Red cell storage, lesion and clearance
Burger, P.

Citation for published version (APA):
Burger, P. (2012). Red cell storage, lesion and clearance

General rights
It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: http://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.
Chapter 4

Collection and storage of erythrocytes with anticoagulant and additive solution with a physiological pH

Patrick Burger
Herbert Korsten
Arthur J. Verhoeven
Dirk de Korte
Robin van Bruggen

Accepted in Transfusion
Abstract

A donation of whole blood is most commonly collected in acidic citrate-phosphate-dextrose (CPD) variants with pH 5.2 to 6.2 as anticoagulants. Previously, we have shown that the initial pH after red cell preparation can have an effect on red cell concentrates (RCC) during storage. First, we investigated the effect of the pH of the anticoagulant on red cell concentrates. Second, we investigated the possibility to decrease the pH of our new additive solution (AS) PAGGGM from pH 8.2 to 7.4 in combination with an anticoagulant with a physiological pH.

Whole blood was collected in CPD (pH 5.6) or trisodiumcitrate (TNC) (pH 7.4) and leukoreduced units were prepared using SAGM as AS. Second, whole blood was collected in TNC (pH 7.4) and leukoreduced units were prepared using PAGGGM (pH 7.4) or PAGGGM (pH 8.2) as AS. During cold storage, several in vitro characteristics were analyzed.

In agreement with our previous findings the initial pH of whole blood has an effect during storage of RCC. In the second part we show that there are no differences between PAGGGM (pH 7.4) and PAGGGM (pH 8.2) units when an anticoagulant with a physiological pH was used.

These results indicate that the pH of the anticoagulant used during whole blood collection has an effect during storage of RCC. When an anticoagulant with a physiological pH is used during whole blood collection, the pH of PAGGGM can be decreased to physiological levels, while maintaining ATP and 2,3-DPG levels.
Introduction

A donation of whole blood is usually separated to yield different products for different transfusion purposes. As anticoagulant, most commonly used are the acidic CPD variants with a pH ranging from 5.2 to 6.2. This low pH is needed, since glucose will caramelize during the sterilization process when a higher pH is used. However, in a recent study, we have shown that the initial pH after red cell concentrate (RCC) preparation can have an effect on red cell in vitro storage parameters throughout the entire storage period. Therefore, from a storage point of view, collecting whole blood in an anticoagulant with a more physiological pH might improve the quality of the stored erythrocytes.

During routine storage of erythrocytes, 2,3-DPG declines rapidly and is depleted within 2 weeks of storage, while the ATP levels decrease more slowly. Both are important for the normal function of erythrocytes, as the levels of 2,3-DPG determine the affinity of hemoglobin to oxygen, while the ATP levels play an important role in maintaining the phospholipid asymmetry of the erythrocyte membrane.

In recent studies published by our group, we have shown that a newly formulated additive solution (AS), phosphate-adenine-glucose-guanosine-gluconate-mannitol (PAGGGM), has improved in vitro storage characteristics as compared to the AS frequently used in Europe, i.e. saline-adenine-glucose-mannitol (SAGM, see table 1 for AS compositions). The composition of PAGGGM is based on the hypothesis of Meryman et al. that a chloride free AS with a high pH (i.e. pH 8.2 compared to SAGM with a pH of 6.2), would result in an increased rate of glycolysis. This resulted in the combined maintenance of 2,3-diphosphoglycerate (2,3-DPG) and adenosine-triphosphate (ATP) levels throughout the storage period. However, we hypothesized that a physiological pH of PAGGGM would be possible if whole blood would be collected in an anticoagulant with a physiological pH.

In the current study we first determined the effect of collecting whole blood in an anticoagulant with a physiological pH. To this end, whole blood was either collected in CPD with a pH 5.6 or trisodiumcitrate (TNC) with a pH of 7.4. Subsequently, leukoreduced red cell concentrates (RCC) were prepared using SAGM as an AS. In line with our expectations, SAGM units collected

