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RESEARCH ARTICLE

Effect of liposome-treated red blood cells in an anemic rat model

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Abstract

Context: Liposomes have been shown to improve human red blood cell (RBC) *in vitro* quality by minimizing membrane damage occurring during 42-d hypothermic storage. Small animal models are necessary to evaluate novel blood products and guide future clinical studies.
Objectives: The aim of this study was to assess the effect of liposome treatments on rat RBC hypothermic storage lesion (HSL) and to examine *in vivo* outcomes of transfusing liposome treated RBCs in a rat model.
Materials and methods: Unilamellar liposomes were synthesized which contained saturated (DPPC:CHOL, 7:3 mol%), unsaturated (DOPC:CHOL, 7:3 mol%), saturated charged (DPPC:CHOL:PS, 6:3:1 mol%), and unsaturated charged (DOPC:CHOL:PS, 6:3:1 mol%) phospholipids. After liposome treatment, rat RBC quality was assessed by percent hemolysis, deformability, aggregation, hematological indices, microvesiculation, and cholesterol/phospholipid concentrations. An anemic rat model of myocardial ischemia and reperfusion (I/R) was used to evaluate the outcomes of transfusing liposome-treated RBCs.
Results: All four liposome treatments resulted in significant decreases in hemolysis, with the most prominent effect seen with DOPC-liposomes (DOPC: 1.6 ± 0.1% versus control: 3.1 ± 0.2%, $p = 0.015$). RBCs treated with uncharged liposomes had lower hemolysis compared with charged liposomes (3.4 ± 0.2% versus 3.9 ± 0.4%, $p = 0.010$). The *in vivo* study showed no significant difference in the hemoglobin levels and infarct size (53.3 ± 13.1% versus 45.3 ± 8.4%, $p = 0.223$) between liposome and control groups.
Discussion and conclusion: Liposome treatment improved *in vitro* quality of stored rat RBCs. However, the changes observed in vitro were not sufficient to improve the in vivo outcomes of myocardial I/R in anemic rats transfused with liposome-treated RBCs.

Keywords

Blood storage, liposomes, red blood cells, transfusion

History

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Introduction

Although the use of additive solutions extends storage length of red blood cell (RBC) units, the quality of stored RBCs progressively decreases during hypothermic storage (1–6 °C). RBCs undergo a series of biochemical and biomechanical changes, collectively known as ''hypothermic storage lesion'' (HSL) (Van De Watering, 2011).

Apart from the use of additive solutions, other approaches to decrease RBC membrane injury during storage have been investigated such as rejuvenating solutions (Koshkaryev et al., 2009), anaerobic storage (Yoshida & Shevkoplyas, 2010) and the use of plasticizers (Draper et al., 2002) without much success. Since the cell membrane plays an important role in post-transfusion survival and oxygen delivery capacity of RBCs (Frenzel et al., 2009; Kiraly et al., 2009; Tsai et al., 2010), our research has focused on the use of liposomes – synthetic lipid vesicles – to mitigate RBC membrane injury during hypothermic storage (Holovati et al., 2008a,b; Stadnick et al., 2010; Stoll et al., 2011a,b). The mechanism of interaction between liposomes and human RBCs involves adsorption of the vesicles to the RBC surface, as well as liposome content incorporation through membrane fusion (Holovati et al., 2008a,b). RBCs are not able to synthesize phospholipids, so the main pathway for membrane phospholipid renewal *in vivo* is through exchange with plasma lipoproteins (Reed, 1968). The motivation for the use of liposomes is based on the transfer of lipids between liposome and RBC membrane (Stoll et al., 2011a,b), as it mimics what happens *in vivo*, and has been previously shown to change the membrane composition of human RBCs, ultimately improving cell deformability, membrane fluidity and reducing hemolysis upon storage (Stadnick et al., 2010; Stoll et al., 2011b). It is unknown whether liposomes can be used to mitigate elements of RBC membrane storage lesion unaddressed by current additive solutions, including microparticle (MP) shedding, phosphatidylserine (PS) exposure, and impaired hemorheology.

