The Preservation of Erythrocytes at Liquid Nitrogen Temperatures with Hydroxyethyl Starch: the Removal of Hydroxyethyl Starch from Erythrocytes after Thawing

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Hydroxyethyl starch (HES) has been shown to protect erythrocytes from freeze-thaw damage when these cells are frozen rapidly in liquid nitrogen and stored in the vapor phase at −145° C (8, 14). This alkylated starch is a product of the reaction of ethylene oxide and amylopectin (7). The viscosity and molecular weight of the material is adjusted by acid hydrolysis. The product used in this study has a weight average molecular weight (MW) of 120,000 and a weight number molecular weight (MN) of 41,000.[1] The large discrepancy between these two figures is explained by the definition of the two terms; the MW describes average particle weight while MN refers to the number of molecules present. The ratio of the two describes the heterogeneity of the polymer.

Because of the high viscosity of erythrocyte–HES mixture, administration to patients is relatively slow. Therefore, washing the cells after thawing appears to be advantageous if such cells are to be more rapidly available for routine clinical use. In our experience, the transfusion of a full unit of frozen thawed erythrocytes, containing 75 gm of HES, to patients produced no adverse effects. However, lowering the viscosity by removal of all or part of the HES after thawing would allow more rapid administration of the product. This paper will describe a method for washing red blood cells that appears promising. To assess the effectiveness of the freeze-thaw cycle, red blood cell saline stability, total red cell recovery, and supernatant hemoglobin levels were determined prior to freezing, after thawing, and after the final wash. In ten of the samples K+ and ATP levels were also determined.

METHODS

Blood was obtained from donors using ACD–A as an anti-coagulant in polyvinyl chloride bags. In some instances, blood bank blood was used instead of fresh donor blood. The blood was centrifuged at 1500*g* to pack the erythrocytes. The plasma was removed, and the cells were washed three times with equal volumes of 0.154 N NaCl. After the last wash the packed cells were suspended in a mixture of NaCl and HES so that the final concentration of HES was 14%.

A 30 ml aliquot of blood–HES mixture was placed in a 9 cm² plastic bag (Hemoflex TM).[2] This plastic is 3/1000 of an inch thick. After sealing the bag it was inserted in an outer envelope of Hemoflex (11 cm² × 3/1000 in thick) and placed in a rubberized spring loaded holder (Fig. 1). The holder was then immersed in liquid nitrogen and agitated at 160 cpm using a BP-2 Blood Processing Machine. This system was designed by the Linde Division of the Union Carbide Corporation, and consists of a compressed air actuated arm driven by a wrist action shaker. The frozen blood–HES mixture was removed from the holder and stored at −145° C.

Prior to thawing, the outer bag was removed and the inner bag containing the frozen blood was then placed in the rubberized holder, and agitated by the machine at 160 cycles per minute for 40 sec in a 47° C circulating water bath. The final temperature of the blood was 20° C.

