Cryobiology 58 (2009) 75–83

Contents lists available at ScienceDirect

Cryobiology

journal homepage: www.elsevier.com/locate/ycryo

Effects of trehalose-loaded liposomes on red blood cell response to freezing and post-thaw membrane quality ☆

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ARTICLE INFO

Article history:
Received 25 June 2008
Accepted 4 November 2008
Available online 14 November 2008

Keywords:
Erythrocytes
LUV
Vesicles
Membrane
Permeabilization
Trehalose
Loading
Cryopreservation
CPA

ABSTRACT

We are investigating the use of liposomes, which are synthetic, microscopic vesicles, for the intracellular delivery of trehalose into mammalian cells. This study focuses on the effects trehalose-containing liposomes improve the recovery and membrane quality of human RBCs following cryopreservation. Unilamellar liposomes consisting of a lipid bilayer composed of DPPC, PS and cholesterol (60:30:10 mol%) were synthesized using an extrusion method. Liposome-treated RBCs (l-RBCs) were resuspended in either physiological saline, 0.3 M trehalose or liposome solution, then cooled with slow (0.95 ± 0.02 °C/min), medium (73 ± 3 °C/min) and fast (265 ± 12 °C/min) cooling rates and storage in liquid nitrogen, followed by a 37 °C thawing step. RBC post-thaw quality was assessed using percent recovery, RBC morphology, PS and CD47 expression. Liposome treatment did not adversely affect the RBC membrane. Post-thaw recovery of l-RBCs was significantly higher (66% ± 5% vs 29% ± 4%) compared to control RBCs (c-RBC, $p = 0.003$). Medium and high cooling rates resulted in significantly higher cell recovery compared to a slow cooling rate ($p = 0.039$ and $p = 0.041$, respectively). The recovery of l-RBCs frozen in liposome solution and trehalose solution was significantly higher than that of l-RBCs frozen in NaCl solution for all three cooling rates ($p = 0.021$). Flow cytometry and morphology assessment showed that liposome treatment resulted in improved post-thaw membrane quality. There was no statistically significant difference in the post-thaw recovery between RBCs treated with liposomes containing trehalose in their aqueous core and RBCs treated with liposomes containing saline in their aqueous core ($p = 0.114$). Liposome treatment significantly improves the recovery and membrane integrity of RBCs following low temperature exposure.

Introduction

When cells are exposed to the ice formation that accompanies low temperatures, they are subjected to major changes in their physical environment, resulting in cell injury. This is the basic principle of cryobiology. Cryoprotective agents (CPAs) can be added to a cell suspension to mitigate cryoinjury and increase cell survival following freezing and thawing. The labour-intensive, technically-demanding nature of the conventional RBC cryopreservation procedures which uses glycerol as a cryoprotectant, provides the rationale for further research into the development of novel cryopreservation approaches. Another approach to RBC cryopreservation involves the use of nonpermeating CPAs, such as hydroxyethyl starch (HES). However, nonpermeating CPAs have been shown to result in delayed hemolysis, occurring after the RBCs are transfused, and are not currently licensed for clinical transfusion [26,28,53].

Studies of natural systems that survive extreme environmental stress, such as freezing and desiccation, have shown that one of the adaptive mechanisms is the accumulation of sugars, such as trehalose [12,13,18]. The mechanism of trehalose protection is an active area of research that includes the interaction of sugars with plasma membranes [14], the role of glassy state [15,24], the effects on cell osmotic responses [10,19], and the unique physico-chemical properties of trehalose [16,49]. Regardless of the mechanism of action, relatively low concentrations of non-toxic trehalose have been reported to act as an effective CPA in a wide variety of biological systems [2,8,21–23,37]. The application of trehalose as a nonpermeating intracellular CPA for RBC cryopreservation would circumvent the necessity for the expensive and tedious deglycerolization procedure, resulting in cryopreserved RBCs available for transfusion immediately upon request. However, for maximum protection efficiency, the presence of extracellular trehalose is not sufficient, as trehalose is required on both sides of cell mem-

☆ This work was funded by the Bayer-Canadian Blood Services-Hema-Quebec Partnership Fund. J.L.Holovati was supported by a Graduate Student Fellowship from the Canadian Blood Services.
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doi:10.1016/j.cryobiol.2008.11.002

brane [2,8,21–23,37]. Our lab is investigating liposomes, which are synthetic, microscopic vesicles, for the intracellular delivery of stabilizing sugars into mammalian cells. Our previous work has described adaptation of existing techniques in the liposomal research to synthesize and characterize trehalose-containing liposomes to be used for the intracellular delivery of this membrane impermeable stabilizing disaccharide [32]. In addition, we have provided evidence that, under specific experimental conditions, trehalose-containing liposomes can fuse with RBCs to deliver micromolar concentrations of trehalose to the cell cytosol, with minimal detrimental effects on RBC membrane quality [34,35]. This study will test the hypothesis that trehalose-containing liposomes improve the recovery and membrane quality of human RBCs following cryopreservation.

Trehalose and liposomes may play an important role in RBC response to freezing, but an additional major factor in determining whether or not cells survive freezing is the rate at which they are cooled [42]. The interpretation of this curve was offered by Mazur et al. in his two-factor hypothesis, which remains a dominant theory in cryobiology: at low cooling rates, the cryoinjury results from exposure to concentrated extra and intracellular solutions at high sub-zero temperatures (solution effects injury); while at high cooling rates, the cell injury is associated with intracellular ice formation (IIF) [43]. Whether a given cooling rate is too high or low for a given cell type largely depends on the water permeability of the cell's plasma membrane and cell surface area to volume ratio, which are cell type dependent properties [43]. An additional parameter affecting the relationship between the cooling rate and cell survival is the composition of the extracellular freezing solution [42,48,41,39]. For example, addition of larger molecules such as sugars, starches and polymers to extracellular freezing solution will broaden the range of optimal cooling rates and reduce cryoinjury [44,45]. It is therefore important to address the roles of cooling rate and extracellular solution when examining the effects of liposome treatment on the RBC response to freezing.