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Small animal models are necessary in evaluating novel manipulations of RBC storage, such as treatment with liposomes and guide future pre-clinical translational and clinical studies (Hu et al., 2012; Solomon et al., 2013). Rat models have been widely used to study effects of transfusion, different blood products, and blood substitutes on the cardiovascular system (D'Almeida et al., 2001; Hu et al., 2010, 2012; Xenocostas et al., 2010). Like human RBCs, rat RBCs are anucleate flexible binconcave discs, with similar structure and biochemical pathways. Similarities also exist in the asymmetry of the RBC membranes, and their phopholipid and protein composition (Benga & Borza, 1995; Matei et al., 2000; Pekiner, 2002). This animal model of anemia/myocardial ischemia provides us with a clinically relevant and objective means to measure the effects of transfusion of RBCs stored in novel conditions. Since the myocardium has a basal oxygen extraction ratio of 55–70% (Hu et al., 2010), it has little capacity to increase oxygen extraction, making it particularly vulnerable to the effects of anemia and an ideal model system to measure injury response to transfusion.

While our previous research on application of liposomes for mitigating the HSL largely focused on the *in vitro* mechanics of RBC and liposome interaction, as well as the quality of rat RBCs in current additive solutions for packed RBC (pRBC), this research investigates the effects of liposome treatment on RBC rheological properties, as well as *in vivo* assesment of efficacy of transfused liposome treated RBCs in a rat model. Considering that chemical characteristics of liposomes (charge, saturation) are important determinants of liposome-RBC interaction (Gottlieb, 1984; Raffy & Teissié, 1999; Tamura et al., 1986), the aim of this study was to assess the effect of different liposome formulations on rat RBC HSL and hemorheology. Finally, we examined the *in vivo* outcomes of transfusing liposome-treated RBCs in an anemic rat model of myocardial ischemia and reperfusion.

Materials and methods

RBC collection and manufacturing

Ethical approval for the animal study was granted by the Canadian Blood Services and the University of Alberta Research Ethics Boards. The Sprague–Dawley rats were all male, between 8 and 9 weeks old with an average weight of 300 g. The blood was collected and processed as previously described (Da Silveira Cavalcante et al., 2015). Briefly, blood was collected after anesthesia of the rats into 10 ml citrate phosphate-dextrose (CPD) anticoagulant vacuum tubes (Haematologic Technologies Inc., Essex Junction, VT) by cardiac puncture. Harvested blood was centrifuged (2200 × *g*, 10 min at 4 °C), and the plasma and the buffy coat were removed by aspiration and the RBCs were leukoreduced at room temperature using a 10 µm Veraspor membrane syringe filter (Pall Corporation, Ann Arbor, MI).

Liposome synthesis

Phospholipids for liposome synthesis were obtained from Avanti Polar Lipids (Alabaster, AL) and cholesterol was obtained from Sigma-Aldrich (St. Louis, MO). Multilamellar vesicles (MLVs) were prepared by the technique of Bangham and Horne (1964). Briefly, chloroform solutions of 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC, 16:0), 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC, 18:1), 1,2-dioleoyl-sn-glycero-3-phospho-L-serine (DOPS, 18:1), and cholesterol were thoroughly mixed, resulting in a 25 mM final lipid solution. Four different formulations were obtained: saturated uncharged (DPPC:cholesterol, 7:3 mol%), unsaturated uncharged (DOPC:cholesterol, 7:3 mol%), saturated charged (DPPC:cholesterol:DOPS, 6:3:1 mol%), and unsaturated charged (DOPC:cholesterol:DOPS, 6:3:1 mol%). Chloroform solvent was evaporated from the lipid mixture using a dry nitrogen stream, and subsequently lyophilized for at least 12 h (Virtis, AdVantage, Wizard-2.0, Gardiner, NY) to create a thin lipid film. Dry lipids were hydrated in HEPES-NaCl buffer containing 135 mM NaCl and 20 mM HEPES (pH 7.4, 276 mOsm). Small unilamellar vesicles were obtained by an extrusion technique (Hope et al., 1985) using a Lipex™ extruder (Northern Lipids Inc, Vancouver, BC) with 200 nm pore size Nuclepore® polycarbonate membranes (Whatman, Clifton, NJ). The mean *Z*-average size (nm) of the liposomes used in this study was determined using a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK).

