In vitro thromboelastometric evaluation of the efficacy of frozen platelet transfusion

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A R T I C L E   I N F O

Article history:
Received 25 March 2015
Received in revised form 3 May 2015
Accepted 30 May 2015
Available online 2 June 2015

Keywords:
Blood transfusion/methods
Cryopreservation
Platelet function test
Platelet transfusion
Thromboelastometry

A B S T R A C T

Background: Although frozen platelets are extensively used in remote locations and military environments, scientific evidence of their efficacy is scarce. The objective of this study was to evaluate the in vitro hemostatic efficacy of frozen versus fresh platelet transfusions by rotational thromboelastometry (ROTEM) to ascertain whether the freezing and thawing process impaired platelet contribution to clot strength.

Methods: An experimental study was performed using platelet in vitro transfusions. Blood samples were collected from 12 patients with non-autoimmune thrombocytopenia. The samples were each transfused with one of 6 pairs of fresh platelet concentrates and platelet concentrates frozen with dimethylsulfoxide. Optical platelet counts, coagulation studies and ROTEM (EXTEM and FIBTEM) were performed for the baseline and the post-transfusion samples.

Results: Only fresh platelet transfusions significantly increased the EXTEM maximum clot firmness (MCF) and maximum clot elasticity (MCE) over baseline (p < 0.001), achieving values within the normal range. The frozen platelet contribution to MCE was negligible. However, the EXTEM clotting time (CT) was significantly shortened after the transfusion of fresh platelets (p = 0.002) compared with the fresh platelet transfusion. The EXTEM clot formation time (CFT) was significantly shortened after the transfusion of fresh platelets compared with the frozen platelet transfusion (p = 0.002).

Conclusion: The ROTEM analysis assessment indicates a dual effect in frozen platelet transfusion: it produces a hypercoagulable state (shortening of CT), and a second, more predominant effect of frozen platelets’ functionality impairment compared with fresh platelets (shorter MCF/MCE and longer CFT).

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⁎⁎ Notes: Preliminary data for this study were presented as a poster presentation at the European Society of Anaesthesiology (ESA) Euroanaesthesia, 9–12 June 2012, Paris.

Financial support and sponsorship: This work has received institutional support from La Paz University Hospital, Madrid, Spain and Armed Forces Transfusion Centre, Madrid, Spain.

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1. Introduction

Exsanguinating haemorrhage is the leading cause of death in military conflicts, and its effective control can improve the survival rates of military casualties. Current battlefield resuscitation practices include aggressive coagulopathy treatment, with a tendency to employ early component therapy using a 1:1:1 ratio of red blood cells (RBCs), plasma and platelets (PLTs). Sending fresh supplies of liquid blood components to combat areas is logistically challenging [1], and it is not possible in the case of platelet concentrates (PCs) due to their short shelf life. While dried platelet products applicable in clinical practice are developed (still under investigation in animal models) [2,3], the medical military corps of several countries have started using integrated fresh-frozen blood banking, and now send red cell concentrates, fresh frozen plasma (FFP) and frozen platelets to combat areas [4–6].

Although previous studies examining the viability and lifespan of frozen platelets have been published [7–9], and extensive use of these frozen components has been adopted by various armies, scientific evidence of their efficacy is scarce.

The objective of this study was to evaluate the in vitro haemostatic efficacy of frozen versus fresh platelet transfusions by rotational...
thromboelastometry (ROTEM; Tem International GmbH, Germany) to ascertain whether platelet contribution to clot strength is significantly impaired by the freezing and thawing process.

2. Materials and Methods

An experimental study was performed using in vitro platelet transfusions. After obtaining institutional review board approval (Ethical Committee of La Paz University Hospital, Madrid, Spain on June 24, 2011. Protocol number HULP: PI-1173) and the patients’ informed consent, 12 patients with non-autoimmune thrombocytopenia (5 non-Hodgkin’s lymphoma, 4 acute myeloid leukaemia, 2 non-secretory multiple myeloma and 1 Hodgkin’s lymphoma) were recruited. Blood (10 mL) was collected in 3 citrate-containing tubes (3.2% citrate, VACUTAINER; Becton Dickinson, Meylan, France).

