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Effect of a glycerol-containing hypotonic medium on erythrocyte phospholipid asymmetry and aminophospholipid transport during storage

Umakant J. Dumaswala [a,*], Michael J. Wilson [b], Thomas José [b], David L. Daleke [b]

[a] *Research Department, Hoxworth Blood Center, University of Cincinnati Medical Center, 3130 Highland Ave., Cincinnati, OH 45267-0055, USA*

[b] *Department of Biochemistry and Molecular Biology, Medical Sciences Program, Myers Hall, Indiana University, Bloomington, IN 47405-4001, USA*

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Abstract

Previous studies from our laboratory have shown that under blood bank storage conditions red blood cell (RBC) ATP and lipid content were better maintained in a glycerol-containing hypotonic experimental additive solution (EAS 25) than in the conventional storage medium Adsol®. The objective of this study was to determine the mechanism of the protective effect of EAS 25, by measuring transmembrane phospholipid asymmetry and the membrane integrity of stored RBCs. Split units of packed RBCs were stored in either EAS 25 or Adsol®. RBCs were analyzed after 0, 42, and 84 days and vesicles shed from stored RBCs were analyzed after 84 days of storage. Phospholipid asymmetry was measured by phospholipase A_2 digestion (RBCs) and activation of the prothrombinase complex (RBCs, vesicles). RBC membrane exhibited a significantly greater ($P < 0.01$) amount of phosphatidylethanolamine externalized after storage in Adsol® than in EAS 25 (44.3% ± 11.7 vs. 25.3% ± 5.7, respectively). Prothrombin converting activities in RBCs were significantly lower than in shed vesicles ($P < 0.001$) suggesting the presence of phosphatidylserine in the outer monolayer of vesicle, but not in RBC membranes. The rates of inwardly-directed aminophospholipid transport in RBCs decreased by ~ 50% and glutathione levels decreased by ~ 50% in both media. RBC cholesterol and phospholipid content of stored RBCs remained significantly greater ($P < 0.01$) in EAS 25 than in Adsol®. The results indicate that despite comparable reduction in the rate of aminophospholipid transport and reduced GSH concentrations, RBC phospholipid asymmetry was better maintained during storage in EAS 25 than in Adsol®. The data suggest that glycerol in the hypotonic EAS helps preserve RBC lipid organization and membrane integrity during storage. © 1997 Elsevier Science B.V.

Keywords: Aminophospholipid translocase; Phospholipid asymmetry; Glutathione; Erythrocyte storage; Erythrocyte membrane vesicle; Glycerol

Abbreviations: AChE, acetylcholinesterase; CPD, citrate-phosphate-dextrose; DLPS, dilauroylphosphatidylserine; EAS, experimental additive solution; HBS, Hepes-buffered saline; PAGGSS, phosphate-adenine-guanosine-glucose-saline-sorbitol storage buffer; PBS, phosphate-buffered saline; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; RBC, red blood cell; SM, sphingomyelin

* Corresponding author. Fax: + 1 513 5581522; E-mail: umakant.dumaswala@ucbeh.san.uc.edu

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1. Introduction

In normal red blood cells (RBCs) phospholipids are asymmetrically distributed across the membrane bilayer. Choline-containing phospholipids, phosphatidylcholine and sphingomyelin, are enriched in the outer leaflet and the aminophospholipids, phosphatidylethanolamine (PE) and phosphatidylserine (PS), are concentrated in the inner leaflet of the lipid bilayer [1–4]. Maintenance of this non-random distribution of phospholipid has been attributed to the action of an aminophospholipid-specific, ATP-dependent aminophospholipid translocase [5–10], the interaction of phosphatidylserine with cytoskeletal proteins [11–14], and the inherently slow passive transbilayer movement of phospholipids [15,16]. Disruption of the normal asymmetric lipid distribution results in altered biophysical properties of the erythrocyte membrane including changes in membrane fluidity, lipid packing, and cell surface charge [17–22]. Macrophages recognize these perturbations and enhanced phagocytosis ensues [23–27]. Aging and oxidant damage are also known to perturb the membrane and enhance phagocytosis [22,23]. It is, therefore, important to understand and prevent these changes to improve the quality of stored blood.

