An In Vitro Evaluation of Ionized Calcium Levels and Clotting in Red Blood Cells Diluted With Lactated Ringer’s Solution

William H. King, M.D.,* Ethel D. Patten, M.D.,† David E. Bee, Ph.D.‡

The addition of lactated Ringer’s solution which contains calcium (RL) to citrated blood products poses a potential risk whenever the level of ionized calcium reaches a concentration capable of catalyzing the coagulation cascade. RL solution is used extensively as both a replacement and a maintenance fluid in the operating room. However, major surgical procedures often require replacement of blood components as well, during the operation. This study examines the in vitro propensity for coagulation when red blood cells (RBC) are diluted with RL. Seven mixtures with different ratios of RBC to RL were prepared from each of 23 units of RBC. These mixtures were analyzed for ionized calcium, total calcium, and pH, and were checked for any indication of coagulation. Nineteen additional RBC units were split into two parts which were mixed with equal volumes of normal saline (NS) or RL. These mixtures were filtered (40 micron) and the weight gain of the paired filters compared. From these studies, the authors identified a threshold value for ionized calcium (0.23 mM/L) below which the probability of clot formation is less than 0.01. This concentration is not reached if the RBC to RL volume ratio is 2:1 or greater. As much as 100 mL of RL can be added to a unit of RBC without exceeding the threshold value. Although not advocating the routine use of RL as a diluent for RBC, the authors conclude that, within the guidelines described, calcium containing salt solutions may be used to dilute blood products. (Key words: Blood, anticoagulant, citrate. Blood, replacement. Calcium, ionized. Hemodilution. Lactated Ringer’s solution. Transfusion, intraoperative.)

The American Association of Blood Banks recommends the use of normal saline (NS) to dilute red blood cells and specifically prohibits the use of lactated Ringer’s solution (RL). In clinical practice, RL solution is sometimes used as a diluent for RBC, despite the recommendation to the contrary. No clinical complication resulting from this practice has been reported.

The purpose of this study was to determine in vitro under what conditions RL could be used as a diluent for RBC. The study was divided in two parts: 1) measurement of the level of ionized calcium (Ca++) in various mixtures of RBC and RL while observing the mixtures for evidence of clot formation; and 2) comparison of the amount of particulate matter recovered from RBC diluted 1:1 with RL with the amount of particulate matter recovered from RBC diluted 1:1 with NS.

Materials and Methods

Twenty-three units of packed RBCs with adjusted hematocrits (Hct) between 67% and 87% were used. The units were 7–28 days old. Seven samples were taken from each unit (fig. 1) and diluted to create RBC/RL volume ratios of 5:1, 4:1, 3:1, 2:25:1, 2:1, and 1:1, respectively. Ionized calcium and pH were measured using a Radioneter ICA-1 ionized calcium analyzer (Radiometer America, Westlake, OH). Total calcium was measured using an alizarin sulfonate calcium procedure (Encore® Centrifugal Analyzer, Baker Instruments Corp., Allentown, PA). All samples were stirred intermittently over 1–2 h to detect any evidence of clot formation. The presence of fibrin strands was reported as cloting. Excess CaCl2 was added to a separate undiluted specimen from each RBC unit and all specimens clotted.

Nineteen additional RBC units were used to compare the amount of particulate matter recovered from RL versus NS diluted RBC. These units were collected in anticoagulant citrate phosphate dextrose adenine solution (CPDA-1). The RBC were filtered three times using desiccated Fall 40 micron screen filters (fig. 2). Filtration was performed on the undiluted RBC to remove accumulated microaggregates (filter 1). Each unit was divided into two aliquots of 110 mL, each of which were diluted with 110 mL of either NS or RL. The RBC were stirred manually during dilution to insure mixing. Filtration was performed on the diluted RBC before warming (filter 2) and after warming (filter 3). Fenwal blood/fluid warmers heated the mixtures from 22° C to 37° C. The two mixtures were filtered at the same time in parallel systems to eliminate the “standing time” variable. Infusion pumps were used to maintain a constant flow (504 mL/h) which resulted in a maximum time from mixing to complete infusion of 30 min. The
Pair-wise associations among ionized calcium concentration, total calcium concentration, pH, dilution, and hematocrit were evaluated using Pearson’s linear correlation coefficient. For those calculations, dilution specific hematocrits were calculated using the starting hematocrit of the undiluted RBC unit. A linear logistic model (see Appendix) was used to estimate the probability of clotting and to assess the predictive significance of potential explanatory variables of the clotting phenomenon.