Postthaw parameters included the following determinations: hematocrit, supernatant hemoglobin (15), the stability of the cells in 0.154 N NaCl (13), and the immediate postthaw recovery of the intact erythrocytes (8). The saline
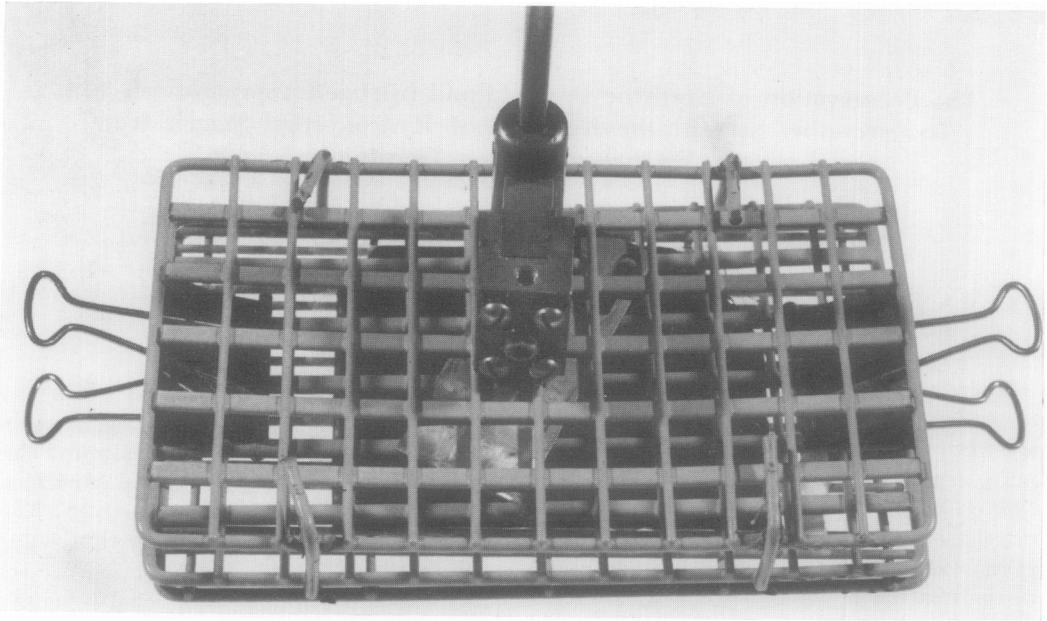
Fig. 1. Rubberized spring loaded holder containing a 30 ml aliquot of blood–HES mixture placed in a 9 cm plastic bag.

stability was determined in the following manner: A small volume of thawed blood (0.4 ml) was diluted 1:40 with 0.154 N NaCl. Two milliliters of this suspension were lysed in 8 ml of 0.1% Na_2CO_3. After 30 min, the saline suspension was centrifuged and the hemoglobin level was determined on the supernatant and compared to the completely lysed suspension to determine the percentage of stable cells as calculated in the following equation:

$$\frac{Hgb(NaCl) \times 40}{Hgb(Na_2CO_3) \times 200} \times 100$$

The percentage of cells recovered immediately postthaw was determined by lysing 0.1 ml of blood in 19.9 ml of 0.1% Na_2CO_3. A whole blood sample was then centrifuged, and 0.5 ml of plasma was added to 9.5 ml of 0.1% Na_2CO_3. The plasma hemoglobin was compared to the total hemoglobin and recovery was calculated thus:

$$100 - \frac{Plasma\ Hgb \times 20}{Total\ Hgb \times 200} \times 100 - HCT$$

The final recovery of washed blood was calculated from the total amount of hemoglobin contained in the mixture prior to washing, and the amount of hemoglobin recovered in the washes as expressed in the next equation.

$$\frac{(Total\ Hgb) - Hgb\ in\ wash}{Total\ Hgb} \times 100$$

The hemoglobin levels used to calculate recovery and saline stability were determined with a Coleman Jr. spectrophotometer at 540 nm. Supernatant hemoglobin levels after thawing were determined as described by Shinowara (15) using a double beam Hitachi-Perkin Elmer recording spectrophotometer. The absorbancy at 575 and 560 nm were compared to determine the supernatant hemoglobin content of washed cells. If the cells were not washed an extra reading at 450 nm was added to correct for bilirubin content (15)

After thawing, a 5 ml aliquot of packed cells was suspended in 10 ml of Normasol-R. This is a balanced solution containing 9×10^{-3} M NaCl, 2.7×10^{-3} M sodium acetate, 2.3×10^{-3} M sodium gluconate, 5×10^{-4} M potassium chloride, and 1.5×10^{-4} M magnesium chloride. The pH is adjusted to 7.4 by the addition of HCl to a final concentration of 1×10^{-3} M. The cell suspension was then centrifuged for 10 min at 2,000g. The supernatant was removed and saved, and the

cells were washed four additional times with 10 ml of Normasol-R. The hemoglobin content of the pooled washes was determined to calculate the total recovery of erythrocytes after processing.