Studies from our laboratory and others have demonstrated that under blood bank storage conditions RBCs lose ATP, have altered transmembrane phosphatidylethanolamine asymmetry, and shed band 3- and band 4.1-enriched microvesicles [28–36]. The molecular mechanisms responsible for RBC vesiculation are still controversial. Loss of phospholipid asymmetry, disruption of membrane skeleton, and calpain-mediated degradation of cytoskeletal protein are some of the factors associated with the vesiculation process [37–41]. It is likely that long term storage alters one or more of these mechanisms, resulting in a loss of lipid asymmetry, since lipid scrambling has been associated with vesiculation, either by cause [8,42–44] or effect [45–47].

In contrast, storage in a hypotonic experimental additive solution (EAS) containing 10–50% ammonium chloride and 10–40% phosphate (P_i) in addition to adenine, glucose, mannitol and sodium chloride, partially preserved RBC ATP levels, improved in vivo survival and reduced storage mediated changes [48,49]. However, the substitution of glycerol (0.69% final concentration) for ammonium chloride in the RBC preservation medium (EAS 25) better maintains ATP levels and the content of protein bands 3 and 4.1 when compared with the conventional storage medium, Adsol®. Traditionally, glycerol at a final concentration of 20–40%, is used for preservation of frozen RBCs. The rationale for substituting glycerol in place of ammonium chloride is because it is pharmacologically inert and permeates membranes freely. The concentration of glycerol in EAS 25 (0.69%) is within the amount permitted in previously frozen, deglycerolized RBCs and allows the preservation of RBCs in a liquid, rather than frozen, state [50].

We hypothesized that compared to Adsol®, EAS 25 conserves phospholipid asymmetry and thus, the membrane and cellular integrity of the stored human RBCs. The present results show that despite comparable reduction in the rate of phosphatidylserine transport and reduced glutathione (GSH) concentration during storage in both media, RBC transmembrane phospholipid asymmetry was better maintained in EAS 25 than in Adsol®. These data suggest that glycerol in the hypotonic EAS protects membrane integrity.

2. Materials and methods

2.1. Samples

Blood donors acceptable by the American Association of Blood Banks and Food and Drug Administration criteria were used. The protocol was approved by the University of Cincinnati's Institutional Review board. Standard units of blood (450 ± 45 mL) were collected into polyvinyl chloride (PVC) bags (PL 146, Baxter Healthcare Corporation, Deerfield, IL) containing 63 mL citrate-phosphate-dextrose (CPD) anticoagulant and were centrifuged (RC-3B, Sorval, Dupont, Wilmington, DE) for 10 min at $835 \times g$. The platelet-rich plasma was expressed into a satellite bag. The packed RBCs were divided into two aliquots by weight in 300 mL PVC transfer bags (code 4R2001, Fenwal Laboratories, Deerfield, IL), using a sterile connection device (SCD 312, Haemonetics, Braintree, MA). One aliquot was stored with 100 mL Adsol®/unit and the other aliquot was stored in an equal volume of EAS 25 (prepared as previously

Table 1
Composition of additive solutions (mM)

	Adsol®	EAS 25
Adenine	2	2
Dextrose	122	110
Mannitol	42	55
Glycerol	–	150
NaCl	154	50
pH at 25°C	5.86	7.15
Milliosmolality (mosmol/kg)		
total	475	406
nonpenetrating [a]	322	145

[a] Nonpenetrating milliosmolality represents components which do not penetrate the cell.

described [35]). The composition of the additive solutions are shown in Table 1. All units were stored at 1–6°C and thoroughly mixed (10 min) on a platelet agitator (Meddev Corporation, Los Altos, CA) before sampling at 0, 28, 42, and 84 days of storage. Sterility was confirmed by inoculating 1.0 mL of the final sample into tubes of thioglycolate and tryptic soy broth.

2.2. Preparation of erythrocytes and microvesicles

RBCs were isolated from the stored blood samples by centrifugation for 10 min at $2000 \times g$ and washed three times in phosphate-buffered saline (PBS: 138 mM NaCl, 5 mM KCl, 6.1 mM Na_2HPO_4, 1 mM $MgCl_2$, pH 7.4), stored on ice, and processed within 24 h for phospholipase treatments, measurement of prothrombinase converting activity, lipid analysis, and lipid transport assays.

