Synergistic effects of liposomes, trehalose, and hydroxyethyl starch for cryopreservation of human erythrocytes

ARTICLE *in* BIOTECHNOLOGY PROGRESS · MARCH 2012

Impact Factor: 2.15 · DOI: 10.1002/btpr.1519 · Source: PubMed

4 AUTHORS, INCLUDING:

Jelena L Holovati
University of Alberta
41 PUBLICATIONS **218** CITATIONS

SEE PROFILE

Synergistic Effects of Liposomes, Trehalose, and Hydroxyethyl Starch for Cryopreservation of Human Erythrocytes

Christoph Stoll
Institute of Multiphase Processes, Faculty of Mechanical Engineering, Leibniz Universität Hannover, Hannover D-30167, Germany

Jelena L. Holovati and Jason P. Acker
Dept. of Laboratory Medicine and Pathology, University of Alberta, Edmonton, Canada T6G 2B7
Canadian Blood Services, Research and Development, Edmonton, Canada T6G 2R8

Willem F. Wolkers
Institute of Multiphase Processes, Faculty of Mechanical Engineering, Leibniz Universität Hannover, D-30167 Hannover, Germany

DOI 10.1002/btpr.1519
Published online February 9, 2012 in Wiley Online Library (wileyonlinelibrary.com).

Red blood cells (RBCs) can be cryopreserved using glycerol as a cryoprotective agent, but one of the main disadvantages is the time-consuming deglycerolization step. Novel cryopreservation strategies for RBCs using nontoxic cryoprotective agents are urgently needed. The effect of DMPC, DOPC, and DPPC liposomes on survival of RBCs cryopreserved with trehalose and HES has been evaluated. DMPC caused hemolysis before freezing and affected RBC deformability parameters. DMPC treated RBCs displayed a strong increase in trehalose uptake compared to control cells, whereas DOPC treated liposomes only displayed a slight increase in trehalose uptake. High intracellular trehalose contents were observed after cryopreservation. The recovery of cells incubated with trehalose and liposomes, frozen in HES ranged between 92.6 and 97.4% immediately after freezing. Recovery values of RBCs frozen in HES, however, decreased to 66.5% after 96 h at 4°C compared to 77.5% for DOPC treated RBCs. The recovery of RBCs incubated and frozen in trehalose medium was 77.8%. After 96 hours post-thaw storage recovery of these cells was 81.6%. DOPC and DPPC treated RBCs displayed higher recovery rates (up to 89.7%) after cryopreservation in trehalose compared to control RBCs. Highest survival rates were obtained using a combination of trehalose and HES: 97.8% directly after thawing and 81.8% 96-h post-thaw. DOPC liposomes, trehalose and HES protect RBCs during cryopreservation in a synergistic manner. The advantage is that the protective compounds do not need to be removed before transfusion. © 2012 American Institute of Chemical Engineers *Biotechnol. Prog.*, 28: 364–371, 2012
Keywords: cryobiology, cryopreservation, liposomes, red blood cell deformability, membrane phase behavior, red blood cells

Introduction

One of the major challenges of blood banking is to deal with the increasing demand of red blood cell (RBC) transfusions. Blood transfusions are used for trauma victims, surgery, organ transplants, and patients receiving treatment for leukemia, cancer or other diseases such as sickle cell disease and thalassemia.

The vast majority of donated RBC units are hypothermically stored. During hypothermic storage molecular reactions are suppressed by reducing the storage temperature to below the physiological temperature but above the freezing temperature. Although hypothermic storage (HS) minimizes RBC injury, cellular metabolism is not completely suppressed.[1] The various detrimental biochemical and biomechanical effects of in vitro RBC hypothermic storage are collectively termed the "hypothermic storage lesion" (HSL). HSL components include: (i) lipid loss through microvesiculation, which leads to progressive spheroechinocytosis; (ii) altered RBC rheological properties; (iii) phosphatidylserine (PS) exposure on the membrane surface; and (iv) decreased expression of the CD47 antigen on the membrane surface.[2,3] HSL takes place during extended storage periods and is associated with adverse clinical outcomes.[4]

RBCs can be cryopreserved for transfusion using glycerol as a cryoprotective agent.[5–7] However, being a technically demanding and labor-intensive process, RBC cryopreservation is limited to rare blood groups, antibody problems, as well as civil emergency and military applications.[2] A lot of effort has been put in the development of new cryopreservation strategies for RBCs. RBCs have been frozen among others in extracellular cryoprotectants such as hydroxyethyl starch (HES)[8,9] or trehalose.[10] Trehalose is particularly suitable because it protects during freezing as well as during drying.[11] Under normal conditions, however, trehalose does not

Correspondence concerning this article should be addressed to W. F. Wolkers at wolkers@imp.uni-hannover.de.

easily cross cellular membranes. A number of procedures have been developed to overcome the impermeability of cellular membranes for trehalose.[11–14]

Cellular membranes are one of the primary sites of injury during chilling, freezing and subsequent thawing. Damage is caused by lipid peroxidation products, which decrease membrane integrity.[15,16] In addition, lipid phase transitions alter membrane structure and lateral organization.[17,18] Freezing results in both temperature-dependent and dehydration-induced membrane phase changes, which are thought to result in lateral phase separation of membrane components and increased membrane permeability for solutes.[19]

The stabilizing effect of liposomes during freezing and thawing of cells has first been discovered in sperm.[20] Specifically, unsaturated lipids such as DOPC were found to have cryoprotective properties. Recently, liposomes have been tested for their ability to stabilize red blood cells during hypothermic storage,[21] cryopreservation,[22] and freeze-drying.[23] Unsaturated phosphatidylcholine lipids such as DOPC were found to be most effective in suppressing hemoglobin leakage during freeze-drying.[23] Interestingly, DOPC is not effective during hypothermic storage. It actually increases hemolysis during prolonged hypothermic storage.[21] The same study showed that DPPC decreases hemolysis during hypothermic storage. DPPC-DPPS—cholesterol cholesterol liposomes protect RBCs during freezing.[24] We have previously shown that certain liposomes remove cholesterol from RBC membranes,[25] which we suggested to play a role in their cryoprotective action. Cholesterol depletion causes several alterations in the membranes properties, e.g., changes in deformability and rigidity[26] and in the permeability for hydrophilic solutes.[27]

