The Effect of the Plasticizer Di-2-Ethylhexyl Phthalate on the Survival of Stored RBCs

By James P. AuBuchon, Timothy N. Estep, and Richard J. Davey

Recent in vitro studies have shown that di-2-ethylhexyl phthalate (DEHP) inhibits the deterioration of RBCs during refrigerated storage in containers that use this compound as a plasticizer. The experiments described in this report were designed to assess whether this in vitro protective effect of DEHP would result in a prolonged in vivo survival of RBCs infused into normal human recipients. Whole blood collected from ten normal donors was stored for 35 days in citrate-phosphate-dextrose-adenine (CPDA-1) anticoagulant contained in polyvinylchloride (PVC) bags plasticized with DEHP or a trimellitate compound that is known to have low leachability. Aliquots of RBCs from each container were then labeled with chromium-51 and were rein infused into the original donors. For blood stored in DEHP-plasticized PVC bags, 24% more red cells survived in vivo 24 hours after reinfusion than was observed when the blood had been stored in trimellitate-plasticized bags (P < .001). Whole blood stored in glass bottles showed a similar improvement in in vivo survival when DEHP was added in weekly increments to mimic the accumulation of this plasticizer seen during storage in plastic containers. Survival of packed red cells stored in the presence of DEHP increased by 14% compared with storage in trimellitate-plasticized bags (P < .05). In agreement with previous studies, hemolysis and microvesicle formation were also reduced in the presence of DEHP. These results suggest that proposed new storage systems lacking DEHP should be carefully evaluated to determine whether adequate post-transfusion survival of RBCs may be achieved.

This is a US government work. There are no restrictions on its use.

MATERIALS AND METHODS

Research subjects entered the study with approval of the human use committees of the National Heart, Lung, and Blood Institute of the National Institutes of Health (NIH); the University of Rochester, and the New York State Department of Health; and after giving informed consent. All donors met the requirements of the American Association of Blood Banks and of the American Red Cross Blood Services with the exception that deferrals related to the transmission of infectious agents were waived.

This study was conducted in three phases (Table 1). In each phase the effect of storage containers on red cells was analyzed in a paired fashion, with each subject donating blood for storage in containers with and without DEHP. At each donation 450 ± 45 mL blood were collected into a collection vessel containing 63 mL of citrate-phosphate-dextrose-adenine-1 (CPDA-1) and stored at 4°C for 35 days with weekly gentle inversion. The sequence of storage containers to be used was determined by a randomized selection process. Donations were at least 8 weeks apart.

Plasma to be used for analytic determinations was separated from an aliquot of whole blood (note 1, Table 1) by centrifugation at 1,100 g for 10 minutes. Plasma to be made free of microvesicles was then spun again at 50,000 g for 60 minutes at 4°C. The purity of this microvesicular preparation was confirmed by transmission electron microscopy, courtesy of Dr William Mayers, NIH. Hemoglobin determinations were performed on plasma and ultracentrifuged plasma by spectrophotometric analysis. Other units were also used to investigate the rate of DEHP accumulation in stored blood. Nine units of blood drawn into PL146 packs in CPDA-1 for therapeutic purposes (hemodiosis-8, polycythemia-1) from other donors were stored at 4°C and sampled weekly for DEHP content.

RBC survival was assessed by the infusion of 51Cr-labeled cells into the original donor. 51Cr survivals were performed on day 35 on aliquots taken from systems A to F according to standard, single-label methodologies. Red cells (1 to 2 mL) were incubated at room temperature for 30 minutes with 20 µCi 51Cr before two washes with saline. Samples were taken at 0, 5, 7.5, 10, 15, 30, and 60 minutes and 24 hours after injection. These methods were validated previously in this laboratory by the evaluation of RBC survivals under conditions used by other investigators and gave survival values that were consistent with published data.

Direct antiglobulin tests were performed on aliquots of phase III units after 35 days of storage using anti-C3 reagents (American Red Cross, Bethesda, MD).