Microvesicles were isolated as previously described [35]. Briefly, the supernatant was collected after centrifugation for 10 min at $2000 \times g$ and passed through 0.8 μm nitrocellulose filters (Nuclepore Corporation, Pleasanton, CA) in a commercial filtration system at 5–10 lbs/in^2 N_2 pressure. The filtered supernatants were centrifuged for one hour at $38\,000 \times g$ and the isolated vesicle pellet was washed and suspended in PBS containing 2 mM EDTA and 1 mM phenylmethylsulfonyl fluoride.

2.3. Erythrocyte and vesicle membrane lipid determination

Lipid extraction and analysis of phospholipids and cholesterol were performed as previously described [35]. Cholesterol and phospholipid concentrations were expressed as μmol/mL RBC and molar fractions of cholesterol/phospholipid were calculated.

2.4. Phospholipase treatments

Transmembrane phospholipid asymmetry was determined by phospholipase digestion of outer monolayer phospholipids following a modification [36] of the method of Wilson et al. [51]. This procedure of outer monolayer digestion has been shown to produce $< 3\%$ hemolysis, $50 \pm 5\%$ hydrolysis of the total phospholipid pool, and produce no net shape change [36]. These requirements were met by $> 93\%$ of the samples analyzed; samples not meeting these criteria were discarded.

2.5. Phospholipid analysis

Erythrocytes treated with phospholipase A_2s were lysed in Tris buffer (10 mM Tris base, 2 mM EDTA, pH 7.4), and the membranes were collected by centrifugation (10 min at $16\,000 \times g$). This procedure was repeated 4–6 times until the supernatant was clear. Phospholipids were extracted by a modification of the procedure of Comfurius and Zwaal [52], with the addition of two chloroform washes of the aqueous phase. The combined organic phases were dried under a stream of nitrogen and reconstituted in 20 μL of chloroform/methanol (4:1, v/v). Lipids were separated by two-dimensional thin-layer chromatography [51] and quantified by phosphate analysis [53] of spots scraped from each plate.

2.6. Prothrombin converting activity

Erythrocyte prothrombin converting activity was assayed according to Wilson et al. [51]. Released thrombin was measured using the chromogenic substrate sarcosine-pro-arg-*p*-nitroanilide (500 μM final concentration) [54].

2.7. Phosphatidylserine transport

Phosphatidylserine transport was measured using short chain phospholipids and the cell morphological method of Daleke and Huestis [6,7]. Briefly, cells were treated with sonicated vesicles composed of dilauroylphosphatidylserine (DLPS). At appropriate

time intervals, aliquots of the cell suspension were fixed in 10 volumes of buffered glutaraldehyde (0.5%) and viewed by light microscopy. Cell shape was scored according to a morphological index scale [7]: discocytes were scored 0, echinocytes were given positive values depending on degree of spiculation, and stomatocytes were given negative values depending on the degree of invagination. The average score of a field of 100 cells was defined as the Morphological Index (MI). Rates of lipid transport were derived from this data by calculating rate of change in MI (ΔMI/min) after the initial incorporation of exogenous lipid ($\sim$ 5 min) [7]. The Morphological Index scale is linear with respect to lipid incorporation in this range [7], allowing a comparison of relative transport rates.

2.8. Miscellaneous assays

Protein assays were performed using the method of Bradford [55]. Since vesicle membranes contain hemoglobin, a correction was made for hemoglobin content [56]. ATP measurements were performed as previously described [35]. Glutathione was measured by the reduction of 5,5′-dithiobis(2-nitrobenzoic acid) as described by Beutler [57].

3. Results

3.1. Effect of storage media on lipid composition and vesiculation

Compared to prestored (fresh) cells, erythrocytes lose a significantly ($P < 0.01$) greater amount of cholesterol and phospholipids during storage in Adsol® than in EAS 25 (Fig. 1). RBC lipid loss during storage can be attributed to vesiculation. Analysis of vesicular cholesterol and phospholipid content suggests that the degree of vesiculation was proportional to the RBC lipid loss. The C/P ratios were not significantly different for RBCs or vesicles in either media, but the cholesterol and phospholipid content of the vesicles shed by RBCs stored in EAS 25 was significantly lower compared to those in Adsol® ($P < 0.001$) (Figs. 1 and 2). RBCs stored in EAS 25 shed significantly less vesicular membrane proteins ($P < 0.002$) than those stored in Adsol® (Fig. 2).