Liposome treatment

The effects of liposome composition on rat RBC *in vitro* quality

The packed rat RBCs were incubated at 37 °C with either HEPES-NaCl solution (non-treated control) or 2 mM lipid from four different liposome formulations (DOPC 127 nm, DOPC-PS 131 nm, DPPC 178 nm, and DPPC-PS 148 nm). The RBCs were gently rotated during incubation. The *in vitro* quality of DPPC/DOPC/DPPC-PS/DOPC-PS RBCs ($n = 3$ each) and control RBCs ($n = 3$) was analyzed immediately after the liposome treatment and at weekly intervals up to 5 weeks of hypothermic storage and included percent hemolysis, hematological indices, deformability, and cholesterol and phospholipid concentrations, as described below.

The effects of DOPC liposome treatment on rat RBC hemorheology

The packed rat RBCs were incubated for 30 min at 37 °C with either HEPES-NaCl solution (non-treated control) or 2 mM lipid from DOPC liposomes (130 ± 2 nm). The RBCs were gently rotated during incubation. The *in vitro* quality of DOPC RBCs ($n = 7$) and control RBCs ($n = 7$) was analyzed immediately after the liposome treatment, upon 1 week and 6 weeks of hypothermic storage and included percent hemolysis, hematological indices, deformability, aggregation, and microvesiculation, as described below. The 6-week storage time is the maximum storage period that is permitted under regulations for human blood that is used in transfusion medicine.

RBC in vitro quality assessment

Rat RBC quality was assessed by percent hemolysis, hematological indices, deformability, aggregation,

microvesiculation, and cholesterol/phospholipid concentrations. Percent hemolysis was measured using a Drabkin's-based method (Drabkin, 1949). Briefly, the RBCs were diluted in Drabkin's reagent (0.61 mM potassium ferricyanide, 0.77 mM potassium cyanide, 1.03 mM potassium dihydrogen phosphate, and 0.1% triton X-100). Percent hemolysis was calculated as a ratio of the supernatant hemoglobin (Hgb) to the total Hgb, with the hematocrit (Hct), measured by microhematocrit (Sowemimo-Coker, 2002).

RBC hematological indices, including RBC count, the mean corpuscular volume (MCV), mean corpuscular Hgb (MCH), mean corpuscular Hgb concentration (MCHC), Hgb, and Hct were determined using a Coulter Automated Cell Counter (Coulter AcT, Beckman Coulter, New York, NY) (Tchir et al., 2013).

RBC deformability was measured using a laser-assisted optical rotational cell analyzer (LORCA) (Mechatronics, Zwaag, The Netherlands), as previously described (Stadnick et al., 2011). RBCs were diluted 1:100 in a polyvinylpyrrolidone solution, and subjected to increasing shear stress at 37 °C. Deformability curves were linearized using the Eadie–Hofstee method as published by Stadnick et al. (2011). Two parameters were extrapolated using this linearized function: the maximum elongation index (EI_{max}) and the cellular rigidity index (K_{EI}). (Da Silveira Cavalcante et al., 2015; Stadnick et al., 2011).

RBC aggregation was measured by syllectometry using laser-assisted optical rotational cell analyzer (LORCA; Mechatronics, Zwaag, The Netherlands) as previously described (Da Silveira Cavalcante et al., 2015). RBCs were washed with PBS and 400 µL of packed washed cells were mixed with 1% dextran (100 kDa) solution to an optimal Hct of 42–46%. The controls were prepared the same way and mixed with PBS (negative) and 3% dextran (positive). The laser backscatter intensity over time was measured and the following aggregation parameters were generated: aggregation index (AI), amplitude (Amp), and aggregation half-time ($t_{1/2}$) (Hardeman et al., 2001).