Blood samples taken for various analyses were stored either...
EFFECT OF DEHP ON RED CELL SURVIVAL

Table 1. Experimental Protocol

<table>
<thead>
<tr>
<th>Phase</th>
<th>Component Stored</th>
<th>Group</th>
<th>Container*</th>
<th>Additions</th>
<th>Subjects Participating</th>
</tr>
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<tbody>
<tr>
<td>I</td>
<td>Whole blood</td>
<td>A†</td>
<td>PVC-TEHTM‡</td>
<td>None</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B</td>
<td>PVC-DEHP§</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>Whole blood</td>
<td>C†</td>
<td>Glass</td>
<td>None</td>
<td>5‡</td>
</tr>
<tr>
<td></td>
<td></td>
<td>D</td>
<td>Glass</td>
<td>DEHP#</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>Packed red cells (Hematocrit: 75%)</td>
<td>E†</td>
<td>PVC-TEHTM</td>
<td>None</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>F</td>
<td>PVC-DEHP</td>
<td>None</td>
<td></td>
</tr>
</tbody>
</table>

*All blood collected into CPDA-1 anticoagulant and stored for 35 days. Blood collected directly into final storage container. Approximately 60 mL of blood removed on days 0 and 35 for analytic determinations.
†Blood drawn from donor using an aluminum-hubbed needle and polyethylene tubing. Standard blood-drawing sets used for other collections.
‡PVC bag plasticized with tri-2-ethylhexyltrimellitate, a plasticizer of low leachability and red cell effect (PL1240;Fenwal, Deerfield, IL).24,25
§PVC bag plasticized with di-2-ethylhexyl phthalate (PL146;Fenwal).
‖Sterile, vented 1-L glass bottles (Travenol, Deerfield, IL). Bacterial cultures performed on day 28.
These subjects also participated in phase I. Two system D units became bacterially contaminated and were not used.

#In group D samples, 20 mL of plasma were harvested (1, 100 g for five minutes) from the day 0 aliquot, removed as stated above. This was frozen in sterile tubes in 4 mL aliquots. On days 0, 7, 14, 21, and 28, 0.8 mL DEHP (99.9 % purity; Eastman, Rochester, NY) was added to one of these plasma aliquots and incubated at 37 °C for two hours with periodic agitation. The plasma was then centrifuged (500 g for five minutes), and 2 mL of plasma were added aseptically to the storage container with gentle agitation.

Whole blood samples to be analyzed for DEHP content were incubated at 56 °C for 30 minutes to inactivate plasma lipases and then frozen at −65 °C. Chloroform/methanol extracts of each sample were prepared and analyzed by gas-liquid chromatography using an internal standard.23 The limit of detection of DEHP with this technique was 7 μg/mL, below the concentration at which red cell effects have been noted in in vitro studies.13–16

The ATP concentration of whole blood on days 0 and 35 was determined after trichloroacetic acid precipitation of proteins at 4°C (Sigma Chemical Co, St Louis).18

To assess whether DEHP affected the uptake or elution of 51Cr by RBCs, an additional 10 units of whole blood were drawn and divided in half, with one half of each unit stored in a DEHP-plasticized PVC container and the other half stored in a PVC bag plasticized with tri-2-ethylhexyltrimellitate (TEHTM). After 35 days of storage a 30-mL aliquot of blood was removed from each bag and was labeled with 2 μCi 51Cr according to the procedure described above. At the conclusion of labeling the RBCs were centrifuged for five minutes at 1,100 g and were resuspended in normal AB plasma to a hematocrit of 0.40 to 0.45. Each cell suspension was then divided among four containers and the other half stored in a DEHP-plasticized PVC bag for five minutes or 24 hours. The activity of 51Cr was determined by the measurement of radioactivity in the plasma of each of these samples after the centrifugal sedimentation of RBCs.

Data are expressed as the arithmetic mean ± SEM. All survival data were paired to obviate problems associated with donor-to-donor variability.27 Data were analyzed by applying the two-tailed paired t test or the Mann-Whitney rank sum test, with a probability of 0.05 used to reject the null hypothesis.28

RESULTS

RBCs stored in systems containing DEHP exhibited better survival after transfusion than did red cells stored in similar containers that lacked DEHP (Table 2). After storage for 35 days as whole blood, red cells stored in PVC bags with DEHP (system B) exhibited a 24-hour post-transfusion survival of 84.1% ± 2.13%, 17% higher than that of red cells stored as whole blood in PVC bags lacking DEHP (system A: 69.9% ± 2.99%, P < .001). Only three pairs of data from phase II could be analyzed because of inadvertent bacterial contamination of two system D units. Despite this small sample, storage of whole blood in glass bottles to which DEHP was added (system D) resulted in a 24-hour red cell survival, 81.7% ± 2.23%, that was statistically significantly higher than storage in system C, which lacked DEHP.

