Original Article
Changes in platelet function following cold storage of RBC suspensions

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Abstract: Objective: To provide a basis for the cold-storage of human platelets as a way to assess changes in platelet function. Methods: Red blood cell suspensions (11 U and 50 U) were randomly selected at different storage times (3-28 days) and evidence of platelet activation (CD62P) and thromboelastography (TEG) reaction times were investigated. Results: After 21 days of storage at 4 °C, a large number of activated platelets (PAC1+62P+, PAC1-62P+) within the red blood cell suspension (RBCs) retained their function and had TEG-maximum amplitude (TEG-MA) indices in the normal range. Conclusion: We report that platelets in RBC suspensions retain high activity when stored at 4 °C for 21 days. The results provide important information for studies that involve storing platelets under cold conditions.

Keywords: RBC suspensions, thromboelastography, blood platelets, cold storage, hemostasis

Introduction

In a clinical setting, a blood cell transfusion is mainly used to treat anemia; whereas platelets and fresh frozen plasma (FFP) are used in the treatment of thrombocytopenia and coagulopathy. However, some studies report that red blood cells suspensions (RBCs) promote hemostasis by platelet marginalization [1] and reduce the risk of bleeding in patients who received transfusions for anemia and thrombocytopenia [2-9]. This suggests that RBCs play a role in hemostasis.

In our early studies, we found that the hemostatic effect of RBC suspensions required platelets and residual coagulation factors. In order to determine whether platelets remain function in a cold RBC we assessed changes in blood routine, coagulation, and TEG indices at different storage times. Our work could potentially clear the misconception that platelets dis-
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**Preparation of platelet-rich plasma (PRP)**

Platelet-rich plasma (10 ml) from the 11 U RBC suspension (continuous observation) was centrifuged at 1000 × g for 12 min and absorbed. The supernatant and tunica albuginea parts were combined to evaluate TEG and blood routine indices.

**Blood analyses**

Routine blood analyses measured the RBC count, hematocrit (Hct), hemoglobin content (Hb), and platelet (PLT) count using an automatic hemacytometer (Coulter LH 750). The Sysmex CA-7000 coagulation analyzer measured prothrombin time (PT), activated partial thromboplastin time (APTT), international standardization ratio (INR), thrombin time (TT), and plasma fibrinogen levels (FIB). The thromboelastography (TEG) parameters recorded as reaction times (R or R-time), K times (K or K-time), Angle, maximum amplitude (MA), and coagulation index (CI). These measurements were gathered using the TEG 5000 Thromboelastograph Hemostasis Analyzer (Haemoscope Corp., IL). Expression of platelet markers CD61, PAC-1,
and CD62P were detected by flow cytometry (Becman Coulter FC 500) and CXP software.

**Statistical analysis**

Data were analyzed using the SPSS statistical software (version 18.0). Analysis of parametric data was performed by the paired Student’s t-test. An association between categorical variables was tested by the Chi-square test and a repeatable ANOVA. Statistical significance was set at $P < 0.05$.

**Results**

Routine blood indices remained at or above the normal range after cold storage

As shown in Table 1; Figures 1 and 2, when RBC suspensions were stored at 4°C the number of RBCs increased, while the number of platelets gradually decreased over time. Additionally, blood coagulation PT and APTT surpassed the normal range at Day 3 and continued until Day 21. After Day 14, the INR values extended beyond the normal range and FIB levels remained below the normal range. The TEG-reaction times (TEG-R) reached beyond the normal range at Day 21. The TEG K-times (which reflect FIB levels) were beyond the normal range. TEG-Angle (reflects blood coagulation rates), MA values (reflects platelet function), and CI (reflects overall coagulability), were beyond the normal range with low coagulation states.

**TEG indices of platelet-rich plasma (PRP) remain in the normal range for 21 days**

As shown in Table 2, PRP obtained from RBC suspensions stored at 4°C for 14 days had a
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A gradual decrease in the number of platelets. TEG R-times and TEG coagulation indices (TEG-Cl) were within the normal range for 21 days, and then dropped below normal on Day 28. However, TEG K-times TEG-Angle, and TEG-MA levels remained within the normal range for the entire storage period.

**Comparison of TEG indices for RBC suspensions and platelet-rich plasma (PRP)**

As shown in Figure 2, there were no statistical differences between RBCs R-times and PRP R-times Day 28. However, there was a significant difference between RBC K-times and PRP

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**Figure 2.** Comparison of TEG indices of RBC suspensions and platelet-rich plasma. TEG R-times (A), TEG K-times reflect plasma fibrinogen levels (B), TEG-Angle reflect coagulation rates (C), TEG-MA values reflect platelet function (D), and TEG-Cl reflects overall blood coagulation rates (E) for RBCs and platelet-rich plasma. Dotted line represents the normal range. *RBCs versus PRP; ● versus 3 days of the PRP storage; ■ versus 3 days of RBCs storage.