The flow cytometry procedure to measure rat RBC MPs and PS exposure was done on a FACSCalibur (BD Biosciences, San Jose, CA) equipped with a 488 nm argon laser and computer software (CellQuest, BD Biosciences, San Jose, CA) as previously described (Da Silveira Cavalcante et al., 2015). Peridinin chlorophyl protein complex-cyanine 5.5 (PerCP-Cy™ 5.5) anti-rat erythroid cells antibody (BD PharMingen, San Jose, CA) was used as a marker for rat RBCs and MPs. APC annexin V (BD PharMingen, San Jose, CA) was used to label PS according to manufacturer's instructions. Commercial isotype control directed against rat erythroid cells (PerCP-Cy™ 5.5 mouse IgM, k isotype control, BD PharMingen, San Jose, CA) was used to account for any non-specific binding of the antibody.

Density gradient separation using Ficoll-Paque (GE Healthcare Biosciences, Uppsala, Sweden) was performed to separate RBCs from liposomes adsorbed to the RBC membrane, as well as free liposomes in the incubation mixture (Holovati et al., 2008a,b). Lipid extraction was performed according to Rose & Oklander (1965). The EnzyChrom™ AF Cholesterol Assay Kit (E2CH-100; BioAssay Systems, Hayward, CA) was used to determine the amount of cholesterol in the extracts (Stoll et al., 2011a,b). Phospholipids in the extracts were estimated by spectrophotometric phosphorus determination after an acid digestion as previously described (Chen et al., 1956).

It was not within the scope of this work to evaluate morphological assessment of the RBCs, since it has been previously shown that the interaction of liposomes with RBCs does not adversely affect the general morphology of the cells (Holovati et al., 2008b).

Effects of transfusing DOPC-treated rat RBCs in an anemic rat model of myocardial ischemia and reperfusion

Male Sprague–Dawley rats (200–300 g) were used to experimentally evaluate *in vivo* effect of transfusing liposome-treated RBCs. All animals were provided water and food *ad libitum* and housed in a temperature- and humidity-controlled facility with 12-h light and dark cycles. The investigation conforms to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication no. 85–23, revised 1996). Experimental protocols were approved by the Animal Use Committee at the University of Western Ontario. Anemia was induced in rats by a combination of iron-deficient diet (10–20 ppm; TestDiet 5859, Richmond, IN) and phlebotomy (2–3 mL) twice weekly to reach target Hgb levels of 80–90 g/L. Immediately after phlebotomy, rats were infused with an equal volume of 10% pentastarch (Bristol-Myers Squibb Canada, Montreal, Quebec, Canada) (Hu et al., 2010). Myocardial ischemia and reperfusion (I/R) was induced by occlusion of the left descending coronary artery for 45 min, followed by 24 h of reperfusion according to our previous reports (Hu et al., 2010, 2012). Anemic rats were transfused immediately after coronary artery ligation with either non-treated RBCs or DOPC liposome-treated (131 ± 5 nm) RBCs stored for 1 week. After transfusion, Hgb levels, infarct size, and 24 h-survival were measured. Infarct size was determined according to our previous reports (Burger et al., 2006, 2009). Briefly, 3 mL of Evans blue dye was injected into the left ventricle to distinguish between perfused and non-perfused areas of the heart. Hearts were then excised and cut into four transverse slices from the apex to base. Sections were stained with 1.5% triphenyltetrazolium chloride (TTC) for 30 min at 37 °C, and then weighed. The non-ischemic area (blue), area at risk (red), and infarct area (pale) were quantitated using an image analysis system (Sigma ScanPro, Ashburn, VA). Percent weight of non-ischemic, risk, and infarct area were calculated. Infarct size was expressed as a percentage of the weight of the infarct area to the area at risk.

Statistical analyses

Statistical analysis was performed using SPSS 22.0 software (IBM, Armonk, NY). Mann–Whitney's *U* non-parametric test was used to assess differences between liposome-treated rat RBCs and controls, and between anemic rats transfused with liposome-treated RBCs versus non-treated RBCs. Wilcoxon signed-rank test was used to assess differences between liposome-treated rat RBCs and controls over storage