The effect of freezing, thawing and washing erythrocytes in the presence of HES, upon intracellular potassium and ATP levels was determined using methods described by Boutwell (2) and Beutler (1). The stability of ATP during a three hour incubation in Krebs–Ringer Phosphate was also evaluated.

RESULTS

Table 1 shows results obtained from 48 samples of thawed and washed blood. The plasma hemoglobin ranged from 177 mg/100 ml to 820 mg/100 ml, but only nine of these aliquots had hemoglobin levels in excess of 500 mg/100 ml. The postthaw recoveries ranged from 96 to 99% and the saline stability from 77 to 89%. The

postwash recoveries ranged from 76 to 91% closely approximating the saline stability of the red cells obtained immediately postthaw. The supernatant hemoglobin following the last wash ranged from 20 to 57% mg. Sixteen of the 48 blood samples were 5–21 days old. These units all demonstrated high plasma hemoglobins after thawing, and had saline stabilities less than 80%. (It should be noted that in our experience with polyvinylpyrolidone (PVP) as the cryoprotective agent, bank blood over 5 days old did not tolerate the freeze process well). This observation has also been made by other investigators who have frozen erythrocytes protected by glycerol techniques. Tables 2 and 3 compare the data obtained with the samples whose saline stability was above 80% and those aliquots which fell below 80%. The overall recovery immediately postthaw and after the washing process was significantly greater among the group with the high stability than those with a lower saline

TABLE 1

POSTTHAW VALUES FOR ALL SAMPLES PROCESSED

	Mean	Standard deviation	Number of samples
Postthaw plasma Hgb	405 mg/100 ml	168 mg/100 ml	48
Saline stability	82%	4.12%	48
Postthaw recovery	98%	0.85%	48
Postwash recovery	83.5%	4.4%	48
Postwash Hgb	33.9 mg/100 ml	10.6 mg/100 ml	48

TABLE 2

POSTTHAW VALUES FOR SAMPLES WITH SALINE STABILITIES GREATER THAN 80%

	Mean	Standard deviation	Number of samples
Postthaw plasma Hgb	324 mg/100 ml	67 mg/100 ml	32
Saline stability	85%	2.2%	32
Postthaw recovery	98.3%	0.5%	32
Postwash recovery	85.7%	2.7%	32
Postwash Hgb	35.9 mg/100 ml	10.9 mg/100 ml	32

TABLE 3

POSTTHAW VALUES FOR SAMPLES WITH SALINE STABILITIES LESS THAN 80%

	Mean	Standard deviation	Number of samples
Postshaw plasma Hgb	553 mg/100 ml	188 mg/100 ml	16
Saline stability	77.4%	1.5%	16
Postthaw recovery	97.1%	0.86%	16
Postwash recovery	78.9%	3.5%	16
Postwash Hgb	29.9 mg/100 ml	8.8 mg/100 ml	16

stability. The plasma hemoglobins determined immediately after thawing were considerably lower, ranging from 177 to 325 mg/100 ml in the units with higher saline stabilities.

Table 4 shows the recovery and saline stability of cells before and after washing. The recovery as calculated from loss of hemoglobin during wash, compares closely to the saline stability of these cells prior to wash. The washed cells demonstrate saline stabilities in excess of 96% possibly suggesting a stable cell suspension at this point.

Table 5 shows changes in intracellular K^+ after freezing, and after washing. An average

TABLE 4

RECOVERY AND SALINE STABILITY OF WASHED CELLS

	Total Hgb (mg) prior to wash	Hgb (mg) recovered from wash	Recovery %	Saline stability (%)	
				Prewash	Post-wash
1	590	72	87.8	87	98
2	630	76	87.9	87	97
3	590	73	87.6	86	99
4	550	73	86.7	86	96
5	580	97	83.2	80	97
6	605	110	81.8	78	98
7	600	86	86.9	86	96
8	655	74	87.7	88	99