The change in weight of the filter used for RL diluted RBC filtration was compared to the corresponding filter used for NS diluted RBC filtration. The data were analyzed as a two-factor repeated measures design, with each factor at two levels: dilution type (NS, RL) and warming (before, after).

**Results**

Samples from 23 CPDA preserved RBC units were analyzed for total calcium, ionized calcium, and pH at each of seven dilutions, giving a total of 161 samples. Descriptive statistics by dilution are given in Table 1 for each measured variable.

The average total calcium concentration of RBC/RL mixtures decreased from 2.17 mM/l for undiluted samples to 1.64 mM/l for 1:1 diluted samples. This decrease is due to dilution of the plasma calcium concentration (2.2–2.7 mM/l) by RL calcium concentration (1.23–1.50 mM/l).

The ionized calcium concentration (Ca++) increased as the volume of RL increased. The undiluted samples contained 0.089 ± 0.021 mM/l Ca++ (range, 0.05–0.14 mM/l), while the 1:1 diluted samples contained 0.296 ± 0.063 mM/l Ca++ (range, 0.18–0.46 mM/l). The ionized calcium concentration increased more at each dilution in those units with a greater starting Hct because the decreased plasma volume provided less "excess" citrate to chelate the added calcium (fig. 3).

The average pH of all 161 samples was 6.80 ± 0.15 (range, 6.46–7.03). The pH of the undiluted samples was 6.78 ± 0.15 (range, 6.46–7.01). The pH for the 1:1 dilution was 6.85 ± 0.15 (range, 6.53–7.02). The seven samples from a given RBC unit had a maximum difference of 0.25 pH units (data not shown).

Evidence of clot formation was observed in 14 of the 161 RL diluted samples. Ten of the 14 observations occurred in samples diluted 1:1 (starting Hct range, 75–87); two occurred in samples diluted 2:1 (starting Hct = 87); and two occurred in samples diluted 2.25:1 (starting Hct = 87). Ionized calcium concentrations for these 14 samples ranged from 0.27–0.46 mM/l (fig. 3).

There were seven additional samples (1:1 dilution) with ionized calcium values greater than 0.27 mM/l that
showed no evidence of clot formation. The remaining 140 samples had measured values less than 0.27 mM/l, and showed no evidence of clot formation.

Linear correlation coefficients (r) were calculated at each dilution for every pair of variables shown in Table 2. The six possible correlations are plotted by dilution in figure 4. The interrupted lines drawn at r = ±0.41 represent the value of r that must be achieved to declare statistical significance at the 0.05 level for a two-tailed test. The correlation between dilution specific hematocrit and ionized calcium is the only relationship that is consistently significant for the diluted samples, and the significance of the relationship increases as the dilution increases (P = 0.0082 at 5:1 to P < 0.0001 at 1:1). The only other significant correlation was for ionized calcium versus pH at the 5:1 dilution (r = -0.42, P = 0.0449).

No gross clotting was observed in the 19 RBC units which were split, diluted, and filtered. Net weight gain of the filters is shown in figure 2. The particulate matter trapped in filter #1 represents the microaggregates which accumulated in the RBCs during storage. Considerably more particulate matter (mean = 412 mg) was recovered from this filter (undiluted RBCs) than was recovered from either RL or NS diluted RBCs (20–25 mg). The interaction between dilution type and warming was not significant (P = 0.8166), and there was no statistically significant difference between the net weights of filters for RL diluted RBCs and filters used for NS diluted RBCs at 22° C (P = 0.1362) or at 37° C (P = 0.2572).