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**Table 2. TEG indices of platelet-rich plasma with different storage times**

<table>
<thead>
<tr>
<th>Length of storage at 4°C</th>
<th>RBC (× 10^{12}/L, X±s)</th>
<th>Hb (g/L, X±s)</th>
<th>Hct (L/L, X±s)</th>
<th>PLT (× 10^9/L, X±s)</th>
<th>R (min, X±s)</th>
<th>K (min, X±s)</th>
<th>Angle (X±s)</th>
<th>MA (mm, X±s)</th>
<th>CI (X±s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 d</td>
<td>0.25±0.11</td>
<td>5±4</td>
<td>0.022±0.012</td>
<td>525±174</td>
<td>1.5±0.8</td>
<td>3.3±7.2</td>
<td>63.8±6.4</td>
<td>60.3±9.9</td>
<td>-0.3±2.2</td>
</tr>
<tr>
<td>7 d</td>
<td>0.18±0.07</td>
<td>3±2</td>
<td>0.015±0.007</td>
<td>581±146</td>
<td>1.5±0.7</td>
<td>73.3±7.2</td>
<td>58.1±18</td>
<td>50±8.9</td>
<td>-0.8±3.7</td>
</tr>
<tr>
<td>14 d</td>
<td>0.24±0.06</td>
<td>4±2</td>
<td>0.021±0.005</td>
<td>427±186</td>
<td>1.3±0.4</td>
<td>73.5±6.8</td>
<td>64.1±6.5</td>
<td>68.1±11.2</td>
<td>0.4±1.7</td>
</tr>
<tr>
<td>21 d</td>
<td>0.26±0.15</td>
<td>7±3</td>
<td>0.027±0.011</td>
<td>237±123</td>
<td>2.3±1.7</td>
<td>75.1±4.2</td>
<td>57.9±8.5</td>
<td>50.3±4.5</td>
<td>-1.8±4.1</td>
</tr>
<tr>
<td>28 d</td>
<td>0.47±0.23*</td>
<td>10±3*</td>
<td>0.081±0.149</td>
<td>399±152</td>
<td>3.2±1.3*</td>
<td>68.1±4.2</td>
<td>57.9±8.5</td>
<td>60.3±9.9*</td>
<td>-5.3±4.5*</td>
</tr>
</tbody>
</table>

TEG = Thromboelastography; Hb = hemoglobin concentration; RBC = red blood cell count; PLT = platelet count; Hct = hematocrit; R = reaction time; K = kinetics, clot formation time, a (angle) = slope between r and k; MA = maximum amplitude; CI = coagulation index. *Compared with Day 3, P < 0.05. The P-value denoted repeated measurements.
Table 3. Expression level of CD62P RBC suspensions with different storage times

<table>
<thead>
<tr>
<th></th>
<th>&lt; 7 d</th>
<th>8-14 d</th>
<th>15-21 d</th>
<th>≥ 22 d</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAC1*62P (%)</td>
<td>6.84±5.61</td>
<td>3.81±3.93</td>
<td>1.81±3.92</td>
<td>2.36±7.55*</td>
</tr>
<tr>
<td>PAC1*62P' (%)</td>
<td>34.02±18.45</td>
<td>41.74±22.25</td>
<td>33.92±12.91</td>
<td>22.57±8.2</td>
</tr>
<tr>
<td>PAC1*62P'' (%)</td>
<td>42.11±17.11</td>
<td>43.26±25.84</td>
<td>50.37±17.94</td>
<td>60.41±18.65*</td>
</tr>
<tr>
<td>R (min, X±s)</td>
<td>8.8±2.2</td>
<td>9.1±1.2</td>
<td>9.6±1.8</td>
<td>14±5.3*</td>
</tr>
<tr>
<td>K (min, X±s)</td>
<td>4.9±3.4</td>
<td>4.7±2.7</td>
<td>6.1±3.8</td>
<td>10.5±6.8*</td>
</tr>
<tr>
<td>Angle (X±s)</td>
<td>56.9±10</td>
<td>55.5±6.5</td>
<td>53.5±6.8</td>
<td>47.2±7.3*</td>
</tr>
<tr>
<td>MA (mm, X±s)</td>
<td>47.3±13.5</td>
<td>49.4±8.9</td>
<td>43.4±10.9</td>
<td>35.7±10.5*</td>
</tr>
<tr>
<td>CI (X±s)</td>
<td>-5.3±5</td>
<td>-5.2±3.1</td>
<td>-7.3±3</td>
<td>-12.5±6.4*</td>
</tr>
<tr>
<td>PLT (× 10^11/L, X±s)</td>
<td>213±75</td>
<td>199±69</td>
<td>156±41</td>
<td>192±73</td>
</tr>
<tr>
<td>Hb (g/L, X±s)</td>
<td>129±57</td>
<td>139±50</td>
<td>145±52</td>
<td>168±49</td>
</tr>
<tr>
<td>RBC (× 10^12/L, X±s)</td>
<td>4.47±2.28</td>
<td>4.49±1.67</td>
<td>4.62±1.65</td>
<td>5.49±1.64</td>
</tr>
<tr>
<td>Hct (L/L, X±s)</td>
<td>0.413±0.198</td>
<td>0.435±0.161</td>
<td>0.449±0.166</td>
<td>0.545±0.164</td>
</tr>
</tbody>
</table>

