 hours. The PRP-PC production includes a pellet of platelet from hard second centrifugation while in BC-PC production, there is no pellet of platelet step. BC-PC was reported to have a better quality of platelets[14] than PRP-PC and we also confirmed this fact with all our assessment tools. We also noticed the difference in mean platelet concentration between our three different platelet products which BC-PC had a significantly lower platelet concentration than PRP-BC and AP-PC. This may be one explanation for the difference between the three products. Our Siriraj or National Blood Centre standard for blood bank[15] does not indicate the concentration of platelet in the final product, but the European Directorate for the Quality of Medicines & HealthCare (EDQM) indicates the concentration of platelet in the final product which should not exceed 40 mL for 60 $x10^9$ platelets[16]. One unexpected finding is the lower discoid percent in AP-PC than in BC-PC. We produced apheresis platelet using the Trima Accel system, blood from platelet donor was passed through a blood cell separator and centrifuge in the collar to create the platelet layer which was manipulated to collect in the system, but platelets may stay longer in the centrifugation compared with separation of platelet from whole blood. Typical Trima Accel apheresis platelet donation usually takes almost 60 minutes to collect platelets. Another possible explanation is the higher concentration of platelets in AP-PC than in BC-PC. The platelet smear in figure 2 expresses different shape change extent from three different products type. When we look at quantitative percent of discoid data, the average proportion of discoid platelet in BC-PC is the highest among three different platelet products.

To compare the newly introduced proportion of discoid shape platelet with the two current quality assessment parameters of stored platelets, swirling[17] and pH[18]. Swirling is widely used for quality control of platelet products because this test is easy to perform, does not

require any special equipment, and does not require sampling the platelets from a bag. The swirling test can be repeated several times during storage and before being issued for transfusion. The disadvantage of the swirling score is its crude scale and subjectively graded. Initially, swirling was scored as positive, intermediate, or negative[19]. The concordance rating of positive and negative swirling was good, but the discordance often occurred with the intermediate grading of swirling[19]. Later, swirling was graded from zero to three[20].

We observed a moderate positive correlation between the proportion of percent discoid platelets and swirling (r = 0.61). From figure 3(A), a swirling platelet score of 3 had a higher proportion of discoid platelet than lower swirling scores, but the range was variable from 53 to 96%, with the mean around 82% and SD being more than 10%.

The average swirling score of our platelet products on day 3 was 2.8, and 86% had a swirling score of 3. The average percentage of discoid at day 3 was 78%, and BC-PC had higher percent discoid platelets than PRP-PC and AP-PC. Bertolini et al. reported the percent discoid of PRP-PC using phase-contrast microscopy at day one of 59% which is lower than on the third day of storage in our study[19]. Similar to our findings, Fiedler et al. reported the percent discoid of BC-PC by TEM on day 4 of 76%[21].

The correlation between the percent discoid platelet and pH was better than with the swirling score (r = 0.77 vs r = 0.61, Figure 3). pH is measured by a pH meter, which is not subjectively graded by a reader and it is a quantitative measurement so we are not surprised to find a better correlation with the proportion of discoid. However, 3 cases with swirling score 1 were only presented in pH 6.8-7.3. The pH was initially considered to be the cause but later was considered to be the result of platelet storage lesion[22].

It is important to monitor the quality of stored platelets to assure their therapeutic effectiveness. The platelet shape change is an early morphological manifestation of activation. Simple, non-invasive, and low-cost methods are desirable. The swirling score is subjectively grade and crude scale, while pH is measurable by the standardized tool but still a crude scale. Measuring the proportion of discoid platelets may be an alternate method to assess the quality of platelet which we assume that the proportion of discoid platelet of more than 70% will correlate to swirling score 2-3 and pH > 7.0 reflect the good quality platelet product and with the benefit of quantitative assay and in the era of digital photography[23], we can take the photo from camera port of the light microscope and the result can be verified by other readers. The use of a light microscope and Wright's stain is economic and widely available even in resource-limited blood centers.

Conclusion

Wright's stain platelet smear visualized under a light microscope is simple, affordable, and requires limited resources to visualize platelet shape change. The proportion of discoid platelet was correlated with the current platelet QC in a blood bank. This method can be an alternate tool for in-process monitoring or the development of a better method to produce platelets.

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