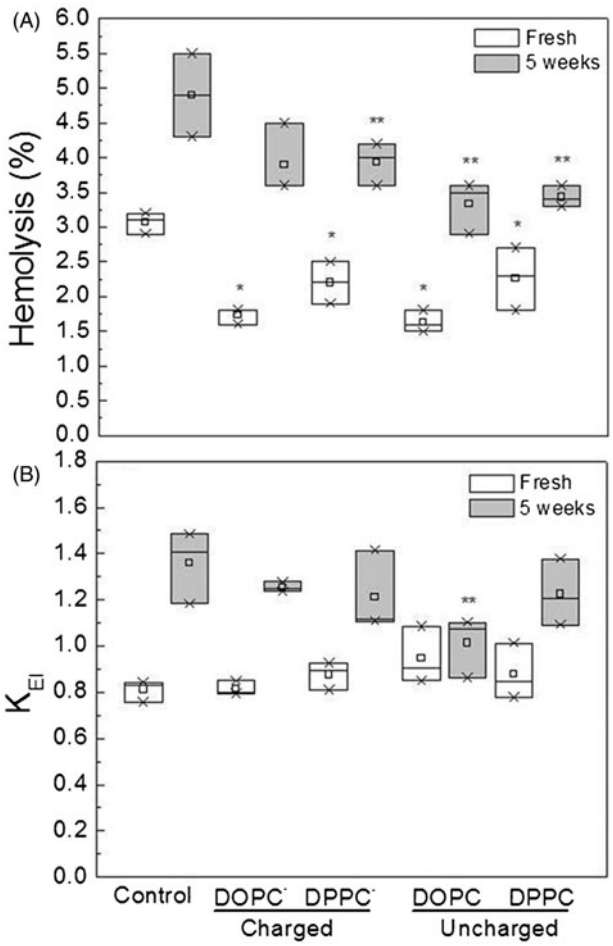

Figure 1. Percent hemolysis (A) and rigidity (B) in rat RBCs treated with charged and uncharged liposome formulations. Shown in the boxplots ($n = 3$) are 99% percentile (higher ×), 1% percentile (lower ×), and mean (□). *Significant ($p < 0.05$) compared with fresh control RBCs; **significant ($p < 0.05$) compared with control RBCs stored for 5 weeks.

time. Data were expressed as mean ± standard deviation and $p < 0.05$ was considered statistically significant.

Results

The effects of liposome composition on rat RBC *in vitro* quality

Immediately after incubation, all liposome treatments resulted in significant decrease in percent hemolysis, with the effect most prominent with DOPC-treated RBCs ($1.6 \pm 0.1\%$ versus $3.1 \pm 0.2\%$, $p = 0.050$) (Figure 1A). Liposome treatment did not significantly alter rat RBC membrane deformability (EI_{max}: control 0.58 ± 0.00; DOPC-PS 0.59 ± 0.01; DOPC 0.57 ± 0.00; DPPC-PS 0.57 ± 0.01; DPPC 0.58 ± 0.00, $p > 0.050$) or hematological indices (MCV, MCH, and MCHC). DOPC liposome treatment resulted in significant increase in rat RBC phospholipid concentration, while DPPC liposome treatment seemed to induce loss of phospholipids from the rat RBC membranes (0.30 ± 0.03 mg versus 0.15 ± 0.01 mg, $p = 0.050$). The cholesterol:phospholipid ratio in DOPC-treated rat RBCs was comparable with control (0.49 ± 0.06 versus 0.51 ± 0.08, $p = 0.800$). All liposome-treated RBCs continued to exhibit decreased hemolysis after 5 weeks of storage compared with the control ($p = 0.050$), with uncharged formulations resulting in lower hemolysis than charged liposomes ($3.4 \pm 0.2\%$ versus $3.9 \pm 0.4\%$, $p = 0.010$) (Figure 1A). In terms of rigidity (K_{EI}), DOPC-treated RBCs remained significantly lower compared with control RBCs even after 5 weeks of storage (Figure 1B). Based on these findings, the DOPC formulation was selected for further studies on hemorheology.