TABLE 5

CHANGES IN INTRACELLULAR K^+ AFTER FREEZING WITH 14% HES, THAWING AND WASHING

Sample	Erythrocyte K^+ (meq/l)				
	Prefreeze	Post-thaw	% Change	Post-wash	% change
1	89	70	21.4	65	27.0
2	81	66	18.6	52	35.9
3	93	80	14.0	70	24.8
4	98	81	17.4	69	29.6
5	86	74	14.0	67	22.1
6	102	91	10.8	82	19.7
7	84	73	13.1	60	28.6
8	93	81	13.0	71	23.7
9	96	82	14.6	74	23.0
10	97	90	7.3	79	18.6
N	91.9	78.9	14.4	68.9	25.3
SD	6.7	8.3	4.0	8.7	5.2
SE	2.1	2.6	1.8	2.8	1.6
11[a]	98	47	48.0	21	78.6

[a] This sample was frozen in 8% HES.

TABLE 6

CHANGES IN INTRACELLULAR ATP AFTER FREEZING, THAWING AND WASHING

	$ATP/\mu M/10^8$ Erythrocytes				
	Unfrozen	Postthaw	Post-wash	% Post-thaw	% Post-wash
1	1.90	1.65	1.50	13.2	21.0
2	1.77	1.60	1.52	9.6	14.0
3	1.72	1.49	1.40	13.4	18.6
4	1.86	1.70	1.61	8.6	13.4
5	1.67	1.58	1.60	5.4	4.9
6	1.93	1.89	1.74	2.1	9.8
7	1.72	1.67	1.62	2.9	5.8
8	1.83	1.71	1.63	6.6	10.9
9	1.91	1.72	1.59	9.9	16.7
10	2.03	1.90	1.79	6.4	11.8
N	1.83	1.69	1.60	7.8	15.7
SD	0.11	0.12	0.11	3.9	5.2
SE	0.04	0.04	0.04	1.2	1.6
11[a]	1.93	1.21	0.91	37.3	53.0

[a] Frozen in 8% HES.

immediate postthaw loss of 14% was observed followed by loss of another 10% after five washes in Normasol-R. The changes in erythrocyte ATP levels are shown in Table 6. An average decrease of 8% postthaw was observed, and washing resulted in the loss of another 8%. The stability of erythrocyte ATP during incubation for three hours was not significantly different in controls, thawed cells and washed cells. This data is shown in Table 7. When 8% HES is used for freezing erythrocytes, a 48% loss of potassium was observed after thawing. The ATP levels of the same cells decreased to 13% of control. These results are shown in the last table, Table 8.

DISCUSSION

In contrast to data presented by Meryman (11) showing an 87% loss of K^+ from erythrocytes frozen in 14% HES, we observed an average 15% loss of this ion immediately after freezing. Washing reduced the K^+ level to 70% of control values and further processing did not affect this level.

Meryman suggests that extracellular agents such as HES or PVP (12) protect erythrocytes from freeze-thaw damage by altering the membrane to allow a reversible efflux and influx of solute, thus preventing the formation of excessive osmotic gradients (11).

TABLE 7

EFFECT OF THREE HOUR INCUBATION UPON THE ATP LEVELS OF UNFROZEN, FROZEN AND THAWED, AND WASHED ERYTHROCYTES

| | ATP (μM/10^8 Erythrocytes) | | | | | | | | |
| | Unfrozen | | | Frozen-thawed | | | Washed | | |
	0	3 Hr	% Change	0	3 Hr	% Change	0	3 Hr	% Change
1	1.90	1.86		1.65	1.59		1.50	1.39	7.3
2	1.77	1.63	7.9	1.60	1.58	1.3	1.52	1.43	5.9
3	1.72	1.68	2.3	1.49	1.40	6.0	1.40	1.21	13.6
4	1.86	1.77	1.1	1.70	1.64	3.5	1.61	1.52	5.6
5	1.67	1.61	3.6	1.58	1.53	3.2	1.60	1.49	6.9
6	1.93	1.88	2.6	1.89	1.80	4.7	1.74	1.65	5.2
7	1.72	1.69	1.7	1.67	1.58	5.4	1.62	1.49	8.0
8	1.83	1.72	6.0	1.71	1.62	5.3	1.63	1.56	4.3
9	1.91	1.86	2.6	1.72	1.51	12.2	1.59	1.48	6.9
10	2.03	1.99	1.9	1.90	1.72	9.5	1.79	1.66	7.3
N	1.83	1.76	3.1	1.69	1.59	5.4	1.60	1.49	7.1
SD	0.11	0.12	2.1	0.12	0.11	3.2	0.11	0.13	2.5
SE	0.04	0.04	0.6	0.4	0.35	1.0	0.35	0.41	0.8
11[a]	1.93	1.84	4.7	1.21	0.46	62.0	0.91	0.12	87.0