In this study, the effect of three different types of liposomes, DOPC, DMPC, and DPPC, on stability and quality of human RBCs before and after cryopreservation has been evaluated. First, the effect of the liposomes on RBC deformability parameters [using ectacytometry (LORCA)] and hemolysis before freezing has been determined. Cryopreservation studies of control and liposome treated RBCs were done using trehalose, HES, and combinations of the two as cryoprotective agents. Trehalose uptake of liposome treated RBCs has been determined after incubation in trehalose and after thawing. Recovery after cryopreservation has been determined immediately after thawing and 96-h post-thaw.

Materials and Methods

Preparation of liposomes

Preparation of liposomes was done as previously described in detail.[25,22] Lipids were obtained from Avanti Polar Lipids (Alabaster, AL). Large unilamellar vesicles (LUVs) were prepared from 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC, 18:1), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC; 16:0) and 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC; 14:0). The lipids were dissolved in chloroform, and the chloroform was evaporated overnight and subsequently lyophilized (Christ® Epsilon 2-10 D) to create a thin lipid film. Dry lipids were hydrated in HBSI (HEPES-buffered saline-intracellular) buffer (10 mM NaCl, 120 mM KCl, 5 mM glucose and 20 mM HEPES [pH 7.4, 293 mOsm]). Unilamellar vesicles were obtained by extrusion using an Avestin mini extruder with 200 nm pore size filters. The lipid concentration after extrusion was 25 mM.

Pretreatment of blood and incubation of blood

Human blood was obtained from the Institute for Transfusion Medicine (Hannover Medical School). Venous blood was collected from healthy adults, with informed consent, according to institutional protocols. Blood was anticoagulated with citrate phosphate dextrose adenine (CPDA) and centrifuged (4,000*g*, 10 min) to remove buffy coat and plasma. Anticoagulated RBCs were stored in 15 mL polypropylene Falcon tubes at 1–6°C and used within a week. RBCs were incubated in the presence of liposomes for 4 h at 37°C. The concentration of lipid ranged from 0.5 to 7 mM. The hematocrit was adjusted to 5% using HBSI buffer. The suspension was gently rotated during incubation. Each measurement was done in triplicate with RBCs from three different donors.

RBC quality assessment

Assessment of Postincubation Hemolysis After incubation RBCs were centrifuged (1,000*g*, 5 min). The post incubation hemolysis was determined spectrophotometrically at 540 nm by comparing the supernatant hemoglobin to the total hemoglobin concentration of the samples, using the cyanmethemoglobin Drabkin's method.[28]

Assessment of Postincubation Deformability RBC deformability was assessed as previously described.[26] Briefly, RBCs were suspended in polyvinylpyrolidone (PVP) and analyzed by ectacytometry (LORCA). The deformability of the RBCs, which is expressed by the elongation index (EI), was determined by the LORCA, from the size of the vertical (L) and horizontal (W) axes of the diffraction pattern according to the formula: EI = (L - W)/(L + W). An increased EI at a given shear stress indicates greater RBC deformability. EI curves were obtained by plotting the calculated values for EI vs. the corresponding shear stress (SS). RBC deformability was determined at shear stresses between 0.95 and 30 Pa. The resulting deformability curve was transformed by Eadie-Hofstee linearization, which gives the deformability parameters EI_{max} and K_{EI} (Eq. 1).[26] EI_{max} is defined as the maximum elongation index predicted at an infinite shear stress, while K_{EI} is defined as the shear stress required to achieve half of EI_{max}.

$$EI = -K_{EI}\frac{EI}{SS} + EI_{max} \quad (1)$$

Environmental Scanning Electron Microscopy Control RBCs and those that were incubated with 1 mM DMPC liposomes were prepared as described above. Subsequently 10 μL sample was dissolved in 500 μL paraformaldehyde (4%). After 20 min, samples were 100-fold diluted in dH_2O and inspected on an environmental scanning electron microscope Quanta 400F (FEI, Eindhoven, the Netherlands). A gaseous secondary electron detector in ESEM modus was used. Acquisition parameters were four purge cycles with pressure between 6 and 13 mbar. The Chamber pressure was 6 mbar, spot size 4.0, high voltage 20 kV and WD 6 mm.

Trehalose Uptake RBCs were incubated with liposomes as described above. After liposome treatment, cells were washed in HBSI buffer to remove hemolyzed cells and liposomes. Twenty-five microliter of RBC concentrate ($\sim 8 \times 10^{12}$ RBCs/L) was transferred in 975 μL trehalose buffer (10 mM HEPES, 0.9% NaCl, 290 mM trehalose) and incubated for 2 h at 37°C with gentle rotation. After incubation, RBC counts and volume were measured by a hematology cell

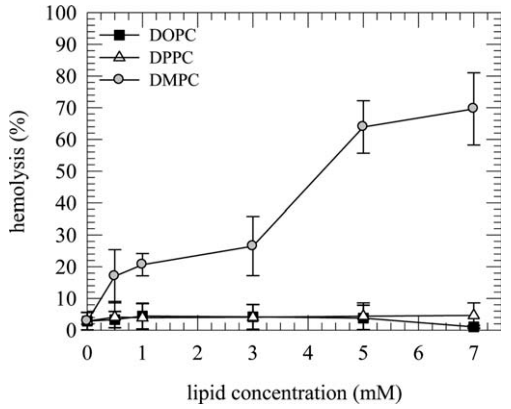

Figure 1. Percent hemolysis of RBCs after incubation with various liposomes (4 h, 37°C, gentle agitation).

