Platelet aggregation and quality control of platelet concentrates produced in the Amazon Blood Bank

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Background: *The study of platelet aggregation is essential to assess in vitro platelet function by different platelet activation pathways.*

Objective: *To assess aggregation and biochemical parameters of random platelet concentrates produced at the Fundação HEMOAM using the quality control tests defined by law.*

Methods: *Whole blood samples from 80 donors and the respective platelet concentrate units were tested. Platelet concentrates were tested (platelet count, aggregation and pH) on days 1, 3 and 5 of storage. Additionally a leukocyte count was done only on day 1 and microbiological tests on day 5 of storage. Collagen and adenosine diphosphate were used as inducing agonists for platelet aggregation testing.*

Results: *Donor whole blood had normal aggregation (aggregation with adenosine diphosphate = 67% and with collagen = 78%). The median aggregation in platelet concentrates with adenosine diphosphate was low throughout storage (18% on day 1, 7% on day 3 and 6% on day 5) and the median aggregation with collagen was normal only on day 1 and low thereafter (54.4% on day 1, 20.5% on day 3 and 9% on day 5).*

Conclusion: *Although the results were within the norms required by law, platelet concentrates had low aggregation rates. We suggest the inclusion of a functional assessment test for the quality control of platelet concentrates for a more effective response to platelet replacement therapy.*

Keywords: *Hemostasis; Quality control; Collagen; Blood component transfusion; Blood banks; Platelet aggregation*

Introduction

Platelets are small discoid anucleated cells, varying from 1 to 4 microns in diameter, which have mitochondria, produce and store adenosine triphosphate and are highly sensitive to their environment.[1] The normal concentration of platelets in the blood is between 150 and 400 x 10^9/L; their main function is to promote a hemostatic surface in blood vessels (primary hemostasis) following the events of vasoconstriction, adhesion, secretion and platelet aggregation. The main inducing agonists in this process are collagen, thrombin, platelet activating factor and adenosine diphosphate (ADP).[2,3] Tissue factor is an activator of coagulation events, and thus part of secondary hemostasis. Some of these substances are released by platelets themselves, promoting the aggregation of other platelets.[4-6] Deficiencies in the number and/or function of platelets may cause abnormalities in coagulation pathways, requiring platelet transfusion.[7,8] This is achieved in transfusion medicine using platelet concentrates (PCs), which allow the infusion of large quantities of platelets in small volumes of fluid; this is especially indicated for thrombocytopenia due to bone marrow failure.

PCs may be obtained through an automated process of blood cell selection, called apheresis, or from whole blood centrifugation by randomization using the platelet-rich plasma (PRP) method or even buffy coat. Variables involved in these procedures cause changes in morphology, activation, aggregation and fragmentation of the PCs.[9,10] Quality control of blood products is used in blood therapy centers to assess the hemostatic potential of PCs and correct possible complications. In Brazil, quality control is regulated by resolutions and by laws which standardize blood therapy services throughout the country. Each service must have written protocols defining the type of control and minimum parameters for each blood component. Thus, PCs obtained from whole blood should have at least 5.5 x 10^{10} platelets/U. It is known that the metabolic activity of platelets continues throughout storage and even with all the procedures that allow platelet hemostatic viability, other factors related to storage and platelet metabolism interfere not

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only in the number of cells, but also in their functional integrity.[11] A study of platelet aggregation is essential to assess the *in vitro* function of these cells in respect to different activation pathways. A citrated platelet-rich plasma (PRP) is continuously stirred by an iron sphere in an instrument called a platelet aggregometer, which allows the measurement of temporal, semiquantitative and qualitative parameters related to *in vitro* aggregation.[12-14]

This study aimed to analyze platelet aggregation and biochemical parameters of PCs produced in the Fundação HEMOAM, through quality control tests required by current norms.

Methods

A cross-sectional study was conducted in the Fractionation Laboratory of the Fundação HEMOAM, in Manaus, Amazonas between August 2009 and April 2010. In this period, 80 samples were collected from blood donors, who signed informed consent forms and whose donations were used to produce platelet concentrates. For this study, we defined sampling as 1% of the monthly production of PCs as demanded by legislation (Directive RDC N° 153) effective at the time of this study.[15] For this study, blood donors were selected using the following inclusion criteria: 1. Male donors between 18 and 65 years old, whose donation took place at the Fundação HEMOAM; 2. Donation candidates with easy venous access; and 3. Donation candidates who gave their informed consent. In the Fractionation Laboratory, the exclusion criteria used were the visual aspect of the PRP units; samples with an appearance of biliverdin, erythrochromia or lipemia or the absence of swirling were discarded.