Table 2. Effect of DEHP on Post-Transfusion Survival of Red Cells

<table>
<thead>
<tr>
<th>Storage System</th>
<th>Container Type</th>
<th>Subjects Studied</th>
<th>24-h Survival (%)</th>
<th>T&lt;sub&gt;50&lt;/sub&gt; (0-10 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phase I</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>PVC-TEHTM (PL1240)</td>
<td>10</td>
<td>69.9 ± 2.99</td>
<td>32.4 ± 5.16</td>
</tr>
<tr>
<td>B</td>
<td>PVC-DEHP (PL146)</td>
<td>84.1 ± 2.13</td>
<td>76.8 ± 12.4</td>
<td></td>
</tr>
<tr>
<td>Phase II</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>Glass, No DEHP</td>
<td>3</td>
<td>73.3 ± 2.69</td>
<td>20.5 ± 2.50</td>
</tr>
<tr>
<td>D</td>
<td>Glass + DEHP</td>
<td>81.7 ± 2.23</td>
<td>22.7 ± 4.56</td>
<td></td>
</tr>
<tr>
<td>Phase III</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>PVC-TEHTM (PL1240)</td>
<td>8</td>
<td>66.5 ± 3.29</td>
<td>42.7 ± 8.32</td>
</tr>
<tr>
<td>F</td>
<td>PVC-DEHP (PL146)</td>
<td>75.8 ± 1.87</td>
<td>61.1 ± 16.1</td>
<td></td>
</tr>
</tbody>
</table>

*T<sub>50</sub> (0-10 min) is the time taken for survival to fall to 50% of original value according to regression line for survival curve during first ten minutes after reinfusion.

*Pairs statistically different by paired t test, P < .001.
†Pairs statistically different by paired t test, P < .05.
(73.3% ± 2.69%, \( P < .05 \)). Survival differences were not attributable to the type of container, PVC vs. glass. The survival of red cells stored as packed red cells was lower than that of cells stored as whole blood. In phase III the presence of DEHP in the storage system was again associated with improvement in post-transfusion survival. Red cells stored in a PVC bag with DEHP (system F) had a post-transfusion survival of 75.8% ± 6.6% vs. 76.8% ± 6.0% for PVC plasticized without DEHP (system E: 66.5% ± 3.29%; \( P < .05 \)).

The difference in survival of red cells after storage was quantitatively attributable to the altered rate of disappearance of red cells from circulation during the first ten minutes after infusion (Fig 1). For example, in phase I, the \( { }^{51} \text{Cr} \) T50 for this period was 32.4 ± 5.16 minutes for red cells stored in PVC plasticized without DEHP (system A) and 76.8 ± 12.4 minutes for the PVC-DEHP system (system B; \( P < .001 \)). The rate of red cell disappearance from circulation from ten minutes onward was not significantly different between storage with and without DEHP (Fig 1).

In agreement with previously reported studies, DEHP accumulated in units of blood stored in PVC bags that used this compound as a plasticizer. During 35 days of storage in system B, the whole blood concentration of DEHP had risen from undetectable levels to 146 ± 12.6 µg/mL. By performing a least-squares linear interpolation of the data derived from the units stored in system B and nine therapeutic phlebotomy units, the accumulation of DEHP in PL146 whole blood units could be expressed as:

\[
24.0 + 3.29 \text{µg/mL} \times (\text{days of storage})
\]

\( n = 49, r = 0.8010, P < .0001 \).

DEHP was successfully solubilized in autologous plasma for addition to the glass bottles of system D. The five additions added 49.0 ± 9.6 mg DEHP to the system. The concentration of DEHP rose throughout storage and was 19.0 ± 0.60 µg/mL on day 35.

Red cells from phase III systems were tested for the presence of C3 on their surface by a direct antiglobulin test using anti-C3 after 35 days of storage. All samples from systems E and F were negative.

The paired in vitro study of red cells stored in PVC with or without DEHP and then subjected to radiolabeling and in vitro elution estimation demonstrated similar uptake and elution of the \( { }^{51} \text{Cr} \) label regardless of the plastic formulation used to store the red cells (Table 3).