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>SAGM for CPD collections</th>
<th>SAGM for TNC collections</th>
<th>PAGGGM pH 7.4 for TNC collections</th>
<th>PAGGGM pH 8.2 for TNC collections</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose (anhydrous) (mmol/L)</td>
<td>50</td>
<td>100</td>
<td>97.5</td>
<td>97.5</td>
</tr>
<tr>
<td>Adenine (mmol/L)</td>
<td>1.25</td>
<td>1.25</td>
<td>1.44</td>
<td>1.44</td>
</tr>
<tr>
<td>Guanosine (mmol/L)</td>
<td></td>
<td></td>
<td>1.44</td>
<td>1.44</td>
</tr>
<tr>
<td>NaCl (mmol/L)</td>
<td>150</td>
<td>150</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>Na-gluconate (mmol/L)</td>
<td></td>
<td></td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>NaH₂PO₄·2H₂O (mmol/L)</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Na₂HPO₄·2H₂O (mmol/L)</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Mannitol (mmol/L)</td>
<td>29</td>
<td>29</td>
<td>55</td>
<td>55</td>
</tr>
<tr>
<td>pH</td>
<td>6.2</td>
<td>6.2</td>
<td>7.4</td>
<td>8.2</td>
</tr>
</tbody>
</table>
in TNC already maintained 2,3-DPG for a longer period compared to SAGM units collected in CPD. However, the 2,3-DPG was still depleted after 3 weeks of storage. In the second part of the study, we explored the possibility to combine anticoagulant TNC with our AS PAGGGM both at physiological pH.

Materials and methods

Isolation and storage of erythrocytes

Whole blood was collected from 12 healthy volunteers. During collection, CPD (pH 5.6) or TNC (pH 7.4) (see Table 2 for anticoagulant compositions) were mixed with whole blood in a final ratio of 1:7 in bottom-and-top blood collection systems with integrated leukoreduction filters used (T3941, Fresenius HemoCare, Emmer Compascuum, the Netherlands; if applicable CPD was replaced by TNC). Leukoreduced RCCs were prepared by centrifugation of whole-blood collections (8 minutes, 2800g), which had been stored for 12 to 18 hours at 20 to 24 °C. After removal of the buffy coat, 110 mL of AS (sterilized by filtration over 0.2-mm filters, stored at room temperature for maximal 2 weeks, variable composition; see table 1) was added, and filtration of the erythrocyte suspension was carried out to remove residual white blood cells (WBCs). To adjust for the difference in glucose levels between CPD and TNC, units manufactured from whole blood collected in TNC had an additive solution with a higher glucose concentration (see table 1). The difference in pH between PAGGGM (pH 7.4) and PAGGGM (pH 8.2) was obtained by adding less 5 M NaOH while manufacturing PAGGGM. The resulting PAGGGM ASs had a similar osmolarity. The resulting RCCs had a volume of 275 to 320 mL, a hematocrit (Hct) of approximately 60 percent (vol/vol) and contained fewer than 1*10⁶ WBCs per unit (as determined with a Nageotte hemocytometer), whereas platelet counts were below detection limit (determined with an Advia 2120, Siemens Medical Solutions Diagnostics, Breda, the Netherlands). After preparation, the erythrocytes were stored in 600-mL polyvinylchloride storage bags (Fresenius Hemocare) at 2 to 6 °C in a standard blood bank refrigerator.

2,3-DPG, ATP and lactate measurements in erythrocytes

2,3-DPG, ATP and lactate were measured as described elsewhere. In short, extracts were made by diluting 600 µl erythrocytes with 900 µl PBS and then acidified with 60 µl perchloric acid (70% weight/vol). After 30 minutes on ice, the extracts were centrifuged for 5 minutes at 4 °C at 6,000g and 56 µl 5 M K₂CO₃ was added to 1 mL deproteinized supernatant for neutralization. Samples were kept frozen till analysis.

2,3-DPG was measured with the 2,3-DPG kit from Roche (Mannheim, Germany). Lactate was analyzed with the kit from Trinity Biotech (St Louis, MO). ATP was analyzed with the glucose/hexokinase reaction as described elsewhere.
**Hemolysis**

Hemolysis was determined as described previously. Briefly, free hemoglobin was determined by absorbance measurement of cell supernatant at 415 nm or 514 nm by a spectrophotometer (Rosys Anthos ht3, Anthos Labtec Instruments GmbH, Salzburg, Austria), with correction for plasma absorption if necessary. Hemolysis was expressed as a percentage of total hemoglobin present in RCC after correction for Hct.

**Measurement of intracellular pH**

Intracellular pH (pHi) was measured as described. Briefly, 1 mL erythrocyte samples were centrifuged for 5 minutes at 21,000g after which the supernatant was removed. Subsequently, the dry pellet was frozen in liquid nitrogen. After thawing, 300 to 350 µl deionized water was added and the pH of the resulting lysate was measured in a Rapidlab 860 (Siemens Medical Solutions Diagnostics, Breda, the Netherlands).