The first tube was used to take a baseline measurement of the patients’ complete blood count and perform the following coagulation studies: prothrombin time (PT), prothrombin activity (PA), activated partial thromboplastin time (aPTT) and fibrinogen concentration (PT-based assay).

Using ROTEM we assessed clot formation via the extrinsic pathway (EXTEM test) using recombinant tissue factor and phospholipids as triggers. To perform the baseline ROTEM analysis, 300 μL samples were obtained from the second and third test tubes (EXTEM and FIBTEM tests, respectively). Afterwards, the same volume (300 μL) of fresh standard 22 °C stored platelets or thawed frozen platelet concentrate was added to the second and third tubes, respectively, using the automated pipetting system of the ROTEM. This was approximately equivalent to a transfusion of 1.3 U/kg of body weight, considering that the total volume contained in the test tubes after platelet transfusion was 3 mL, the estimated blood volume of an adult is approximately 70 mL/kg and the volume of a single donor platelet unit is approximately 60 mL.

After the addition of the platelet concentrates to the second and third test tubes, the platelet count, coagulation tests and determination of fibrinogen concentration were performed prior to the ROTEM studies (Fig. 1). Six pairs of standard platelet concentrates and frozen platelets were compared, each pair for two patients. Both the standard and the frozen platelet concentrates were used before their expiration date: 5 to 7 days and 8 to 12 months, respectively.

The standard platelet concentrates were obtained from a single blood bank and they were either leukocyte-reduced pooled platelets (5) or apheresis platelets (1), filtered and conserved in Composol-PS (Fresenius HemoCare, Emmer-Compascuum, Netherlands) and InterSol (Cerus, Amersfoort, Netherlands) additive solutions, respectively. The frozen platelets were obtained from two different blood banks to rule out processing deficits. All were leukocyte-reduced platelet pools frozen at −80 °C, using 5% dimethyl sulfoxide (DMSO) as the cryoprotective agent, and reconstituted in plasma. The thawing procedure was performed by trained personnel in a 30 m water bath at 37 °C according to established institutional blood bank standards.

The platelet counts were measured by a CELL-DYN Sapphire analyser (Abbott Diagnostics Division, Santa Clara, CA), using optical measurement techniques to avoid possible impedance measurement inaccuracies due to electrical noise by cell fragments or microcytic red cells.

The ROTEM analysis was performed by an experienced operator following the manufacturer’s instructions. Clotting time (CT = time to reach 2 mm amplitude from the beginning of the test, in s), clot formation time (CFT = time from CT to 20 mm amplitude, in s, which reflects the dynamics of the coagulation process), and maximum clot firmness (MCF, in mm) were recorded. To assess the contribution of the platelets to clot kinetics, a platelet-inhibited FIBTEM test was performed and compared with the EXTEM test for all collected parameters.

The maximum clot elasticity (MCE) was calculated from the EXTEM and FIBTEM MCF to accommodate for Hooke’s law. The MCE, a parameter calculated from MCF values, is intended to reflect the actual physical properties of clot strength and to allow a better interpretation of this parameter in case of high MCF amplitudes.

\[
\text{MCE} = \frac{(100 \times \text{MCF})}{100 - \text{MCF}} \text{dyne cm}^{-2}
\]

The platelet contribution to clot strength is then obtained by subtraction [10]:

\[
\text{MCE platelets} = \text{MCE EXTEM} - \text{MCE FIBTEM}
\]

The contribution of transfused platelets to clot strength can also be calculated:

\[
\text{MCE (Frozen/Fresh) Platelets} = \text{MCE (post-transfusion) platelets} - \text{MCE (baseline) platelets}
\]

The MCE platelets were adjusted to the platelet count in calculating the “Platelet Index” (PI):

\[
\text{PI} = \frac{\text{MCE platelets}}{\text{Platelet count} \times 10^9/L}
\]