Data are mean values with corresponding standard deviations determined from three donors, each measured in triplicate.

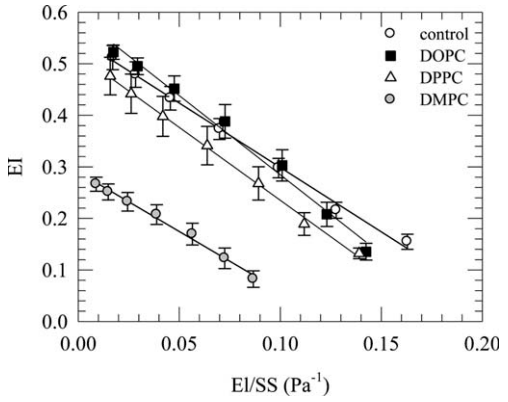

Figure 2. Effect of liposomes on RBC deformability measured by LORCA.

The data reflect an Eadie-Hofstee plot of control RBCs (open circles) and of RBCs treated with DOPC (filled squares), DPPC (open triangles) or DMPC (gray filled circles) liposomes (4 h, 37°C, gentle agitation). Data are mean values with corresponding standard deviations determined from three donors, each measured in triplicate.

analyzer (Beckman Coulter AcT, New York, NY). Cells were washed twice with HBSI buffer (4°C) to remove the extracellular trehalose. For calculations, the mean value of the RBC count and volume were determined for each liposome treatment. Subsequently, cells were hemolyzed by resuspension in double-distilled H_2O, frozen in liquid nitrogen and thawed at room temperature. The trehalose concentration of the suspension was measured spectrophotometrically using a commercially available enzymatic assay (Megazyme International Ireland, Wicklow, Ireland). The intracellular trehalose concentration was calculated based on the values of measured trehalose in solution, RBC count and volume, and corrected for the osmotically inactive volume fraction (Vb) value of RBCs (Vb = 0.423 × Vo) as previously described in detail.[24] Intracellular trehalose contents of cryopreserved RBCs were also determined after thawing.

Freezing of cells after liposomal-trehalose incubation in presence of trehalose and HES

Since RBC quality can be maintained at 37°C only for a limited time, the incubation with liposomes and trehalose was done in one step (4 h, 37°C) in a mixing block at 300 rpm (Bioer MB-102). The concentration of lipid ranged from 0.5 to 7 mM. The other incubation parameters were the same as those described earlier.

Immediately after incubation RBCs were centrifuged (1,000*g*, 5 min) and supernatant removed. Afterwards the cells were washed one more time in trehalose buffer (1,000*g*, 5 min). Subsequently, samples were cryopreserved either in trehalose buffer or HES. Samples cryopreserved in trehalose were prepared by diluting 20 μl of the RBC pellet in 400 μl trehalose buffer according to Holovati et al.[22] Samples cryopreserved with HES were prepared using a RBC/HES ratio of 1/1 as described by Sputtek et al.[9]: 30 μl of RBC pellet was mixed with 30 μl HES solution (MW 200,000 g/mol, 24.5% w:w). Samples were rapidly frozen by plunging into liquid nitrogen and stored at −150°C. Thawing was done by gently rotating the samples in a water bath of 37°C. Hemolysis was assessed immediately after thawing and 96 h post-thaw storage. RBCs were stored in 150 μl HBSI buffer at 1–6°C. Before storing, RBCs were washed twice with HBSI buffer (1,000*g*, 5 min). Each measurement was done in triplicate using six donors.

Statistical analyses

Mean values with corresponding standard deviation of the experimental data have been calculated from three experiments with either three different donors (incubation hemolysis, deformability, trehalose uptake) or six different donors (freezing experiments). Statistical analysis of the data was performed using a two-tailed student *t* test assuming unequal variances. Probability values (*P*-values) of less than 0.05 were considered significant.

Results

Post incubation hemolysis

Figure 1 depicts hemolysis of RBCs after incubation in the presence of liposomes (4 h, 37°C, gentle agitation). Hemolysis in the absence of liposomes was 2.8% ± 2.8%. DPPC liposomes had no effect on the hemolysis of RBCs. Application of 7 mM DOPC liposomes significantly reduced hemolysis to 1% ± 0.5% ($P < 0.05$). DMPC, however, caused a significant hemolysis, indicating that it is toxic to the cells. Even at 0.5 mM DMPC, hemolysis was found to be 17.0% ± 8.4%, significantly higher compared to control RBCs. Hemolysis further increased to 69.6% ± 11.4% at 7 mM DMPC.

Deformability

Figure 2 depicts Eadie-Hofstee plots of RBC deformability in the presence and absence of 3 mM DOPC, DPPC or DMPC liposomes. Particularly DMPC affects the y-intercept in the Eadie-Hofstee plot, whereas the slope is less affected. The EI_{max} of control RBCs is 0.5 ± 0.11, and K_{EI} is 2.1 ± 0.95 Pa. Figure 3 shows EI_{max} and K_{EI} values of RBCs in the presence of DOPC, DPPC, or DMPC liposomes. Incubation with liposomes resulted in detectable changes in EI_{max} and K_{EI} values. DPPC does not affect maximum elongation, but K_{EI} increases to a maximum of 3.3 ± 0.6 Pa at 5 mM. DOPC liposomes cause an increase of EI_{max}, i.e., to 0.59 ± 0.015 at 3 mM. The most drastic changes on EI_{max} were observed with DMPC liposomes, whereas K_{EI} of DMPC treated RBCs was little affected. EI_{max} decreases to 0.3 ±