**Potassium, sodium, glucose and extracellular pH measurements**

Potassium, sodium, glucose and extracellular pH (pHe) were measured with a Rapidlab 860 (Siemens Medical Solution Diagnostics).

---

**Figure 1:** Effect of physiological pH of the anticoagulant on pH, potassium concentrations and hemolysis of RCCs in standard SAGM during storage. Whole blood was collected in CPD (▼) or in TNC (●). Subsequently, RCC units were prepared with SAGM as additive solution as described in material and methods. Results shown represent mean ± 1 SD of 3 units. * p<0.05; ** p<0.01; *** p<0.001
Chapter 4

Statistical analysis

Data was analyzed using Graphpad Prism 5.01 for Windows (GraphPad Software, La Jolla, CA). Statistical analysis was performed by 2-way ANOVA tests with the Bonferroni post-tests to compare means over time.

Results

In a previous study, we found that depending on the method of preparation, 2,3-DPG levels were better maintained when the units had an initial higher pH_i when compared to units with an initial lower pH_i. This suggested that there might be a memory effect, which influences glycolysis for a long period. To further explore the effect of a difference in the initial pH during blood collection and storage, we collected whole blood units with CPD (pH 5.6) or with TNC (pH 7.4) as an anticoagulant. After leukodepletion, SAGM was added to the red cell concentrates. During the first 2 weeks of storage, the TNC units had both a significantly higher pH_i and a significantly higher pH_e (figure 1a and 1b) (p<0.05). After 2 weeks of storage the potassium leakage was higher in the TNC units (figure 1c) (p<0.05), although there was no difference in hemolysis, which remained well below the limit of international standards (figure 1D). The consumption of glucose and production of lactate showed no differences (figures 2a and b). Also, the ATP levels remained similar throughout storage (figure 2c). However, the 2,3-DPG levels were significantly higher in TNC units than in CPD units during the first 2 weeks of storage (p<0.01) (figure 2d).
This seems to correlate with the increased pH seen during the first 2 weeks of storage. Next, we tested the feasibility of lowering the pH of our new additive solution PAGGGM "alkaline pH" to a physiological pH, in combination with an anticoagulant with a physiological pH. We collected whole blood in TNC (pH 7.4) and RCC units were prepared with PAGGGM with a pH of 7.4 (□) or PAGGGM with a pH of 8.2 (∆). Results shown represent mean ± 1 SD of 3 units.

SAGM and PAGGGM (pH 7.4 and pH 8.2) units prepared from whole blood collected in TNC were compared. Compared to the SAGM units manufactured from TNC whole blood, the PAGGGM units showed the same pH_i and pH_e values (figure 5a and b). The potassium leakage was higher in SAGM units in week 2 and 3 of storage (P<0.05), but was similar after that (data not shown). There were no differences in the levels of hemolysis either, which remained minimal over the whole storage period (data not shown). The glucose levels were decreased throughout storage in PAGGGM units, but only significantly different from week 3 onwards (p<0.001) and the lactate levels were increased from week 2 in PAGGGM units when compared to SAGM units (p<0.01) (data not shown). Despite this, the ATP levels were increased in PAGGGM units from week 3 on (p<0.05) and the 2,3-DPG levels were increased from week 1 on (p<0.01) in...
The glucose consumption and lactate production (per week of storage) were only significantly increased during the first week (figure 6a and b) in PAGGGM units.

Discussion

For a long time now whole blood is collected in CPD, CPD-A or CP2D, all with a low pH. The main reason for this is that glucose can only be sterilized at a low pH. Sterilizing glucose at a pH of 6 or above will result in caramelization. However, for storage of RCC, the glucose does not need to be present in the anticoagulant, as glucose is present in the additive solutions used to supplement the erythrocyte suspension. When the glucose normally present in CPD would be added to the additive solution, the glucose would no longer be needed in the anticoagulant. This would make the use of an anticoagulant with a higher pH possible. Moreover, in our previous study we found evidence that the initial pH in RCCs might have an effect throughout the entire storage period of erythrocytes. Therefore, we hypothesized that collecting whole blood in an anticoagulant with a higher pH than CPD might improve the quality of RCCs.