PAC1* = an activated complex on the glycoprotein II b/III a; CD62P = P-selectin; Hb = hemoglobin concentration; RBC = red blood cell count; PLT = platelet count; Hct = hematocrit; R = reaction time; K = kinetics, clot formation time, a (angle) = slope between r and k; MA = maximum amplitude; CI = coagulation index. *Compared with Day 7, P < 0.05. The normal range for PAC1*62P goes as follows: PAC1*62P < 10%; PAC1*62P' < 10%; PAC1*62P'' < 4%.

K-times. The RBCs K-time was above the normal range while the PRP K-times were within the normal range. The TEG-Angle values, which reflected the blood coagulation rate, as well as the PRP-Angle and RBCs-Angle values were in the vicinity of the normal range on both sides and there was no statistical difference. As for the MA values (PRP-MA), which reflect platelet function, and the CI value (PRP-CI), which reflects whole blood coagulation rates, they were in the normal range. As for RBCs, the coagulation indices (CI) and maximum amplitude levels (MA) were below the normal range in a low coagulation state.

Platelets remain unaffected by a 3-week storage period under cold conditions

The expression levels of activated platelet marker, PAC1*62P', in suspended red fell below 50% when stored for less than 14 days at 4°C (Table 3 and Figure 3). By Day 22 CD62P expression levels increased. This data suggests that platelets remained active.

Blood smear of RBC suspensions reveal sustained presence of platelets

Wright-Geima staining revealed that a large number of platelets remained in the RBC suspension at Day 21 and decreased by Day 28 (Figure 4).

Discussion

The goal of this study was to validate the presence of high platelet activity in suspended red blood cells stored under cold storage conditions. Our data would provide some important information for studies on the cold storage of platelets. Previous reports show that researchers believed that cold storage of red blood cells could cause the platelets to be rapidly eliminated in vivo, so they suggested the platelets should be preserved in 20-24°C oscillating conditions [10, 11-20]. Further improvement of platelet additive solutions and bags has extended the platelets storage time to 5-7 days [21-26]. In our study, the platelets in suspended red blood cells retained their aggregate function in a highly activated state in cold storage for 21 days. Additionally the platelets retained certain functions after 28 days of cold storage. These results provide the basis for 4°C cold-storage platelets.

A large number of platelets contained in suspended red blood cells, the amount of platelets (288±69 × 10^9 cells/l) remained in the RBC suspension after Day 3 and gradually decreased (194±46 × 10^9 cells/l) after Day 28. Furthermore, morphological examinations revealed that platelets maintained their cellular integrity until Day 21. In order to analyze the changes in platelet function, we prepared platelet-rich plasma (PRP) from suspended RBCs stored at 4°C for varying lengths of time. TEG index showed that the R-times, K-times, and Angle values, which reflect blood coagulation, were in the normal range during 21 days of storage. MA and CI values, which reflect platelet function and overall blood coagulation rate, respectively, were also in the normal range after 21 days of storage.
Our results are consistent with previous findings from Stiegler et al., and Josefsson et al., in which the research groups believed that platelets retained certain activities under cold storage conditions, and suggests that storing platelets at 4°C is more beneficial than storage at 22°C, particularly for patients with acute bleeding [27, 28]. The underlying mechanism by which platelets retain their functional abilities is unclear, but Frederick et al., speculated that a specific molecule within the RBC supernatant inhibits platelet aggregation [29]. Additionally, the blood coagulation indices (PT, APTT, INR, and FIB) were in a low coagulation state. This is due to the dilution of coagulation factors and decreased activity during the preparation process. In this process many blood coagulation factors were removed as a considerable
amount of RBC maintenance solution was added. The TEG index in a low coagulation state, and the R, K, Angle, MA, and CI values were out of the normal reference range. This may be related to the decreased concentration of coagulation factors or may result from changes in red and white blood cells after cold storage.

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Disclosure of conflict of interest

None.

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