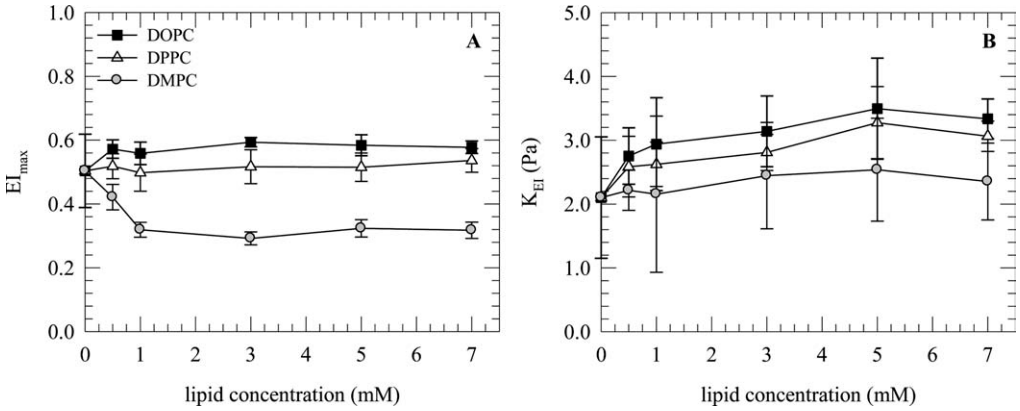

Figure 3. Maximum elongation index (EImax) (A) and shear stress at half maximum deformation (K_{EI}) (B) after treatment of RBCs treated with DOPC (filled squares), DPPC (open triangles) or DMPC (gray filled circles) liposomes (4 h, 37°C, gentle agitation).

Data are mean values with corresponding standard deviations determined from three donors, each measured in triplicate.

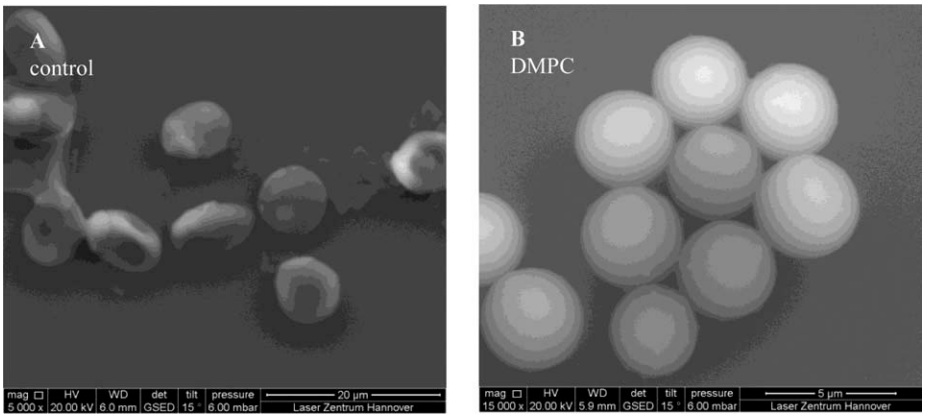

Figure 4. Environmental scanning electron micrographs of RBCs.

The image on the left side depicts control RBCs, while RBCs on the right side were previously incubated with DMPC liposomes (4 h, 37°C, gentle agitation).

0.02 at 3 mM DMPC, and K_{EI} is 2.5 ± 0.8 Pa. Even at 0.5 mM DMPC, EI_{max} significantly decreases compared to control RBCs.

Environmental scanning electron microscopy (ESEM)

ESEM images were used to further investigate the effects of DMPC on RBC deformability and hemolysis. Figure 4 depicts ESEM images of RBCs before (A) and after incubation with DMPC liposomes (B). It is evident that DMPC has major effects on RBC shape. RBCs show a typical spherocytic shape after incubation with DMPC.

Intracellular trehalose after incubation

RBCs in trehalose buffer were incubated in the presence and absence of liposomes to determine uptake of trehalose. The cell volume of control RBCs was determined to be 90.3 ± 5.2 μm^3. The cell volume of RBCs after DOPC treatment and trehalose incubation was 89.5 ± 6.3 μm^3 with 0.5 mM of DOPC and 89.5 ± 6.1 μm^3 with 7 mM DOPC, which implies that cell volume is not affected by DOPC. DPPC liposomes did not affect cell volume either (data not shown). A measurable decrease in cell volume was observed after incubation with DMPC: RBC volume decreased to 81.7 ± 2.2 μm^3 at 7 mM DMPC. The intracellular trehalose concentration of RBCs that were not incubated with liposomes was determined to be 5.1 ± 6.6 mM. DPPC treated RBCs showed a drop in intracellular trehalose concentrations after incubation (Figure 5). DOPC treated RBCs show a moderate increase in trehalose uptake. The loading efficiency (ratio of intracellular and extracellular trehalose), however, remains relatively low (<10%). By contrast, DMPC treated RBCs show a strong increase in trehalose uptake, which increases in a dose dependent manner up to 80.5 ± 27 mM at 7 mM DMPC.

Cryopreservation of liposome-treated RBCs using trehalose and HES

To evaluate the effects of liposomes on RBC freezing response, RBCs were incubated in the presence of liposomes and trehalose and frozen using trehalose or HES in the cryopreservation medium. Figure 6A depicts the recovery after freezing and thawing in HES. The recovery of the control cells, not incubated with liposomes, was 94.5% ± 5%. In the presence of DOPC liposomes recovery increases up to 97.4% ± 2.6% ($P = 0.05$). DMPC caused a slight (not

significant) decrease in recovery (min. 92.6% ± 2.5%), whereas DPPC liposomes slightly increased recovery (max. 95.8% ± 2.3%). The effect of liposome treatment is better visible after 96-h post thaw storage (Figure 6B). The recovery of frozen control RBCs dropped to 66.5% ± 12.2% (recovery of nonfrozen control RBCs was determined to be 98.2% ± 1.2% after 96 h), whereas DMPC (7 mM) treated RBCs showed a sharp drop in recovery after 96-h to 15.1% ± 6.6%. The 96 h recovery values of DPPC treated RBCs were similar to those of control RBCs. DOPC treated RBCs, however, displayed a significantly ($P < 0.05$) increased recovery compared to control RBCs of up to 77.5% ± 7.3% at 0.5 mM DOPC.