The samples were collected in citrated tubes and after blood collection they were taken to the Hemostasis Laboratory for platelet aggregation testing. The collected units were taken to the Fractionation Laboratory for processing in accordance with the standard operational procedure. The PCs were collected in five-day triple bags (Macopharma®), containing 100 mL of Sag-Manitol preservation and anticoagulant solution.

After obtaining the PRP, the units were submitted to swirling analysis by shaking them in front of a light source, with the results expressed as presence or absence of birefringence. The units of PCs produced with a volume of 50-70 mL were left to rest for one hour and placed in a linear shaker (C-Mar®) at 70 rpm at a controlled temperature of 22 ± 2ºC (71.6 ± 3.6ºF).

The tests of PCs (platelet count, platelet aggregation and pH) were carried out on days 1, 3 and 5 after processing; the leukocyte count was performed only on day 1 and the microbiological control was performed only on the 5th day of storage. In order to obtain aliquots from samples of PCs, a sterile connection (Haemonetics®) was used which ensured the integrity of the environment. Platelet aggregation was achieved using the turbidimetric aggregometry technique using a dual-channel Chronolog (Crono-Log Corporation®) within four hours of blood collection. For this, the PRP was initially obtained through light centrifugation at 1000 rpm for five minutes, and then the autologous platelet-poor plasma (PPP) was centrifuged at 3000 rpm for fifteen minutes (Eppendorf®). PRP samples were subjected to a platelet count in an automatic counter (Human Count®) and adjusted for a mean value of 250×10^9 platelets/L.

Platelet aggregation

After adjusting the platelet concentration, aggregation was evaluated using different concentrations of inducing agonists: collagen 2.0 µg/mL and ADP 7.0 µg/mL (Crono-Log Corporation®). For each test, 400 µL of PRP and 400 µL of PPP were used, each one in a different cuvette after waiting for spontaneous aggregation. The aggregation curve was observed after five minutes of stimulation by inducing agonists, and soon after, aggregation was measured and expressed as a percentage according to the curves formed during the tests. The result of the test is commonly expressed as a percentage of aggregation by the quantity of light transmitted through the test solution; aggregation is classified as normal, low or high.

Platelet and leukocyte concentrations

The platelet and leukocyte counts were attained in an automatic counter on days 1 (donor whole blood and PCs), 3 (PCs) and 5 (PCs), while the leukocytes were counted only on day 1. The PPP was used as the diluent with the electrical charge of platelets remaining unaltered.

pH analysis

Samples of 5 mL of PCs were placed in laboratory beakers and tested in pH meters (Tecnal®).

Microbiological control

The bacteriological analysis was achieved through the inoculation of 10 mL samples in blood culture bottles (Hemoprov - NewProv®) incubated at 37ºC (98.6ºF) for seven days.

Statistical analysis

The results were analyzed using the Epi-Info computer program. Statistical significance of experimental data was analyzed using the ANOVA and Barlett tests. Due to the non-normality of the data, the median and the Kruskal-Wallis non-parametric test were used.

Results

Visual aspect and volume of PCs

The presence of swirling was observed in all PCs assessed. Only four PC units presented erythrochromia.

Table 1 - Distribution of laboratory findings of platelet concetrates throughout storage

Tests	Day			Reference values
	1	3	5	
ADP	18%	7%	6%	≥ 40%
Collagen	54.5%	20.5%	9%	≥ 50%
Platelet count	5.45 x 10^{10} / 70 mL	5.80 x 10^{10} / 70 mL	5.76 x 10^{10} / 70 mL	≥ 5.5 x 10^{10} / 70 mL
Leukocyte count	0.1/ 70 mL	-	-	< 10^{8}/ 70 mL
pH	7.42	7.72	7.73	≥ 6.2
Microbiology	-	-	Negative	Negative

Lipemia and biliverdin were not seen in the PCs. The mean volume of PCs produced was about 60 mL.

Platelet aggregation of PCs

Platelet aggregation of PCs with ADP showed significantly low platelet aggregation on the first day of storage (18%) which dropped to 7% on day 3 and 6% on day 5.

The mean value of platelet aggregation with collagen was 54% on day 1, 20.5% on day 3 and 9% on day 5 (Table 1).

Platelet count of PCs

The concentration of platelets of PCs showed a slight increase of 5% from the first to the third day of storage; from 5.45 x 10^{10} platelets/U on day 1 to 5.80 x 10^{10} platelets/U on day 3 (Table 1).

Leukocyte count of PCs

The median leukocyte concentration in PCs was 0.1, with quartile deviation (QD) of 0.2 (n=51). Evaluation was only performed on day 1 of storage (Table 1).

pH of PCs

On the first day of analysis, the median pH was 7.4, with quartile deviation (QD) of 0.18. This was statistically different ($p < 0.001$) from the third and fifth days (Table 1). There was an increase in the pH from 7.4 on day 1 to 7.7 from day 3.

Microbiological control

All PCs had negative results for microbiological control.