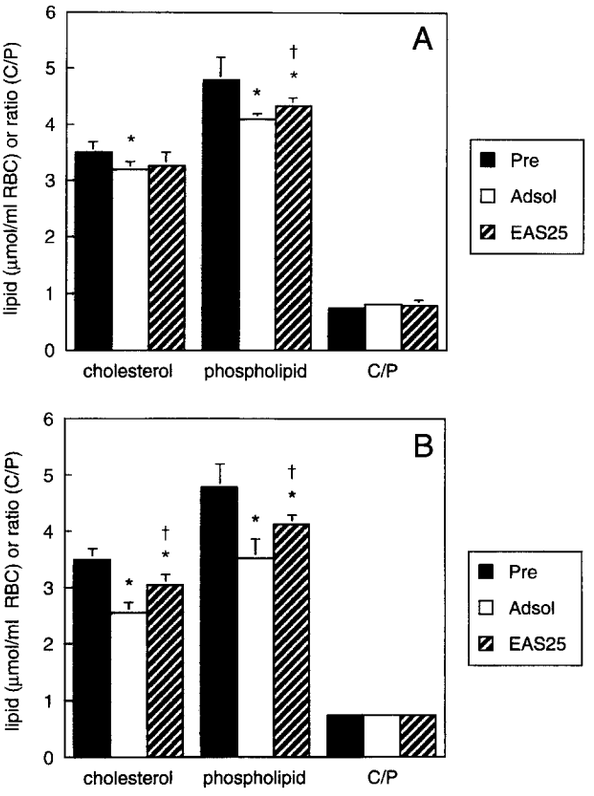

Fig. 1. Erythrocyte cholesterol (C), phospholipid (P) and molar cholesterol:phospholipid ratios (C/P) after storage in Adsol® and EAS 25 for 42 days (A) or 84 days (B). Cholesterol and phospholipid were measured as described in Section 2. Data are expressed as means ± S.D. (μmol/mL RBC); $n = 9$; * $P < 0.01$ (pre-storage vs. Adsol®/EAS 25); † $P < 0.01$ (Adsol® vs. EAS 25).

3.2. Phospholipid asymmetry and aminophospholipid transport

RBCs stored for 42 days in Adsol® expressed significantly greater amounts of PE in the outer leaflet compared to those stored in EAS 25 ($P < 0.001$), as assayed by phospholipase accessibility (Fig. 3). The accessibility of other phospholipids to exogenous phospholipases was not affected significantly. Erythrocyte prothrombin converting activity of RBC membranes was similar in both media (Table 2), supporting a lack of externalization of PS. In contrast, prothrombin converting activity of the vesicular membranes was significantly greater than RBCs indicating at least a partial loss of PS asymmetry in vesicular membranes. One of the mechanisms postu-

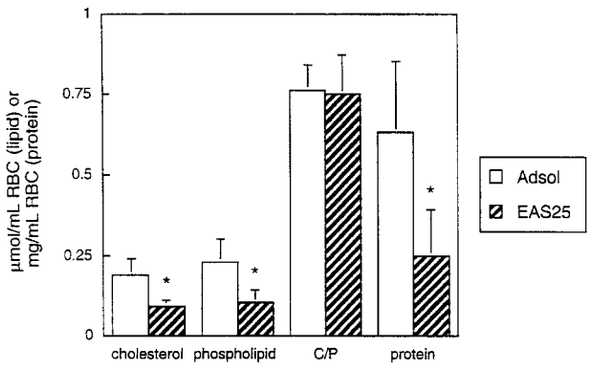

Fig. 2. Vesicle membrane cholesterol (C), phospholipid (P), cholesterol:phospholipid ratios (C/P) and total protein after 84 days storage in Adsol® and EAS 25. Cholesterol, phospholipid, and total protein content were measured as described in Section 2. Data are expressed as means ± S.D. (lipids: µmol/mL RBC; protein: mg/mL RBCs); $n = 14$; * $P < 0.001$ (Adsol® vs. EAS 25).