The effects of DOPC liposome treatment on rat RBC hemorheology

Table 1 describes the hemorheology parameters of liposome-treated and control RBCs, both immediately after treatment (fresh) and after 6 weeks of hypothermic storage. DOPC liposome treatment resulted in significant changes in fresh liposome-treated RBCs compared with control RBCs, such as a decrease in percent hemolysis ($1.7 \pm 0.2\%$ versus $2.3 \pm 0.3\%$, $p = 0.018$), MCH (22.0 ± 1.0 versus 22.3 ± 0.9, $p = 0.018$), and MCHC (355 ± 11 versus 375 ± 7, $p = 0.046$) an increase in rigidity (K_{EI}: 0.84 ± 0.10 versus 0.77 ± 0.07, $p = 0.043$) and Hct ($51.7 \pm 2.3\%$ versus $49.8 \pm 1.3\%$, $p = 0.041$). After 6 weeks of hypothermic storage, liposome-treated RBCs continued to differ from control RBCs by exhibiting an increased Hct ($47.1 \pm 1.7\%$ versus $45.0 \pm 1.5\%$, $p = 0.028$) and lower rigidity (K_{EI}: 1.03 ± 0.18 versus 1.16 ± 0.13, $p = 0.043$). Flow cytometry analysis also showed increased PS exposure in liposome-treated RBCs (1.1 ± 0.2 versus 0.8 ± 0.2, $p = 0.018$), while PS exposure in MPs was significantly lower (55.8 ± 6.5 versus 34.8 ± 8.5, $p = 0.03$) (Figure 2). In terms of aggregation parameters, there was no significant difference between control and liposome-treated RBCs. However, control RBCs showed a significant decrease in aggregation index (AI: $41.9 \pm 7.2\%$ versus $29.9 \pm 4.1\%$, $p = 0.018$) and an increase in aggregation half time ($t_{½}$: 6.5 ± 2.9 s versus 12.5 ± 2.8 s, $p = 0.018$) after 6 weeks of storage that was not observed in liposome-treated RBCs (Figure 3). The only change observed in liposome-treated RBCs was a decrease in aggregation amplitude (Amp: 12.9 ± 1.7 au versus 10.9 ± 1.4 au, $p = 0.018$).

Effects of transfusing DOPC-treated rat RBCs in an anemic rat model of myocardial ischemia and reperfusion

The Hgb levels prior to transfusion were similar in both control and liposome-treated groups (84.2 ± 7.1 versus 83.5 ± 5.3 g/L, $p = 1.000$). The *in vivo* study showed that the 24 h-survival after myocardial I/R was the same in both liposome-treated (seven out of seven) and control groups (seven out of seven). No significant difference was observed in the Hgb levels (control: 99.8 ± 3.0 versus lipo: 101.9 ± 2.5 g/L, $p = 0.197$) and infarct size between the two groups (control: 53.3 ± 13.1 versus lipo: $45.3 \pm 8.4\%$, $p = 0.223$) (Figure 4). Note that two rats from the liposome-treated group had to be excluded from the statistical analysis of infarct size due to very small infarct sizes attributed to the surgical procedure.

Discussion

A membrane-based approach to preserve RBC quality, such as the use of liposomes, may mitigate MP shedding, PS

Table 1. Hemorheology parameters of DOPC-treated RBCs and control RBCs. Mean values ± SD for DOPC-treated and control rat RBCs immediately after treatment and after 6 weeks of hypothermic storage are shown.