2.1. Statistical Analysis

The data were transferred from the ROTEM device to Microsoft Excel (Microsoft Office 2007, Microsoft Corporation, Redmond, WA, USA), and

![Fig. 1. Diagram showing how the experiments were performed.](image)
the statistical calculations were performed with SPSS Version 17.0 for Windows (SPSS, Chicago, IL). The ROTEM data between the experimental and baseline samples were distributed normally (tested using the Kolmogorov-Smirnov test and the Shapiro-Wilk test) and were analysed by multiple linear regression, adjusting and not adjusting for the platelet count.

Statistical significance was set at p < .05. The data are presented as means (± SD), numbers and percentages, as appropriate.

Sample size calculations showed that a t-test with a type I error (two-sided) of 0.05 would have 87.5% power to detect a 10% difference in MCF EXTEM values (after a transfusion of fresh platelets vs. frozen platelets, compared with baseline) with a sample size of 12 in each group.

3. Results

The patients’ mean baseline platelet count was 13.58 ± 6.32 x 10^9/L. The mean platelet count of the fresh and frozen platelet units was 690.83 ± 73.60 x 10^9/L and 1119.83 ± 451.98 x10^9/L, respectively (p = .004).

The increase in the mean post-transfusion platelet count was greater with frozen platelets than with fresh platelets; 123.25 ± 31.52 x 10^9/L and 90.42 ± 16.66 x10^9/L, respectively (p = .005).

The mean patient baseline MCF was 40.92 ± 5.13 and 25.83 ± 6.03 mm in the EXTEM and FIBTEM tests, respectively.

After in vitro transfusion of fresh platelets, the EXTEM MCF mean value increased to 63.75 ± 4.41 mm, reaching values within the reference range (50–72 mm) that were statistically significant (p < .001). However, the increase in the mean EXTEM MCF following the transfusion of frozen platelets was poor; 44.08 ± 5.13 mm, achieving only a slight improvement over baseline values. Similar results were obtained in the EXTEM MCE (Table 1), in which the transfusion of fresh platelets doubled the EXTEM MCE baseline value, whereas the transfusion of frozen platelets did not affect this parameter (p < .001).

Fig. 2 shows the contribution of baseline and transfused platelets to MCE (MCE platelets). The contribution to MCE of frozen transfused platelets (MCE frozen Tx platelets) (4.33 ± 15.23) was negligible in contrast to the important contribution of the fresh transfused platelets (MCE fresh Tx platelets) (107.79 ± 31.13), which was 3 times the mean MCE of the patients’ baseline platelets (MCE Platelet) (34.77 ± 13.16). This difference was statistically significant (p < .001). In fact, fresh platelet transfusion normalised MCF and MCE values in all 12 cases, whereas frozen platelet transfusion did so in only 1 of 12 cases.

The platelet index was reduced after both the fresh and frozen platelet transfusions. However, the reduction was greater and was statistically significant after the frozen platelet transfusion (p < .001).

The patients’ mean baseline EXTEM CFT (211.25 ± 93.62 s) was above the reference range (34–159 s). This parameter was shortened by transfusions of both fresh and frozen platelets, but the shortening was more pronounced and was statistically significant after the fresh platelet transfusion (p = .002), reaching values within the reference range.

There was no improvement in FIBTEM MCE, MCF or CFT; no significant difference was measured between post-transfusion values of fresh and frozen platelets, as had been expected in a test in which the platelets are inactivated. The FIBTEM MCF baseline mean values (25.83 ± 6.03 mm) were above the normal range (9–25 mm), in concordance with the high baseline fibrinogen levels found in the patients. The EXTEM CFT value was shortened from baseline (73.25 ± 11.77 s) after a transfusion of both fresh and frozen platelets, but it was significantly (p < .001) shorter after the frozen platelet transfusion (44.00 ± 4.31 s) compared with the fresh platelet transfusion (61.75 ± 8.32 s). This was in accordance with the statistically significant (p = .024) shortening of the aPTT ratio after the frozen platelet transfusion. No statistical differences were found in the other variables (Table 1), and there were no statistically significant differences found in relation to the blood bank where the frozen platelets had been processed or in connection with their expiration date.