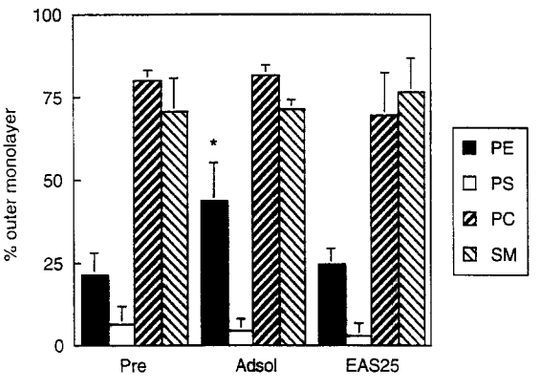

Fig. 3. Outer monolayer phospholipid composition of erythrocytes stored for 42 days in the indicated additive as determined by phospholipase hydrolysis. Samples were assayed as described in Section 2. Data are expressed as means ± S.D. (%); $n = 6$; * $P < 0.01$ (Adsol® vs. pre-storage or EAS 25).

lated for maintaining aminophospholipid asymmetry is via an ATP-dependent flippase [5–7]. We observed that after 42 days of storage, the initial rate of DLPS transport decreased ~ 50% in both groups ($\Delta MI/min = -0.06$) compared to fresh samples ($\Delta MI/min = -0.11$; Fig. 4). In addition, the morphological index achieved after 60 min in the control cells was slightly less then that in the stored cells. To determine the equilibrium value of the morphological index, the data describing the transport phase (> 5 min) were fit to a first-order kinetic equation. This analysis indicated that cells stored in EAS 25 displayed a 1% increase in equilibrium morphological index, while those stored in Adsol showed a 9% increase, compared with control cells. These data indicate that the equilibrium transmembrane asymmetric distribution of DLPS was only slightly less pronounced in the stored cells.

Table 2
Prothrombin converting activity of erythrocytes and shed vesicles after 84 days of storage [a]

	Pre	EAS 25	Adsol®
Erythrocytes	6.24 ± 0.2	4.74 ± 1.6	6.48 ± 0.9
Vesicles *	–	13.1 ± 8.2	11.4 ± 3.2

Released thrombin was measured using the chromogenic substrate sarcosine-pro-arg-*p*-nitroanilide (500 µM final concentration) [44].

[a] Results are expressed as nM thrombin produced per min per µg phospholipid. Data are expressed as means ± S.D.; $n = 3$ or 4. * $P \leq 0.05$ (vesicles vs. erythrocytes).

3.3. RBC ATP and glutathione

RBC ATP levels declined during storage; however this decline was significantly greater in Adsol® than in EAS 25 after 84 days of storage ($P < 0.01$; Table 3). Interestingly, RBC ATP values were similar in both media after 42 days of storage (2.34 ± 0.67

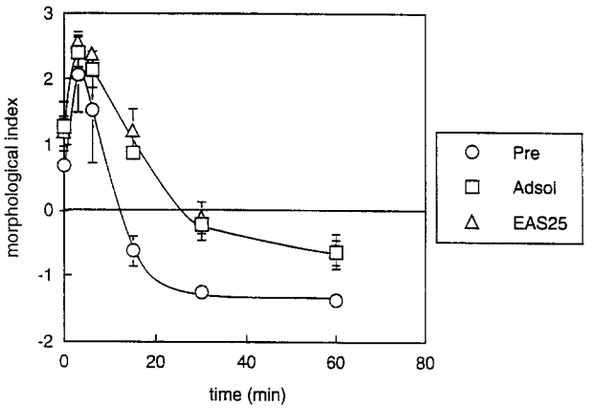

Fig. 4. Aminophospholipid transport activity in erythrocytes stored for 42 days. Erythrocytes were stored in the indicated additives as described in Section 2. Following storage, erythrocytes were washed and incubated with DLPS liposomes (250 µM). At the indicated time points, an aliquot of RBCs was fixed in glutaraldehyde, examined by light microscopy and a Morphological Index (MI) was calculated.