Platelet aggregation of donor whole blood

To evaluate platelet aggregation of donor whole blood, the inducing agents, adenosine phosphate (ADP) and collagen, were used separately. The median aggregation with ADP was 67% and for collagen it was 78% according to the percentages established by the aggregation curves. Therefore, the results demonstrate that 80% of donors present with normal aggregation.

Discussion

When assessed with ADP, platelet aggregation of PCs showed low aggregation from the first day of storage; this continued until the fifth day.

The results of aggregation with collagen were considered normal on the first day, with a progressive decrease in platelet function during the five days, when the aggregation was considered low.

These results are in agreement with other studies which state that low aggregation of PCs occurs after 24 hours of storage. However, the details of the relationship between these induction agents and the decrease in platelet aggregation are still unknown.[16]

A comparison of the aggregation of donor whole blood (normal aggregation) with that of PCs prepared from this whole blood (low aggregation) shows that the platelet metabolism becomes critical when kept *in vitro*, even when the quality standards defined by the Ministry of Health are followed. Di Minno et al.[17] report that when aggregation is tested using pairs of agonists, the results are better than when tested with these agonists in isolation. This suggests that the physiological function of platelets is possibly maintained *in vivo* due to the interaction of multiple biological factors.

It is known that ADP induces platelet aggregation in citrated PRP and that this is considered to be the main cause of morphological changes in platelets, which is essential for platelet adhesion.[18] ADP molecules, also found in platelet granules along with thromboxane A2 which is synthesized during platelet activation, lead to the formation of the primary hemostatic plug. Collagen is regarded as a potent agonist as it is related to the activation of several intracellular metabolic systems with different receptors in platelet membranes.[19] Thus, it binds to von Willebrand factor (vWF), creating an adherence bridge between collagen and the platelet glycoprotein Ib receptor. Granule content secretion is triggered by platelet surface receptor agonists. Moreover, some drugs can irreversibly interfere in platelet function; these drugs include antimicrobial and anti-inflammatory drugs, and, in particular, cyclooxygenase inhibitors such as acetylsalicylic acid, which block the

synthesis of thromboxane A2, a potent platelet aggregation activator.[20] In our study, the results of platelet aggregation in donor whole blood, both with ADP and collagen, were considered normal, showing that the blood collection process did not significantly affect platelet function and also that donors had not been taking drugs that had an effect on these cells.

Jilma-Stohlawetz et al.[20] showed that low platelet function can be reverted *in vivo* 24 hours after transfusion. On the other hand, limited storage time is associated with better hemostatic capacity *in vivo*. In our study, the findings related to platelet normality were seen in the first 72 hours.

We observed that the other quality control criteria of the PCs were within acceptable ranges according to current technical standards. The platelet count on the first day of storage was normal (5.5×10^{10}/70 mL). However, by the third day there was an increase in the number of platelets, suggesting platelet fragmentation[21] with subsequent stabilization by the fifth day of storage. Quality control of PCs is the tool available to assess results and, consequently, to adjust production techniques when necessary. Despite this, these parameters are limited. According to Bowbrick et al.,[22] the platelet count of PCs does not indicate normal, partial or absent functional activity. It is necessary to determine not only the cell content, but also the functional integrity. Given this, the platelet count is an assessment of the centrifugation process used to obtain PRP, together with the presence of swirling.

In the analysis of the leukocyte content of PCs, we obtained a value of 0.1 mL/unit, which indicates normality. As reported by Cardigan,[21] contaminating leukocytes in PCs compete for available oxygen. In a similar study, Henschler et al.[23] demonstrated the importance of quantifying leukocytes in the quality control of PCs.

In the evaluation of pH, there was an increase between the first and the third days of storage, which may have occurred due to changes in gas concentrations, which are usually at high levels at the beginning of storage, but which stabilize later, as reported in the literature.[24] The pH is an important marker for the quality of PCs *in vitro* since at values below 6.8, platelets become spherical; this change in shape becomes irreversible when the pH drops below 6.2. Platelet metabolism ceases completely when pH values drop below 6.0.[25]

The presence of infectious agents was not observed in the PCs, possibly due to the relatively small number of samples. According to the literature, one in every 2000 PCs (random and apheresis) is positive for bacterial contamination.[26] According to Walther-Wenke,[27] bacterial contamination of units may lead to sepsis and other complications after transfusion.

The focus of this study was to analyze the variance in hemostatic function of PCs during the period of storage. In this work there was a progressive decrease in platelet aggregation throughout the 5-day period. Many studies indicate that the loss of platelet function is directly associated with factors related to the use of anti-aggregating drugs and to the complex metabolism of these cells when kept *in vitro*. A review of the currently established quality control criteria is suggested, in order to define a method of evaluating functional analysis of PCs for a more effective response in platelet replacement therapy.

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