In addition, to the well-described biostabilizing properties of trehalose, liposomes themselves have been shown to protect cells under stress conditions, other than freezing. Recently, Kheirolomoom et al. have demonstrated that membrane lesion caused by RBC lyophilization can be reduced by adding liposomes to the lyophilization buffer [36]. During lyophilization and rehydration, RBC membranes lose phospholipids through microvesiculation, resulting in the deterioration of RBC membrane composition, morphology, and rheology [36,56,52]. Small unilamellar liposomes added to the lyophilization buffer preserved membrane integrity and reduced hemolysis of lyophilized RBCs [36]. Similar RBC membrane damage has been reported by Yamaguchi et al. when RBCs were subjected to high-pressure and hypotonic conditions [59]. This study also showed that the addition of aminophospholipid vesicles, in combination with amphiphatic drugs and hypertonic conditions, markedly reduced pressure-induced RBC membrane damage [59]. Liposomes were also used to reduce the chilling injury of bovine spermatozoa and oocytes [60]. As stress conditions are also associated with ice formation that accompanies low temperatures, this study will also investigate whether delivering of liposome phospholipids through liposome incorporation into RBC membranes may provide an effective approach for reducing cell cryoinjury.

RBC post-thaw quality is traditionally assessed by percent hemolysis, as this is the only measure of *in vitro* quality stipulated by current regulatory bodies in transfusion medicine [30,31,4]. Strong evidence has recently indicated that RBC viability, defined by post-transfusion RBC survival, is closely related to the structure of the membrane and metabolic status of the RBC [57,27,6,7,29]. More subtle membrane changes, such as phosphatidylserine exposure from disruption of the asymmetric distribution of the RBC membrane and loss of CD47 antigen expression, have been shown to induce post-transfusion erythrophagocytosis, and therefore act as potentially significant predictors of RBC *in vivo* survival and function [6,54,3,11,25,50]. Our previous study investigated the quality of conventionally cryopreserved RBCs using novel indicators of RBC membrane lesion and correlated them to traditional markers of RBC injury [33]. A similar approach will be taken here—in addition, to percent hemolysis, the novel markers of subtle membrane injury will be included in evaluating post-thaw quality of liposome-treated RBCs.

Therefore, the purpose of this study is to assess the effects of liposome-delivered trehalose on RBC post-thaw survival and quality using conventional and novel markers of RBC membrane injury. This study will address several key questions, including: does liposome treatment improve RBC recovery after freezing; what is the effect of different extracellular solutions and cooling rates on liposome-treated RBC response to freezing, are the effects seen due to trehalose or membrane phospholipid delivery resulting from liposome fusion; and what are the effects of freezing on liposome-treated RBC membrane quality?

Materials and methods

Liposomes and lipids

Unilamellar trehalose-containing liposomes were synthesized using an extrusion procedure, as previously described [32]. The liposome lipid bilayer was composed of 1,2-dipalmitoyl-*sn*-glycero-3-phosphacholine (DPPC, Sigma–Aldrich, St. Louis, MO), phosphatidylserine (PS, Sigma–Aldrich, St. Louis, MO) and cholesterol (Avanti Polar Lipids, Alabaster, AL) in 60:10:30 mol% ratio, resulting in a 25 mM final lipid solution. After lyophilization, DPPC:PS:cholesterol lipid film was either hydrated with trehalose buffer (300 mM α-D-glucopyranosyl-α-D-glucopyranoside, 10 mM HEPES, pH 7.4, 307 mOsm, Sigma–Aldrich, St. Louis, MO) or saline buffer (140 mM NaCl, 10 mM HEPES, pH 7.4, 298 mOsm, Sigma–Aldrich, St. Louis, MO). Therefore, these negatively charged liposomes contained either trehalose or saline in their aqueous cores. Synthesized liposomes were characterized for lipid content, size and trehalose content, as previously described [32]. In addition, to liposome synthesis, negatively charged lipids were suspended in trehalose solution without further liposome synthesis steps. To evaluate the effect of liposome charge on RBC post-thaw recovery, uncharged liposomes were synthesized using the same protocol, but only to contain DPPC and cholesterol in their membrane (70:30 mol%). These liposomes were hydrated with 0.3 M trehalose buffer.

Incubation with RBCs

Human whole blood (7 mL) was collected from healthy volunteers into vacutainer tubes containing citrate anticoagulant (BD, Fanklin Lakes, NJ) using standard phlebotomy. The sample was centrifuged at 1500g for 10 min at 4 °C (Eppendorf Centrifuge 5810R, Westbury, NY). Plasma supernatant and buffy coat were removed and the RBC pellet was washed three times with HBS physiological buffer. After the last wash, RBCs were resuspended in 5 mL of HBS buffer, counted on a hematology cell analyzer (Beckman Coulter AcT, New York, NY), and diluted to an appropriate count for future experiments. Phlebotomized blood samples were stored refrigerated (4–6 °C) for up to 3 days until needed. RBCs were incubated with either charged/uncharged liposomes or lipids (4 mM) in 300 mM trehalose buffer, at 37 °C for 4 h on a laboratory shaker with low speed setting (VWR International, West Chester, PA) [34]. After incubation, density gradient separation was used to separate RBCs from unbound liposomes that were adsorbed to the RBC membranes, along with the free liposomes in the incubation mixture [32,35]. After the separation, the supernatant containing liposome layer