Also in agreement with previous studies, the presence of DEHP did not alter the disappearance of ATP from red cells during the storage period. It did mitigate the normal increase of plasma hemoglobin accumulation, however (Table 4). The relative increase in plasma hemoglobin concentration in blood stored without DEHP was accompanied by an increase in microvesicle content, but the latter could not quantitatively account for the total increment of plasma hemoglobin concentration.

### DISCUSSION

The post-transfusion survival of RBCs stored under refrigeration for 35 days in CPDA-1 anticoagulant in DEHP-plasticized PVC containers was an average 17% to 24% higher than the survival of cells stored in containers plasticized with TEHTM. Enhanced survival was also observed when DEHP was added to whole blood stored in glass bottles, offering further evidence that the improved post-transfusion survival was indeed attributable to the presence of plasticizer. This improvement was not due to an effect of DEHP on \( { }^{51} \text{Cr} \) uptake or elution, since there was no difference in the efficiency of uptake or rate of elution of the radiolabel after storage with or without DEHP.

The smaller effect of DEHP on red cells stored in glass bottles compared with PVC containers is probably related to the relatively lower concentration of DEHP achieved in the glass containers. However, a confounding, detrimental effect of glass per se on RBCs during storage cannot be excluded in light of the historic evidence for such a phenomenon. The lower DEHP concentration achieved on addition to glass

<table>
<thead>
<tr>
<th>Storage Container</th>
<th>Labeling Efficiency</th>
<th>Proportion of Radiolabel Eluting (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PVC-TEHTM (PL1240)</td>
<td>96.9% ± 0.36%</td>
<td>0.28 ± 0.052 0.52 ± 0.20 0.54 ± 0.068 1.7 ± 0.13</td>
</tr>
<tr>
<td>PVC-DEHP (PL146)</td>
<td>96.4% ± 0.51%</td>
<td>0.33 ± 0.057 0.39 ± 0.066 0.61 ± 0.087 1.9 ± 0.19</td>
</tr>
</tbody>
</table>

Whole blood from 11 single donations was split between PVC bags lacking (PL1240) or containing (PL146) DEHP and stored for 35 days in CPDA-1. After \( { }^{51} \text{Cr} \) labeling, red cells were held in AB plasma at 37°C, and plasma radioactivity was determined as a percentage of the radioactivity originally taken up by the red cells. There were no statistically significant differences in the uptake or loss of the radiolabel between red cells stored in the two bag types.
bottles may have been due to hydrolysis of the phthalate ester during the incubation of plasma at 37°C necessary to effect rapid plasticizer dissolution.29,30 (This method of plasticizer dissolution was selected over incubation of plasma in PVC-DEHP bags to avoid dissolution of other components of the plastic bag that might leach into plasma.)

As has been demonstrated in other in vitro studies, the presence of DEHP in clinically relevant quantities does not directly affect the internal metabolism of red cells.31,34,36 We documented no effect of DEHP on ATP levels; previous studies have also shown that 2,3-DPG levels and intracellular electrolyte concentrations are not affected by the presence of DEHP.34

While other mechanisms cannot be completely excluded, the enhanced post-transfusion survival of red cells stored in the presence of DEHP probably reflects a protective effect of this compound with regard to the deterioration of the cell membrane. Previous in vitro studies have demonstrated that RBCs stored in the presence of DEHP exhibit more normal morphology, less hemolysis, lower osmotic fragility, and better filterability than do comparable cells stored in the absence of this plasticizer.31,34,36 Each of these parameters is believed to reflect the integrity of the cell membrane. Cell morphology, in particular, has been correlated closely with in vivo cell survival.31,32,33 The results reported in this communication demonstrate that the improvement observed during RBC storage in the presence of DEHP as assessed by in vitro measurements is indicative of an improvement in in vivo cell survival.

It is interesting to note that improved RBC survival due to the presence of DEHP is manifested in blood storage systems that are currently in widespread use. This suggests that the removal of DEHP from such systems could result in decreased red cell post-transfusion survival if no compensatory additives were used to replace the protective effects of DEHP. This in turn suggests that cell viability should be carefully evaluated in any new storage system in which DEHP is eliminated or reduced.

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