[a] Frozen in 8% HES.

The initial 15% loss of intracellular erythrocyte K^+ observed by us corresponds to the K^+ loss from glycerolized cells reported by Valeri (17, 18). This suggests that mechanisms other than "solute leak" may be involved in the cryoprotection of erythrocytes by HES, such as hydrogen bonding (5), and stabilization of the lattice structures (6). Both mechanisms could enhance structuring of extracellular water (10), decreasing the tendency to form hexagonal ice during rapid freezing and thawing. Another attractive mechanism would be stabilization of the cell membrane by surface deactivation somehow preventing propagation of ice crystals through the membrane (3). This mechanism has been suggested by us (16) since 14% HES prevents the unmasking of a membrane (Ca^{2+} + Mg^{2+}) − ATPase, and damage to this structure results in activation of this enzyme with ATP loss (19). We have shown that when concentrations of 12–14% HES are used, this enzyme is not activated, but in concentrations less than 12%, marked ATPase activation occurred (16).

Rinfret pointed out a correlation between the postthaw saline stability of erythrocytes preserved in the PVP, and the number of cells that would circulate upon transfusion (13). Erythrocyte survival data from our laboratory suggest an identical correlation with red cells frozen with HES. Further indications that the stability

TABLE 8

THE EFFECT OF FREEZING ERYTHROCYTES WITH 8% HES UPON K^+ AND ATP LEVELS

| K^+ (meq/1) | | ATP (μM/10^8 cells) | |
Prefreeze	Postthaw	Prefreeze	Postthaw
101	49	2.02	0.23
91	47	1.96	0.21
98	46	1.87	0.20
101	53	1.99	0.25

of thawed cells in saline measure *in vivo* recovery are seen in the fact that the saline stability test predicted the percentage of cells lost during the washing procedures.

The development of a process utilizing hydroxyethyl starch, modified by the manufacturer at our suggestion, to provide an acceptable extracellular cryoprotective agent was initiated to make available erythrocytes that could be administered without postthaw process. However, the high viscosity of the HES-packed cell mixture limits large volume use of blood without first processing to remove a portion of the HES. The results reported in this paper suggest that hydroxyethyl starch can be effectively removed from red cells by washing in a balanced salt solution.

Normasol-R was chosen as a preliminary

washing agent since it is an approved parenteral solution. This is not an ideal solution for use in existing automated blood washing units since these systems require initial high density wash solutions such as 15 or 20% mannitol, but results obtained using the balanced salt solution demonstrate that HES can be washed out of the packed cells thus effectively lowering the viscosity. Other systems must be explored with particular emphasis on further reducing loss of intracellular K^+. Since HES is nontoxic and nonantigenic (9), and has not in our hands shown any adverse affects upon coagulation, it may be only necessary to partially remove it from the cells to obtain the required viscosity for rapid transfusion.

SUMMARY

Packed human erythrocytes were frozen in liquid nitrogen using hydroxyethyl starch as a cryoprotective agent. Upon thawing, the cells were washed with a balanced salt solution. The washed cells were stable when resuspended in 0.154 N NaCl. The postthaw saline stability approximated the total recovery after washing the cells. Intracellular potassium loss was 15% immediately postthaw, and an additional 15% loss occurred after washing. These results indicate that HES can be removed from frozen-thawed cells by washing, and that freezing erythrocytes protected by HES does not result in significant losses of intracellular potassium.

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