Discussion

Calcium in blood exists in a dynamic equilibrium between the ionized fraction which is active in coagulation and the bound fraction. CPDA anticoagulated blood contains more citrate than is necessary to chelate the ionized calcium in the unit of blood. The amount of excess citrate is a function of the donor’s blood calcium level and the total amount of blood collected in the unit (450 ± 45 ml in 63 ml of CPDA solution). The molar ratio of citrate to ionized calcium, as reported in the literature, ranges from 12.5:1 to 8:1.7,8 Historically, this excess was considered necessary, since there was occasional clot formation when lesser amounts of citrate were used for blood stored in glass containers. Mishler

![Ionized Cal./Hct./Dilution](image-url)

**FIG. 3.** Starting hematocrit and post-dilution ionized calcium concentration were plotted at various dilutions of RBC with RL. Linear fit lines were calculated for the 23 samples at each dilution. (r:0.531-0.751; P < 0.05). Clotted samples (c) are shown. The rectangle in the lower left corner is bounded by the threshold ionized calcium concentration and the maximum standard hematocrit for RBC. No samples clotted within these limits.
TABLE 2. Estimated Coefficients ($\beta$) and the Likelihood Ratio Statistic (LRS) for the Linear Logistic Model under Five Combinations of Explanatory Variables

<table>
<thead>
<tr>
<th>Model</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Variable</td>
<td>$\beta$</td>
<td>$[t]$</td>
<td>$\beta$</td>
<td>$[t]$</td>
<td>$\beta$</td>
</tr>
<tr>
<td>Hct (undil)</td>
<td>0.5399</td>
<td>3.34</td>
<td>0.2644</td>
<td>1.53</td>
<td></td>
</tr>
<tr>
<td>Dilution</td>
<td>22.7795</td>
<td>4.05</td>
<td>3.5787</td>
<td>0.40</td>
<td></td>
</tr>
<tr>
<td>Hct (actual)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ionized Ca</td>
<td>43.1377</td>
<td>2.43</td>
<td>56.9466</td>
<td>3.63</td>
<td>3.5860</td>
</tr>
<tr>
<td>Total Ca</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH Constant</td>
<td>-54.1897</td>
<td>-35.9388</td>
<td>-36.6942</td>
<td>-7.4939</td>
<td></td>
</tr>
<tr>
<td>LRS</td>
<td>58.31</td>
<td>67.96</td>
<td>67.25</td>
<td>33.89</td>
<td>64.20</td>
</tr>
<tr>
<td>d.f.</td>
<td>2</td>
<td>2</td>
<td>4</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>$P$</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>False Negatives*</td>
<td>5</td>
<td>3</td>
<td>4</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>False Positives†</td>
<td>2</td>
<td>3</td>
<td>3</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Total Error Rate</td>
<td>4.96%</td>
<td>3.73%</td>
<td>4.35%</td>
<td>8.70%</td>
<td>4.35%</td>
</tr>
</tbody>
</table>

* Sample did clot with probability <0.5 of clotting.
† Sample did not clot with probability ≥0.5 of clotting.

et al. demonstrated the adequacy of "half strength" citrate anticoagulant solutions in polyvinyl chloride collection bags. Reports of transient hypocalcemia with rapid transfusion of blood further highlight the presence of excess citrate. No minimum citrate concentration has ever been defined.

An evaluation of the "protecting effect" of the excess citrate must recognize the fact that citrate is dissolved in the plasma of the donor unit rather than the cells. Thus, as the hematocrit (per cent cells/volume) increases, the volume of plasma decreases resulting in a smaller absolute amount of plasma citrate to interact with any additional calcium. The Hct of RBCs prepared from blood collected in CPDA-1 should be no higher than 80. Twenty per cent plasma is necessary to ensure adequate glucose for cellular metabolism during storage and enough buffering material to maintain acceptable pH levels.

Blagdon and Gibson reported clot formation when whole blood was diluted 1:3 with "compound sodium lactate" solution, which contains 2 mM Ca++/L. They also observed clot formation when plasma reduced blood (RBC) was diluted 1:2 with "compound sodium lactate" solution. Their dilution is greater than any evaluated in this study. Barkoff et al., in an abstract, published data which showed no significant difference in microaggregate numbers or size when RBCs were reconstituted with normal saline versus lactated Ringer's solution or Plasmalyte A. Their volume ratio was 1.8:1 (180 ml of RBC to 100 ml of RL). Our results agree with these two studies. Ryden and Oberman reported trace amounts of clot formation at a whole blood:RL volume ratio of 1:1, and frank clotting at a blood:RL ratio of 1:5. Shackford et al. compared the administration of RBC diluted with 150–250 ml of RL to whole blood administration in patients undergoing aortic reconstructions. Fourteen patients received 7.1 ± 0.6 RBC units which were reconstituted with RL and infused through a 125-micron filter. They were com-

Pairwise Linear Correlations by Dil.