When we consider the 2,3-DPG levels of erythrocytes collected either in CPD or TNC and stored in SAGM, we can indeed conclude that the higher pH of TNC has a positive effect on the in vitro quality parameters of the RCCs (figure 1). It could be that this is caused by the increased intra- and extracellular pH of the TNC units at the start of the study (figure 2). However,
Figure 5: Comparison of units collected in TNC (pH 7.4) and stored in SAGM, PAGGGM (pH 7.4) and PAGGGM (pH 8.2). For comparison, data from SAGM units collected in TNC (●) and PAGGGM (pH 7.4) (□) and PAGGGM (pH 8.2) (△) units collected in TNC were merged. Statistical analyses were performed comparing SAGM units with PAGGGM units and were only considered different when both PAGGGM ASs were significantly different from the SAGM units. Results shown represent mean ± 1 SD of 3 units. * p<0.05; ** p<0.01; *** p<0.001

Figure 6: Glucose consumption and lactate production in RCCs stored in PAGGGM media with different pH. The same units as described in the legend of figure 3, where whole blood was collected in TNC (pH 7.4) and RCC units were prepared with PAGGGM with a pH of 7.4 (□) or PAGGGM with a pH of 8.2 (△), were analyzed. Glucose consumption and lactate production were calculated from the glucose and lactate levels measured once every week during storage. For comparison, data are also shown from units collected in TNC with SAGM as additive solution (●). Results shown represent mean ± 1 SD of 3 units. * p<0.05
although the TNC units maintained 2,3-DPG for a longer time, 2,3-DPG was still depleted after 3 weeks of storage.

The units collected in TNC (pH 7.4) and stored in PAGGGM (pH 7.4) maintained 2,3-DPG and ATP levels much better than during storage in SAGM (figure 5c and 5d). This is comparable, both in absolute levels as in kinetics to what we observed in previous studies with PAGGGM (pH 8.2) units collected in CPD. Surprisingly, although chloride depletion has been proposed to increase the pH, we observed no differences in pH or pH between PAGGGM and SAGM units (figure 5a and 5b). As PAGGGM does show an effect on 2,3-DPG and ATP levels, chloride depletion probably does not exert its effect via an increase in pH, but via another, yet unknown, way. In line with this hypothesis is the absence of differences in pH or pH in PAGGGM (pH 7.4) and PAGGGM (pH 8.2) units.

By using an anticoagulant and AS with a physiological pH several issues arise. First of all, as stated previously, the main reason for using an acidic anticoagulant and additive solution is that this allows heat-sterilization of the glucose present in the solutions. At a higher pH, such as present in TNC and PAGGGM, glucose will caramelize during heat-sterilization. However, several manufacturers have produced and published divided systems, in which the glucose is kept separate from the other components of the additive solution. By keeping the glucose separate, heat sterilization of the solution will be possible.

Secondly, whole blood collected with TNC will have a lower glucose concentration than whole blood collected with CPD and will be less acidic. Therefore, one must take into account the effect this will have on the other isolated blood components, namely plasma and platelets. Plasma is often collected via plasmapheresis, in which TNC is used as an anticoagulant. Therefore, we do not expect any negative impact on the quality of plasma when we collect whole blood with TNC as anticoagulant.

Unlike plasma, platelets need glucose which is normally supplied by CPD added during whole blood collection. When TNC is used as an anticoagulant, platelet concentrates can only use glucose already present in the plasma. In plasma, glucose levels range from 4 to 6 mM. Under normal conditions, platelet concentrates use 5 mM of glucose during their 7 days of storage, which suggests that the glucose present in the plasma might not be enough and should be added during preparation. A possibility could be to use an additive solution, which contains glucose and/or other metabolites like acetate, for storage of the platelet concentrates. Further analysis of platelet concentrates obtained from whole blood collected with TNC as anticoagulant would be required to show the feasibility of this approach.

In conclusion, the present study shows that the pH of the anticoagulant does have an effect that influences RCC in vitro parameters throughout the storage period. The results show that an increased initial pH in SAGM units maintains 2,3-DPG for a longer period, but it is not enough to maintain 2,3-DPG during 5 weeks of storage. However, the combination of both anticoagulant and PAGGGM medium with a physiological pH is able to maintain ATP and 2,3-DPG throughout storage. Even though this does not result in large changes in both intra- and extracellular pH from SAGM units collected in CPD, the metabolic parameters of these units are far better. A next logical step would be to test in vivo if this improved metabolic quality will also result in a better post-transfusion recovery and survival of the stored erythrocytes.
References

1. de Korte D, Kleine M, Korsten HG, Verhoeven AJ. Prolonged maintenance of 2,3-diphosphoglycerate acid and adenosine triphosphate in red blood cells during storage. Transfusion 2008;48:1081-1089.