Figure 7 depicts the post thaw recovery of RBCs frozen in 290 mM trehalose medium. Control RBCs had a recovery of 77.8% ± 15.1%. Recovery of DOPC and DPPC treated RBCs increased compared to control cells. The highest recovery values were achieved with 5 mM DOPC (89.7% ± 3.4%) and 3 mM DPPC (87.8% ± 2.0%). The 96-h recovery of DPPC and DOPC treated RBCs, however, is only slightly higher compared to that of control cells. DMPC treated RBCs show a drop in recovery down to 61.5% ± 9.2% at 3 mM directly after thawing, which further drops down to 17.7% ± 11.5% 96 h post-thaw (Figure 7B) compared to 81.6% ± 3.4% for control cells.

Synergistic Effects of Trehalose and HES Samples that were frozen in HES as described above have some residual trehalose in the cryopreservation medium from the trehalose incubation step. Therefore, we also cryopreserved RBCs in HES that have not been in contact with trehalose, and used a combination of trehalose and HES as cryopreservation medium. Figure 8 shows a comparison of recovery values between; RBCs that have not been incubated with trehalose and frozen in HES, RBCs frozen in HES with residual trehalose, and RBCs frozen in a HES-trehalose mixture. Hemolysis was assessed immediately and 96-hours post-thaw. RBCs frozen in HES that were not incubated in trehalose display recovery values of 83.8% and 42.5%, respectively, directly after thawing, and 96-hours post-thaw. RBCs that were incubated in trehalose medium and cryopreserved in HES display higher recovery rates. Compared with RBCs that were not in contact with trehalose, this leads to a significant increase in recovery of 10.7% directly post thaw and 24.0% after 96 hours. Freezing RBCs, incubated with trehalose, in a combination of HES and trehalose further increased recovery to 97.8% ± 0.7% post-thaw and 81.8% ± 6.2% 96 hours after post-thaw storage.

Post-Thaw Intracellular trehalose Concentration To investigate if trehalose is taken up by RBCs during freezing and/or thawing, intracellular trehalose contents of thawed cells were determined (Figure 9). Interestingly, trehalose concentrations after thawing were found to have increased by an order of magnitude (>100 mM) after freezing and thawing (compare trehalose contents with Figure 5), which indicates that RBCs have taken up trehalose during the cryopreservation procedure. Intracellular trehalose contents of frozen DPPC and DOPC treated cells did not change with increasing lipid concentration and were similar compared to control cells.

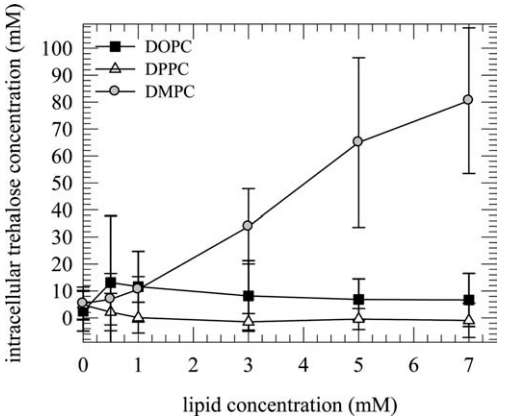

Figure 5. Trehalose uptake of liposome treated RBCs.

RBCs were incubated with DOPC (filled squares), DPPC (open triangles) or DMPC (gray filled circles) liposomes (4 h, 37°C, gentle agitation) and subsequently incubated in 290 mM trehalose buffer (2 h, 37°C, gentle agitation). Data are mean values with corresponding standard deviations determined from three donors each in triplicate.

Discussion

Liposomes are thought to modify cell membranes by lipid and cholesterol exchange, which alters physical properties and renders cells to be more (or less) stable at reduced temperatures. Several studies have demonstrated lipid and

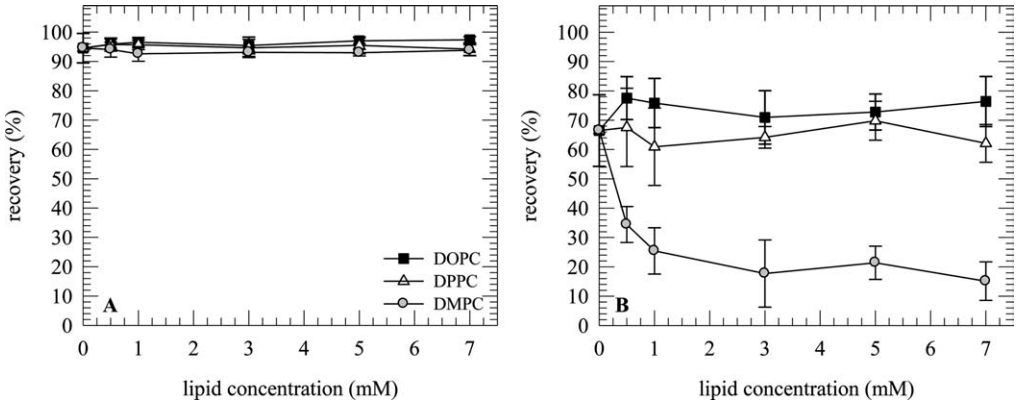

Figure 6. Cryopreservation of liposome treated RBCs in HES.

RBCs have been incubated in a liposome trehalose medium (4 h, 37°C, gentle agitation), washed in trehalose medium and frozen in HES. (A) Recovery immediately after thawing and (B) after 96 h post thaw storage in HBSI buffer (B). Data are mean values with corresponding standard deviations determined from six donors, each measured in triplicate.