Fig. 4. Correlation coefficients were calculated for pairs of explanatory variables (1. Ion. Cal./Hct; 2. Ion. Cal./Tot. Cal.; 3. Hct/pH; 4. Tot. Cal./pH; 5. Tot. Cal./Hct; 6. Ion. Cal./pH). Interrupted lines ($r = \pm 0.41$) represent the minimum "r" value needed for statistical significance at the 0.05 level in a two-tailed test. The correlation between ionized calcium and hematocrit (1) is statistically significant at all dilutions.

Dilution Level: Vol (RL)/Vol (RL + RBC)
pared to 14 patients who received 6.2 ± 0.6 units of whole blood. No change in pulmonary capillary wedge pressure, cardiac index, or shunt fraction occurred. The colloid osmotic pressure was decreased in patients who received RBC and RL. Pulmonary vascular resistance was calculated, and no evidence of pulmonary hypertension was found.§

Dilution of RBCs is often necessary in the operating room when rapid administration of blood is required. Calkins et al.17 showed that dilution decreases viscosity and decreases hemolysis, and recommended at least 100 ml of diluent per unit of RBCs.

Two critical questions have been asked to determine the efficacy of using RL as a diluent for RBC: 1) Does a threshold level for ionized calcium exist below which clotting will not occur? and 2) Is there a clinically useful method for estimating ionized calcium levels when RBCs are diluted with RL?5

Various logistic models were investigated to determine which explanatory variable or combination of variables could reliably predict the probability of a clot (table 2). Models, using 1) ionized calcium (Model 5), 2) starting hematocrit and dilution level (Model 1), or 3) these three variables combined (Model 2), have significant likelihood ratio statistic (LRS) values18 (see Appendix), which indicated that at least one of the explanatory variables for each model is a statistically significant risk factor for clotting ($P < 0.0001$).

Probability nomograms for Model 1 are shown in figure 5. For example, if the undiluted Hct is 90 and the dilution level is 3:1, then the probability of a clot is about 0.40. If the dilution level is changed to 2:1, the probability of a clot increases to about 0.80. When the Hct is 80, the probabilities of a clot are <0.01 and 0.025, respectively. The probability nomogram for Model 5, which uses ionized calcium as a singular explanatory variable, is shown in figure 6.

Our results indicate a range of usefulness for lactated Ringer’s solution as a diluent for red blood cells which does not produce statistically significant increases in microaggregate filter weight when compared with samples similarly diluted with normal saline. In addition, our data show that an ionized calcium value above 0.23 mM/L is needed to activate coagulation, and that the critical value is not exceeded when RBC units are diluted with lactated Ringer’s solution in a RBC/RL volume ratio greater than 2:1.

We conclude that lactated Ringer’s solution can be used as a diluent for red blood cells in those situations where dilution and administration proceed within 30 min. When a unit of red blood cells is diluted with 100 ml of lactated Ringer’s solution, mixed well during dilution, and rapidly administered, the probability of achieving an ionized calcium concentration which will activate the coagulation cascade in the bag is minimal.

§ Personal communication.

FIG. 5. Probability nomogram: The probability of clot formation can be determined by using the starting hematocrit and dilution level. The diamond marks the maximum expected hematocrit and the maximum recommended dilution level (RBC:RL = 2:1). Formulas for calculating probability lines are given in the Appendix.

FIG. 6. Probability nomogram: The probability of clot formation can be determined by using ionized calcium as the sole explanatory variable. The formula for calculating probability is given in the Appendix.
The authors gratefully acknowledge the work of Mrs. Sara Patel, MT(ASCP)SBII, blood bank technologist; Ms. Pat Huber, anesthesia technician; and the UTMB Clinical Chemistry laboratory staff under the supervision of Dr. Gary Graham.