4. Discussion

Recommendations for platelet administration and monitoring in clinical practice are often made based on platelet count. For ethical reasons, no clinical trials were performed comparing platelet therapy with placebo before the introduction of platelet transfusion in the 1950s; therefore, the appropriate dosing of platelets is uncertain. In a similar manner, frozen platelets have been used in military operations in the Middle East by the Dutch [5], Australian [1] and Spanish [6] armies for several years despite limited evidence of their effectiveness.

An adequate and appropriate test for evaluating the haemostatic function of a stored platelet product is not currently available, although a thromboelastography (TEG)-PC assay sensitive to storage effects has recently been developed [11]. To date, the few published studies have

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Baseline and post-transfusion results. Statistical significance.</th>
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</thead>
<tbody>
<tr>
<td>-</td>
<td>RV Baseline PT (M ± SD)</td>
</tr>
<tr>
<td>PLT-CC (x10^9/L)</td>
<td>-</td>
</tr>
<tr>
<td>PT PLT-c (x10^9/L)</td>
<td>180-450</td>
</tr>
<tr>
<td>EXTEM-CT (s)</td>
<td>38-79</td>
</tr>
<tr>
<td>EXTEM-CFT (s)</td>
<td>34-159</td>
</tr>
<tr>
<td>FIBTEM-CFT (s)</td>
<td>-</td>
</tr>
<tr>
<td>EXTEM-MCF (mm)</td>
<td>50-72</td>
</tr>
<tr>
<td>FIBTEM-MCF (mm)</td>
<td>9-25</td>
</tr>
<tr>
<td>EXTEM-MCE (dyne cm^-2)</td>
<td>117-257</td>
</tr>
<tr>
<td>FIBTEM-MCE (dyne cm^-2)</td>
<td>9.9-33</td>
</tr>
<tr>
<td>MCE Platelets (x10^9/L)</td>
<td>-</td>
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<tr>
<td>MCE Tx platelets</td>
<td>-</td>
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<tr>
<td>Platelet Index</td>
<td>-</td>
</tr>
<tr>
<td>PT (s)</td>
<td>11.5-13.5</td>
</tr>
<tr>
<td>PA (%)</td>
<td>75-120</td>
</tr>
<tr>
<td>INR</td>
<td>0.8-1.3</td>
</tr>
<tr>
<td>aPTT (s)</td>
<td>27-40</td>
</tr>
<tr>
<td>aPTT ratio</td>
<td>0.8-1.3</td>
</tr>
<tr>
<td>Fibrinogen (mg/dL)</td>
<td>180-450</td>
</tr>
</tbody>
</table>