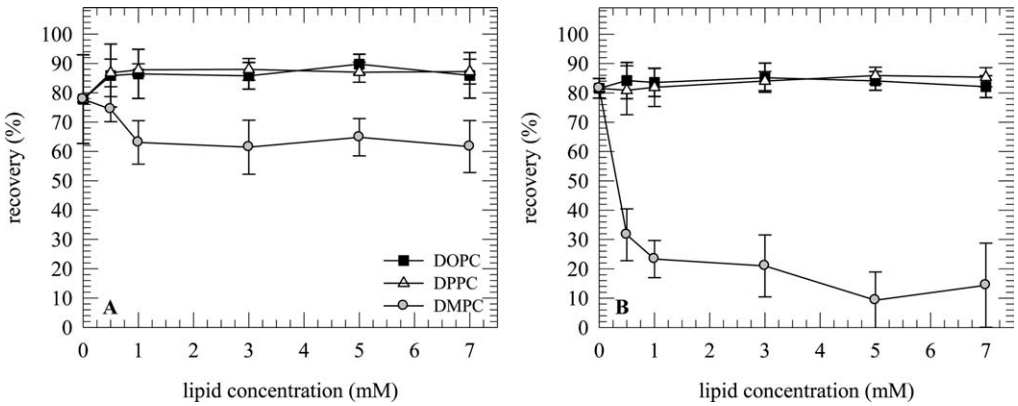

Figure 7. Cryopreservation of liposome treated RBCc in trehalose.

RBCs have been incubated in a liposome trehalose medium (4 h, 37°C, gentle agitation), washed in trehalose medium and frozen in trehalose medium. (A) Recovery immediately after thawing and (B) after 96 h post thaw storage in HBSI buffer. Data are mean values with corresponding standard deviations determined from six donors, each measured in triplicate.

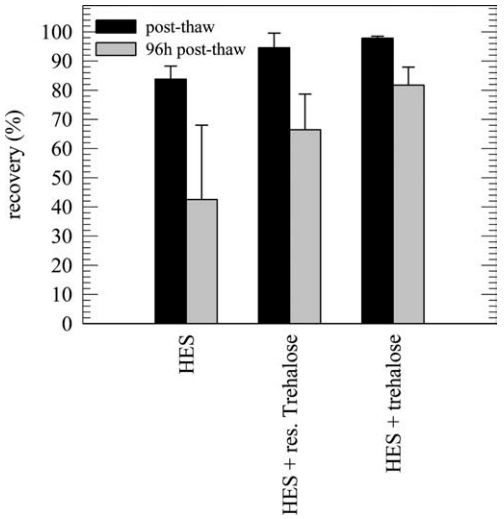

Figure 8. Recovery of RBCs frozen in HES-trehalose mixtures.

RBCs were: (1) not incubated with trehalose and cryopreserved in HES (left), (2) incubated and washed with trehalose medium and cryopresreved in HES (with residual trehalose from washing procedure) (middle), or (3) incubated and washed in trehalose medium and frozen in a mixture of HES and trehalose. Data reflect recovery immediately after thawing (black) and after 96 h post thaw storage in HBSI buffer (gray). Data are mean values with corresponding standard deviations determined from six donors, each measured in triplicate.

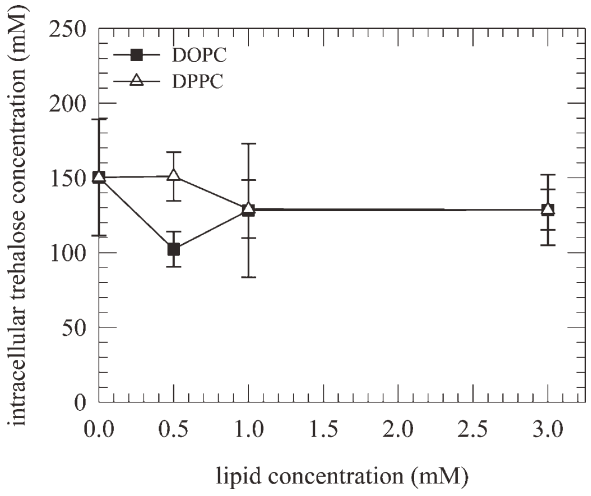

Figure 9. Intracellular trehalose after freezing and thawing of liposome treated RBCs.

Data indicate a 30-fold increase in intracellular trehalose content compared to post incubation (pre-freeze) values. Data are mean values with corresponding standard deviations determined from three donors, each measured in triplicate.

cholesterol exchange between liposomal and cellular membranes. In a recent study we have shown that liposomes composed of saturated lipids, similar as the ones that were studied here, display both lipid and cholesterol transfer with erythrocyte membranes.[25,29] DLPC (12:0) shows rapid lipid and cholesterol exchange with RBCs, which causes complete disintegration of the cells. DMPC (14:0), which has two additional CH_2 groups, displays much slower lipid and cholesterol exchange with RBCs. DPPC (16:0) hardly interacts with RBCs. Cholesterol depletion fluidizes RBC membranes and affects the permeation of solutes into the RBCs.[27] DMPC increases trehalose uptake, but it also causes significant hemolysis. The increased hemolysis and uptake of extracellular compounds is likely due to a combination of cholesterol depletion, and lipid addition. DMPC treated cells are spherical in shape, have a reduced cell volume, and a very low EI_{max}. The sharp decrease in EI_{max} suggests an inability of the RBCs to deform, even at high shear stresses. Interestingly, DMPC was shown to protect RBCs during freeze-drying.[23] Here, DMPC treatment was found to decrease RBC recovery after freezing and thawing. There is probably a fine balance between the advantageous increased permeability of DMPC treated RBCs for cryoprotective agents and the adverse effects of DMPC treatment on RBCs, such as decreased deformability and hemolyis. In a recent study, we have shown that DMPC possesses cryoprotective properties for bull sperm, whereas DPPC neither positively nor negatively affected cryosurvival.[30]