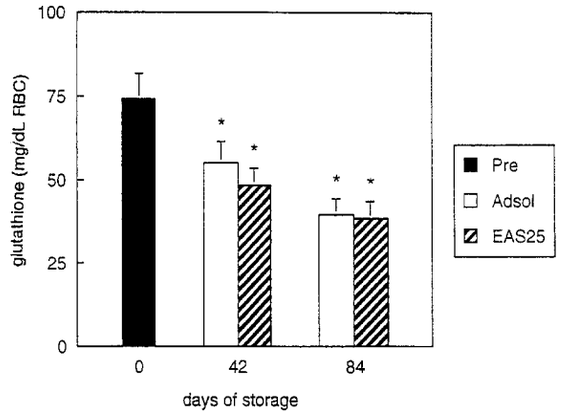

Fig. 5. Erythrocyte reduced glutathione content after storage in Adsol® and EAS 25. GSH was measured as described in Section 2. Data are expressed as means ± S.D. (mg/dL of RBC); $n = 5$; * $P < 0.01$ (pre-storage vs. Adsol®/EAS 25).

μmol/g Hb and 2.41 ± 0.56 μmol/g Hb for Adsol® and EAS 25, respectively). Erythrocyte glutathione content decreased by ~ 50% after 84 days of storage in both groups (Fig. 5).

4. Discussion

The specific aim of the current study was to identify storage-induced lesions in erythrocyte membranes and to gain an insight into mechanisms involved in the preservation of RBC membrane lipid asymmetry during storage. These results indicate that storage of human RBCs in a glycerol-containing hypotonic additive prevents flipping of PE from the inner- to the outer-leaflet of the membrane.

Several studies have shown that an ATP-dependent aminophospholipid translocase contributes significantly to the maintenance of PS and PE transmembrane asymmetry [5–9]. Loss of translocase activity may contribute to a loss of lipid asymmetry during storage. Geldwerth et al. [58] reported a progressive loss of translocase activity and the equilibrium asymmetric distribution of exogenously added synthetic phospholipids after storage of RBC in CPD or PAG-GSS. These defects were attributed largely to severe depletion of ATP. In the present study, erythrocyte ATP levels did not decline below 0.2 mM in any of

Table 3
In vitro characteristics of RBCs after storage in various media

	Adsol®	EAS 25 [a]	EAS 44 [b,c]	EAS 45 [b,c]
MCV [d] (fL)				
1 h	90.4 ± 3.8	114.5 ± 3.3	112.5 ± 4.5	101.7 ± 3.4
84 days	87.5 ± 3.0	99.9 ± 4.1	95.3 ± 2.7	95.1 ± 6.7
ATP (μmol/g Hb)	1.25 ± 0.30	1.71 ± 0.29	1.64 ± 0.13	2.10 ± 0.29
Vesicle analyses (84 days)				
Cholesterol (μmol/mL)	0.23 ± 0.07	0.09 ± 0.04	0.08 ± 0.03	0.08 ± 0.04
Phospholipid (μmol/mL)	0.34 ± 0.18	0.13 ± 0.05	0.16 ± 0.10	0.14 ± 0.07
Total lipid (μmol/mL)	0.58	0.22	0.24	0.22
Total protein (mg/mL)	0.97	0.35	0.3	0.4
Band 3.0 content [e]	+ +	+	+ +	+ +
Band 4.1 content [e]	+ +	+	+ +	+ +
GSH (mg/mL; 84 days) [f]	0.39 ± 0.05	0.38 ± 0.05	0.45 ± 0.04	0.53 ± 0.07

[a] $n = 14$; [b] $n = 5$; ± S.D.
[c] Dumaswala et al. [36].
[d] Mean Cell Volume.
[e] Vesicular band 3 and band 4.1 were detected by immunoblotting and quantitated by densitometric scanning [35] and expressed as relative concentrations.
[f] Pre-storage value: 0.750 ± 0.076 mg/mL.
Total lipid = Cholesterol plus Phospholipid.

the media, the minimal level of ATP required for translocase activity [36,59], yet a 50% reduction in transport activity was observed. The equilibrium transmembrane distribution of exogenously added PS decreased only slightly in both our study (1–9% decrease) and that of Geldwerth et al. [58] (17% decrease). The greater perturbation of equilibrium distribution observed by Geldwerth et al. can be attributed to a higher loss of ATP [58]. Though no substantial loss of endogenous lipid asymmetry was found by Geldwerth et al. [58], a minor amount of redistribution of PC and PE occurred within 60 h and was attributed in part to oxidative damage of the cytoskeleton during storage. In the present study, a much greater loss of endogenous PE asymmetry is observed. These differences are likely the result of different storage conditions. The medium used by Geldwerth et al. contained 16 mM sodium phosphate in addition to adenine, guanine, sorbitol, glucose and sodium chloride. Both media used in the present study (Adsol and EAS 25) did not contain phosphate, guanine or sorbitol. Since a loss of PE asymmetry was observed in Adsol but not in EAS 25, the protective effect could be attributed to glycerol and/or hypotonicity.