Patient = PT; mean and standard deviation = M ± SD; platelet concentrate count = PLT-CC; patient platelet count = PT PLT-c; clotting time = CT; clot formation time = CFT; maximum clot firmness = MCF; maximum clot elasticity = MCE; MCE EXTEM-MCE FIBTEM = MCE Platelets; MCE (post-transfusion) platelets-MCE (baseline) platelets = MCE Tx Platelets; MCE platelets/Platelet count (x10^9/L) = Platelet Index; prothrombin time = PT; prothrombin activity = PA; International Normalized Ratio = INR; activated partial thromboplastin time = aPTT; *statistically significant (p < .05); RV = reference values of the local laboratory and ROTEM (Lang et al., 2009); 1Adjusted for platelet count; 1Not adjusted for platelet count.
shown morphological and functional defects in previously frozen platelets when these cells were tested in vitro. Frozen platelet adhesion was significantly decreased in relation to fresh platelets and platelets that had been stored for 5 days [12]. Recovery, survival [13] and other *in vitro* function markers, such as stimulus–response coupling [14], aggregation [15], granule release and pH were also impaired in frozen platelets. However, an acceptable life span and a superior *in vivo* immediate haemostatic function of frozen platelets have been reported in animal [16] and human [17] research. The latter is the only prospective, randomised clinical trial performed to date with postcardiopulmonary bypass (CPB) bleeding as the primary outcome. This study showed that patients transfused with cryopreserved platelets had significantly lower nonsurgical blood loss and transfusion of blood products compared with the patient group that received liquid-preserved platelets. Despite the excellent design of that study, some criticism can be made. Twenty patients of 73 were excluded from the study after random assignment, and the statistical power was not stated. Furthermore, mean CPB time was 22 m longer in liquid-preserved platelet transfused patients. Although this difference was not statistically significant (*p* = .12), it could be clinically relevant because CPB platelet-induced mechanical damage is proportional to CPB time. This could be why liquid-preserved platelet transfused patients received a significantly higher number of platelets than the cryopreserved group (*p* = .0001); no reason was given in the text. The fibrinogen level was significantly higher in the patients receiving cryopreserved platelets (*p* = .024), which could also have influenced non-surgical blood loss. Despite these criticisms, other studies measuring bleeding time as the primary outcome support the theory that cryopreservation strengthens the *in vivo* procoagulative activities of platelets [16,18], whereas other *in vitro* measures of platelet quality such as aggregation might not indicate the actual *in vivo* quality of platelet transfusions.

Rotation thromboelastometry (Tem International GmbH, Germany) is based on the original TEG system described by Hartert [19], capable of providing a rapid evaluation of clot formation, strength and lysis, using whole blood. The working principles of ROTEM monitoring and the interpretation of results have been extensively reviewed elsewhere [20–22]. Thromboelastometry trace and parameters are shown in Fig. 3.

To date, thromboelastometry and TEG have been used to investigate the *in vitro* effects of colloid and crystalloid administration [23], hypothermia and acidosis [24], fibrinogen and other coagulation factor substitution [25,26] and drug administration [27] on whole blood haemostasis. TEG has recently been used to evaluate donor-specific and storage-induced responses in PCs. The TEG-PC assay data showed a clear trend to faster reaction times (R-time, K-time and angle) with little variation in maximal amplitude (MA) during the storage time of single-donor PCs [11].

The strength of a clot measured by MCF in a standard tissue factor activated test (EXTEM) is affected primarily by platelets, fibrinogen and Factor XIII. The activation of coagulation in FIBTEM is the same as in EXTEM, but the platelets are blocked by the addition of cytochalasin D. The resulting clot is independent of the count and function of the platelets and depends only on fibrin formation and polymerisation. The platelet contribution to clot firmness can be assessed by comparing the MCF/MCE of the standard test (EXTEM) and the platelet inhibitor test (FIBTEM). CFT represents fibrin polymerisation and the stabilisation of the clot with thrombocytes and factor XIII. CFT value is prolonged in clot polymerisation disorders.

We designed a transfusion model based on the blood samples obtained from patients admitted to the haematology ward with a nonimmune-mediated thrombocytopenia diagnosis of various aetiologies. This diagnosis represents an isolated reduction in platelet count, as opposed to other possible models such as *in vitro* haemodilution that would change all the ROTEM parameters and the global coagulation test. [25] Thus, this model allowed for the acquisition of a consistent set of samples ideal for assessing the efficacy of *in vitro* platelet transfusions.