The combination of an extracellular cryoprotectant with a disaccharide has been investigated in the early years of RBC cryopreservation before cryopreservation with glycerol became the standard method.[31,32] RBCs cryopreserved in lactose combined with either albumin or dextran yielded recovery rates of 98–99% after cryopreservation and 69% 24 hours post transfusion. The authors concluded a synergistic protective effect of lactose and dextran or albumin. The reason why this procedure did not become the standard method for cryopreservation may have been that lactose has been

shown to produce pathological changes in kidneys, liver und lungs post infusion.[33]

We have shown that freezing and thawing in the presence of extracellular trehalose causes a drastic increase in intracellular trehalose content. Trehalose uptake can take place either during freezing when extracellular ice crystallization processes lead to an osmotic imbalance and membrane phase changes,[34] or during thawing. The high intracellular trehalose levels may increase the post-thaw stability of the cells. Our studies using combinations of trehalose and HES suggest that these cryoprotective agents protect RBCs in a synergistic manner. We postulate that the remarkable increase in post-thaw RBC stability in the presence of trehalose is due to intracellular trehalose taken up by the RBCs during freezing and/or thawing. The protective role of trehalose could be protection of membranes and diminishing the formation of injurious intracellular ice.[19] DOPC treated RBCs displayed the highest survival rates after freezing and thawing. The increased recovery is likely not due to the moderate increase in solute permeability of the DOPC treated RBCs, but could be explained by the increased mechanical stability of the cells. The storage stability of the cryopreserved cells is not very good, which is due to the fact that the RBCs in this study are diluted and stored suboptimally in HEPES-NaCl buffer rather than citrate phosphate dextrose anticoagulant and SAGM additive solution. It is therefore not surprising that hemolysis values are much higher than typically observed in blood banks.[35] Storing cryopreserved RBCs under suboptimal conditions, however, allowed us to more easily detect the protective effects of liposome treatment, trehalose or HES on RBC membrane quality.

Taken together, in this study, the effects of various large unilamellar liposomes composed of DPPC, DMPC or DOPC on RBC properties and RBC survival after freezing and thawing have been investigated. DMPC was found to be toxic to the cells and caused hemolysis, whereas DPPC or DOPC did not. RBC deformability parameters that were found here are in good agreement with previous findings.[21] The maximum elongation of DPPC treated RBCs is not affected, but K_{EI} increases at higher DPPC concentrations, suggesting an increase in rigidity. DOPC treated RBCs show an increase in maximum elongation and in K_{EI}, indicating the cells are more flexible. DMPC treated RBCs display a strongly increased trehalose uptake compared to DOPC or DPPC treated RBCs. DOPC treated RBCs show an increase in trehalose uptake. Loading efficiencies, however, remain relatively low for DOPC treated RBCs (<10%). DOPC treated RBCs displayed the highest survival rates after freezing and thawing. The synergistic protective effects of trehalose, DOPC liposomes and HES potentially hold promise for the development of new cryopreservation methods for RBCs. The advantage of such formulations is that these compounds are already used in various pharmaceutical applications and there is no need for time-consuming extensive washing procedures before transfusion. It should be noted that our studies have been done with small sample volumes and low hematocrit values. Scaling up studies with larger volumes and higher hematocrit values are needed to use the formulations in the blood banking practice.

Acknowledgment

This work is supported by the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) for the Cluster of Excellence REBIRTH (From Regenerative Biology to Reconstructive Therapy). This study was partially funded from a grant "Liposomes in Transfusion Medicine: An Approach for Reducing the Red Blood Cell Hypothermic Storage Lesion" to J.A. from the Canadian Blood Services/Canadian Institutes for Health Research. The authors thank Dr. Heuft from the Hanover Medical school blood donation service for providing blood samples for the experiments, and Dag Heineman from the Laser Zentrum Hannover for helping with the ESEM images.

Notation

DOPC = 1,2-dioleoyl-sn-glycero-3-phosphocholine
DMPC = 1,2-dimyristoyl-sn-glycero-3-phosphocholine
DPPC = 1,2-dipalmitoyl-sn-glycero-3-phosphocholine
DPPS = 1,2-dipalmitoyl-sn-glycero-3-phosphatidylserine
pfa = paraformadehyde
HES = hydroxyethyl starch
LORCA = laser-assisted optical rotational cell analyzer