Appendix

The linear logistic model was used to estimate the probability of clotting and to assess the relative significance of potential explanatory variables of the clotting phenomenon. Logistic model parameters were estimated using a computer program which employs the maximum likelihood method of Walker and Duncan. The approach is to find appropriate estimates of the unknown parameters of the vector β in the model

\[ P = \frac{1}{1 + \exp(-X'\beta)} \]

where P denotes probability of success (clotting); exp denotes the exponential constant (e); and X denotes the vector of explanatory or independent variables. After parameters are estimated, a measure of goodness of the resulting model is given by the likelihood ratio statistic (LRS) which has a chi-squared distribution with \( \kappa \) degrees of freedom, where \( \kappa \) is the number of explanatory variables.

Various logistic models were investigated to determine which explanatory variable or combination of variables could predict the probability of a clot with the least number of misclassifications. A misclassification is defined to occur if the predictive model indicates a probability of clotting to be \( \geq 0.5 \), but the sample did not clot, or if the model gives the probability of clotting as \( < 0.5 \), but the sample did clot.

Possible explanatory variables considered in the study were starting Hct, dilution specific Hct, dilution level, ionized calcium, total calcium, and pH. Dilution level when used as an explanatory variable was coded as 0 = undiluted, 1/6 = 5:1, 1/5 = 4:1, ..., and 1/2 = 1:1. The variable Hct (undil) represents the starting hemocrit value for the undiluted sample, and was used in those models which incorporated the dilution level as an explanatory variable. In some models, the dilution specific hemocrit value, denoted by Hct (actual), was calculated and used as the explanatory variable.

Of the many models tested, five were chosen for presentation (table 2). All five models have significant LRS values (\( P < 0.0001 \)) which indicated that at least one of the explanatory variables for each model is statistically significant risk factor for clotting. For example, when Hct (actual), ionized calcium, total calcium, and pH are simultaneously fitted (Model 3), the total fit is highly significant (LRS = 67.25, \( P < 0.0001 \)), but total calcium and pH are relatively insignificant in comparison to ionized calcium and Hct (actual) as determined by the absolute value of \( \Gamma \). The variable pH was used in several models not presented here, and for the narrow range of our pH data showed no value as a risk factor for clotting when used either singularly or in combination with other variables.

Model 2 is the best model in terms of its LRS value (67.96) and in terms of the total number of false negatives and false positives (total error rate = (3 + 3)/100/161 = 3.73%). However, Model 2 requires knowledge of the ionized calcium value to estimate the probability of a clot. In contrast, Model 1, with a total error rate of 4.96% (six false negatives, two false positives), requires only knowledge of the undiluted Hct and the dilution level. Thus, from a clinical standpoint, it appears that Model 1 would be the model of choice.

Parameter estimates \( \beta \) shown in table 2 may be used to estimate the probability of clotting by calculating

\[
\phi = \ln \left[ \frac{P}{1 - P} \right] = \beta_0 + \sum \beta X
\]

for a given sample, where \( \beta_0 \) is the constant and the summation is over the number of explanatory variables in the model. Then

\[
P = \exp(\phi)/(1 + \exp(\phi))
\]

estimates the probability of a clot for the \( j \)th sample. For example, if the undiluted sample has an Hct = 82 and a 4:1 dilution is to be used (coded Dilution = 1/5) then, by Model 1,

\[
\phi = -54.1897 + 0.5339 \text{ Hct (undil)} + 22.7795 \text{ Dilution} = -5.854
\]

and

\[
P = \exp(-5.854)/(1 + \exp(-5.854)) = 0.00286
\]

Thus, the estimated probability of a clot for a sample with an undiluted Hct = 82 at the 4:1 dilution is 0.00286. However, if the dilution level is changed from 4:1 to 1:1 (coded Dilution = 1/2) then (\( \phi \) = 0.97985, and the estimated probability of a clot is increased to \( P = 0.72707 \).

Since \( \phi \) for Model 1 is a linear function of two explanatory variables, probability nomograms may be depicted as straight lines in the undiluted Hct by Dilution plane. For example, Pr(clot) = 0.5 when (\( \phi \) = 0 which, for Model 1, implies that

\[
-54.1897 + 0.5339 \text{ Hct (undil)} + [22.7795 \times \text{ Dilution}] = 0,
\]

and any pair of values for Hct (undil) and Dilution that satisfy this equation would result in the estimate of 0.5 for the probability of a clot.

References