Recently, we have shown [36] that RBC phospholipid asymmetry was also protected in a hypotonic additive medium containing glutamine plus phosphate (P_i) (EAS 45), compared to a glutamine-containing hypotonic medium (EAS 44) or the isoosmolar Adsol®. Specifically, a loss of PE asymmetry was observed in the latter media. PE contains a higher amount of polyunsaturated fatty acids compared with other phospholipids and is therefore, more susceptible to oxidative damage [60,61]. It is possible that storage in Adsol® or in a medium containing glutamine alone does not afford adequate protection against lipid peroxidation. The resulting oxidative damage may induce the flipping of oxidized PE to the outer leaflet either spontaneously or through selective transport of PE by a lipid scramblase activity, similar to the Ca^{2+}-dependent scramblase [15,16,36,51,62]. We reasoned that glutamate, generated by phosphate-dependent glutaminase activity in EAS 45, provides an adequate concentration of glutathione precursors and results in the better maintenance of reduced glutathione, which is required for the protection of membrane constituents from oxidative damage. Indeed, a comparison of all the hypotonic media revealed that even after 84 days of storage, RBCs stored in the glutamine plus P_i-containing hypotonic medium (EAS 45) could maintain higher (25%) GSH levels (Table 3). A paradoxical finding in the present study is that despite a similar decline in GSH levels in glycerol-containing hypotonic additive or Adsol®, aminophospholipid asymmetry was better maintained in EAS 25. These data suggest that EAS 25 may prompt alternate protective mechanisms for preserving RBC membrane phospholipid asymmetry, perhaps through changes in the bulk physical properties of the membrane due to the presence of glycerol.

In an earlier study [35], we demonstrated that the microviscosity of the RBC membrane, an indicator of lipid packing, was less affected in EAS 25 than in Adsol®. Bruckdorfer et al. [63] have postulated that the beneficial effects of glycerol hydroxyl moieties is due to strengthening the association between cholesterol and other membrane constituents and in the regulation of membrane permeability. More recently, Benaim et al. [64] demonstrated that glycerol concentrations less than 5% stimulate the RBC Ca^{2+}-ATPase, activate Ca^{2+}-efflux, inhibit Ca^{2+}-dependent proteases, and better maintain membrane integrity. We observed significant increases in intra- and extra-cellular free amino acid concentrations, indicative of enhanced proteolysis, when erythrocytes were stored in Adsol® compared to EAS 25 (data not shown). These results are also consistent with our previous observation [35] that compared to Adsol®, storage of RBCs in EAS 25 diminished microvesiculation, as measured by loss of bands 3 and 4.1. Recently, Peters et al. [65] have shown that band 3 is required for membrane stability and to prevent surface loss in the form of vesicles. Decreased loss of bands 3 and 4.1 in glycerol-containing additive solutions may contribute to the preservation of membrane integrity and aminophospholipid asymmetry [11–14].

Stretching of the RBC membrane in hypotonic media has also been implicated in preventing vesiculation [66]. The addition of glycerol to the hypotonic medium can synergize this effect by intercalating into the inner leaflet to prevent echinocytosis and ultimately vesiculation [67]. Hypotonicity mediated stress can also stimulate non-protein-mediated mechanisms for lipid transport which may contribute to the maintenance of lipid asymmetry [68,69].

Comparison of a variety of hypotonic media indicates that though they all induce swelling and decrease vesiculation to the same extent, specific additives appear to dictate the biochemical and physical parameters of the stored cells, such as ATP and GSH levels and phospholipid asymmetry (Table 3). How these differences translate into improved in vitro and posttransfusion in vivo survival warrants further investigation and will be subject of future studies.

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