Literature Cited

1. Lion N, Crettaz D, Rubin O, Tissot JD. Stored red blood cells: a changing universe waiting for its map(s). *J Proteomics*. 2010;73:374–385.
2. Hess JR. An update on solutions for red cell storage. *Vox Sang*. 2006;91:13–19.
3. Hess JR, Greenwalt TG. Storage of red blood cells: new approaches. *Transfus Med Rev*. 2002;16:283–95.
4. Tinmouth A, Fergusson D, Yee IC. Clinical consequences of red cell storage in the critically ill. *Transfusion*. 2006;46:2014–27.
5. Smith AU. Prevention of haemolysis during freezing and thawing of red blood-cells. *Lancet*. 1950;265:910–911.
6. Rowe AW, Eyster E, Kellner A. Liquid nitrogen preservation of red blood cells for transfusion: a low glycerol—rapid freeze procedure. *Cryobiology*. 1968;5:119–128.
7. Meryman HT, Hornblower M. Simplified prodecure for deglycerolizing red blood-cells frozen in a high glycerol concentration. *Transfusion*. 1977;17:438–442.
8. Knorpp CT, Merchant WR, Gikas PW, Spencer HH, Thompson NW. Hxdroyethyl starch: extracellular cryophylactic agent for erythrocytes. *Science*. 1967;157:1312–1313.
9. Sputtek A, Singbartl G, Langer R, Schleinzer W, Heinrich HA, Kühnl P. Cryopreservation of red blood cell with the non-penetrating cryoprotectant hydroxyethylstarch. *Cryo Lett*. 1995;16: 283–288.
10. Pellerin-Mendez C, Million L, Marchand-Arvier M, Labrude P, Vigneron C. In vitro study of the protective effect of trehalose and dextran during freezing of human red blood cells in liquid nitrogen. *Cryobiology*. 1997;35:173–186.
11. Wolkers WF, Walker NJ, Tablin F, Crowe JH. Human platelets loaded with trehalose survive freeze-drying. *Cryobiology*. 2001; 42:79–87.
12. Satpathy GR, Torok Z, Bali R, Dwyre DM, Little E, Walker NJ, Tablin F, Crowe JH, Tsvetkova NM. Loading red blood cells with trehalose: a step towards biostabilization. *Cryobiology*. 2004;49:123–136.
13. Quan GB, Han Y, Liu MX, Gao F. Effects of pre-freeze incubation of human red blood cells with various sugars on postthaw recovery when using a dextran-rapid cooling protocol. *Cryobiology*. 2009;59:258–267.
14. Eroglu A, Russo MJ, Bieganski R, Fowler A, Cheley S, Bayley H, Toner M. Intracellular trehalose improves the survival of cryopreserved mammalian cells. *Nat. Biotechnol*. 2001;18:163–167.
15. Aitken RJ, Buckingham DW, Brindle J, Irvine DS. Analysis of sperm movement in relation to the oxidative stress created by leucocytes in washed sperm preparations and seminal plasma. *Hum Reprod*. 1995;10:2061–2071.
16. Neild DM, Brouwers JFHM, Colenbrander B, Aguero A, Gadella BM. Lipid peroxide formation in relation to membrane

stability of fresh and frozen thawed stallion spermatozoa. *Mol Reprod Dev*. 2005;72:230–238.
17. Crowe JH, Hoekstra FA, Crowe LM, Anchordoguy TJ, Drobnis E. Lipid phase transitions measures in intact cells with fourier transform infrared spectroscopy. *Cryobiology*. 1989:26:76–84.
18. Quin PJ. A lipid phase separation model of low-temperature damage to biological membranes. *Cryobiology*. 1985;22:128–146.
19. Stoll C and Wolkers WF. Membrane stability during biopreservation of blood cells. *Transfus Med Hemother*. 2011:38:89–97.
20. Graham JK, Foote RH. Effect of several lipids, fatty acyl chain length, and degree of unsaturation on the motility of bull spermatozoa after cold shock and freezing. *Cryobiology*. 2001;24: 42–52.
21. Stadnick H, Stoll C, Wolkers WF, Acker JP, Holovati JL. The effect of liposome treatment on the quality of hypothermically stored red blood cells. *Biopreserv Biobanking*. doi:10.1089/bio.2011.0023.
22. Holovati JL, Gyongyossy-Issa M, Acker JP. Effects of trehalose-loaded liposomes on red blood cell response to freezing and post-thaw membrane quality. *Cryobiology*. 2008;58:75–83.
23. Kheirolomoom A, Satpathy GR, Torok Z, Banerjee M, Bali R, Novaes RC, Little E, Manning DM, Dwyre DM, Tablin F, Crowe JH, Tsvetkova NM. Phospholipid vesicles increase the survival of freeze-dried human red blood cells. *Cryobiology*. 2005;51:290–305.
24. Holovati JL, Gyongyossy-Issa M, Acker JP. Investigating interactions of trehalose-containing liposomes with human red blood cells. *Cell Preserv Technol*. 2008;6:131–142.
25. Stoll C, Stadnick H, Kollas O, Holovati JL, Glasmacher B, Acker JP, Wolkers WF. Liposomes alter thermal phase behavior and composition of red blood cell membranes. *Biochim Biophys Acta*. 2011;1808:474–481.
26. Stadnick H, Onell R, Acker JP, Holovati JL. Eadie-Hofstee analysis of red blood cell deformability. *Clin Hemorheol Microcirc*. 2011;47:229–239.
27. Kimbelberg HK. Influence of lipid phase transitions and cholesterol on protein-lipid interactions. *Cryobiology*. 1978;15:222–226.
28. Zwart A, van Assendelft OW, Bull BS, England JM, Lewis SM, Zijlstra WG. Recommendations for reference method for haemoglobinometry in human blood (ICSH standard 1995) and specifications for international haemiglobinocyanide standard (4th edition). *J Clin Pathol*. 1996;49:271–274.
29. Stoll C, Holovati JL, Acker JP, Wolkers WF. Liposomes composed of unsaturated lipids for membrane modification of human erythrocytes. *Mol Membr Biol*. 2011;28:454–461.
30. Röpke T, Oldenhof H, Leiding C, Sieme H, Bollwein H, Wolkers WF. Liposomes for cryopreservation of bull sperm. *Theriogenology*. 2011.
31. Strumia MM, Strumia PV. Effect of lactose, dextran and albumin on recovery and survival of frozen red cells. *Transfusion*. 1965;5:399–411.
32. Luyet B, Rapatz G. A review of basic researches on the cryopreservation of red blood cells. *Cryobiology*. 1970;6:425–482.
33. Perth JH, Schork PK, Moore R. Low-temperature preservation of human erythrocytes: biochemical and clinical aspects. *Bibl Haematol*. 1964;19:47–53.
34. Akhoondi M, Oldenhof H, Stoll C, Sieme H, Wolkers WF. Membrane hydraulic permeability changes during cooling of mammalian cells. *Biochim Biophys Acta*. 2011;1808:642–648.
35. Zimrin AB, Hess JR. Current issues relating to the transfusion of stored red blood cells. *Vox Sang*. 2009;96:93–103.

Manuscript received Nov. 10, 2011, and revision received Jan. 6, 2012.