Unlike previous studies that demonstrated a smaller increase in platelet count with frozen platelets than with fresh platelets, our results showed that the increase in post-transfusion mean platelet count was greater with frozen platelets than with fresh platelets and was statistically significant. This result is consistent with the statistically significant greater platelet count of frozen units. Frozen platelet units could have been diluted to compare an equivalent number of platelets, but we decided to avoid sample manipulations that could alter the results, thus we performed the experiment and obtained results from the platelet units as they were when they leave the blood bank and are used in clinical practice. The platelet count did not influence the results, as evidenced after adjustment of the statistical analysis by this parameter (Table 1).
Despite the difference in the number of platelets transfused, which favoured the frozen product, the shortening of EXTEM CFT and the increase in EXTEM MCF/MCE was greater with fresh platelets (p < .01) and was potentially clinically relevant. All the EXTEM MCF/MCE values were normalised in the fresh platelet transfusion group compared with only 1 of 12 in the frozen platelet transfusion group. According to our study, the improvement in platelet contribution to EXTEM MCF/ MCE after the transfusion of frozen platelets is small and clearly inferior to that provided by fresh platelets, suggesting an impairment of platelet function during the process of freezing and thawing. In support of this hypothesis, it has recently been reported that frozen and thawed platelets showed reduced surface expression of GPIIb and GPIIbα and diminished aggregation in response to agonists [28].

Lang et al. demonstrated a positive correlation between the changes in clot strength shown in MCE and increasing platelet counts or fibrinogen concentrations. Surprisingly, the platelet component of clot strength (MCE platelet) increased in a fibrinogen-concentration-dependent manner at a constant platelet count [10]. This outcome could be explained by increased fibrinogen binding to the abundant fibrinogen receptors (GPIIb/IIIa) on a single platelet (40,000-50,000 copies) [29]. Consequently, fibrinogen repletion was found to be superior to the apheresis platelet transfusion according to ROTEM variables and blood loss [30]. Therefore, fibrinogen levels remained unchanged from baseline. Variations in MCE were solely due to platelet transfusion. The analysis of the contribution of transfused platelets to clot strength (MCE Tx Platelets) showed no influence in the case of frozen platelets. Nevertheless, the contribution of fresh platelets to clot strength was so important that it normalised MCE EXTEM and MCE platelets. The platelet index as an index derived from MCE platelets showed reduced surface expression of GPIIb and GPIIbα and diminished aggregation in response to agonists [28].

Therefore, from the results of our study we can conclude that the freezing and thawing process has a dual effect on platelets identifiable by ROTEM. On one hand, it seems that this treatment increases the exposure of phosphatidylserine on the platelet surface, leading to a hypercoagulable state as reflected in the shortening of EXTEM CT and aPTT ratio. On the other hand, platelets contribution to clot strength is decreased with cryopreserved platelets, as reflected by the low EXTEM MCF obtained after in vitro transfusion with frozen-thawed platelets.

In order to ascertain the overall predominant effect of the transfusion of platelets, a more comprehensive CFT could be used. In our study, according to the results obtained with a CFT significantly shortened by fresh platelet transfusion, the damage to platelet function by the process of freezing and thawing predominates over the increased generation of thrombin produced by the transfusion of frozen platelets.

5. Conclusion

The ROTEM analysis assessment indicates a dual effect in frozen platelet transfusion: it produces a hypercoagulable state (shortening of CT), and a second, more predominant effect of frozen platelets' functionality impairment compared with fresh platelets (shorter MCF/MCE and longer CFT). In vivo studies are needed to confirm whether the transfusion of frozen platelets produce better or poorer improvement of platelet haemostatic function than fresh platelets, and if their effectiveness justifies the logistical effort and resources required for them to be sent to combat zones.
Acknowledgements Relating to this Article:

We are grateful to Ana Rodríguez de la Rúa, MD, PhD, (Former Chief of the Department of Haematology, La Paz University Hospital, Madrid, Spain) for her invaluable help in designing the study and providing logistical support for this and numerous other projects. We also thank Eric So (Senior Software Specialist, Mount Sinai Hospital, New York) for his indispensable contribution to the translation of this manuscript.

Author Contribution Statement

APF, RNS, JMPL and JPC conceived the study. AVLL was responsible for the recruitment of patients, the blood, the samples and the fresh platelets. JMPL and JPC provided the frozen platelets. APF, RNS and MJAM acquired the data. JVS analysed the data. NB, RNS, JVS, AVLL and MJAM critically revised the manuscript. APF wrote the paper and takes responsibility for the paper as a whole.

Conflict of Interest

None declared.

References