RBC hemorheology	Control RBCs		DOPC-treated RBCs	
	Fresh	6 weeks	Fresh	6 weeks
Hemolysis (%)	2.3 ± 0.3	4.7 ± 0.7‡	1.7 ± 0.2*	5.0 ± 0.6‡
Hematologic indices				
MCV (fL)	62.1 ± 2.2	64.1 ± 2.5‡	61.9 ± 2.5	64.4 ± 3.0‡
MCH (pg)	22.3 ± 0.9	24.1 ± 1.1‡	22.0 ± 1.0*	24.2 ± 1.2‡
MCHC (g/l)	360 ± 10	375 ± 7	355 ± 11*	376 ± 6‡
Deformability				
EI_{max}	0.56 ± 0.01	0.40 ± 0.02‡	0.56 ± 0.02	0.39 ± 0.02‡
K_{EI}	0.77 ± 0.07	1.16 ± 0.13‡	0.84 ± 0.10*	1.03 ± 0.18*
Aggregation parameters				
AI (%)	41.9 ± 7.2	29.9 ± 4.1‡	34.7 ± 9.2	30.7 ± 6.1
Amp (au)	11.7 ± 1.8	10.6 ± 2.0	12.9 ± 1.7	10.9 ± 1.4‡
$t_{1/2}$ (s)	6.5 ± 2.9	12.5 ± 2.8‡	9.1 ± 4.4	11.4 ± 4.5
Microvesiculation				
MPs/μL	24 663 ± 1529	444 084 ± 103 366‡	26 339 ± 2897	569 258 ± 115 981‡
RBC-PS (%)	0.3 ± 0.0	0.8 ± 0.2‡	0.3 ± 0.0	1.1 ± 0.2*‡
MP-PS (%)	35.1 ± 4.7	24.8 ± 7.8‡	34.3 ± 4.0	28.5 ± 9.3
RBC-MFI	68.3 ± 6.1	59.3 ± 9.2	74.0 ± 17.0	53.3 ± 5.0‡
MP-MFI	146.3 ± 32.3	49.1 ± 8.5‡	131.6 ± 32.5	44.9 ± 2.0‡
MP-MFI/MP-PS%	3.9 ± 0.8	1.8 ± 0.2‡	3.7 ± 0.9	1.5 ± 0.3* ‡

au, arbitrary units.
*$p < 0.05$ compared with control RBCs of corresponding age.
‡$p < 0.05$ compared with fresh RBCs of the same group.

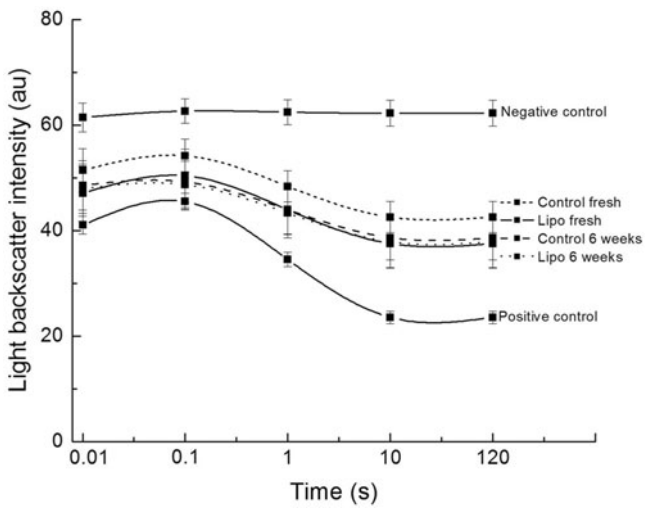

Figure 2. RBC aggregation in control and liposome-treated rat RBCs (fresh and after 6 weeks of storage). Syllectograms (light scatter x time) showing RBC aggregation patterns ($n = 7$). Negative control (PBS) = no aggregation; positive control (3% dextran) = high aggregation.

exposure, and impaired hemorheology. One of the objectives of this study was to investigate the effects of liposome treatment on rat RBC HSL and rheological properties. In addition, the outcomes of transfusing liposome-treated RBCs in a rat model were assessed.

All the liposome formulations tested successfully decreased hemolysis in rat RBCs compared with non-treated controls. The same effect has also been described for human RBCs (Stadnick et al., 2010) treated with DPPC liposomes, considering that preference for saturated or unsaturated fatty acids is dependent on the RBC species (Rindlisbacher & Zahler, 1983). RBCs treated with liposomes exhibited higher Hct and lower supernatant Hgb concentrations, showing that the treatment stabilizes the cell membrane, preventing Hgb leakage. The exact mechanism by which liposomes reduce hemolysis has not been completely elucidated, but based on the *in vitro* mechanisms of liposome-cell interactions previously described, the possible interactions include lipid transfer, adsorption, fusion, or a combination of these (Pagano & Weinstein, 1978; Stoll et al., 2011b). The DOPC liposomes used in this study, which were small unilamellar vesicles (SUV), neutral (not charged), and fluid (always kept above the *Tc* of the lipid), had the most prominent effect in reducing hemolysis of rat RBCs. Based on those characteristics, the most probable RBC–liposome interaction types are membrane fusion and lipid transfer (Pagano & Weinstein, 1978; Stoll et al., 2011a). Renooij & Van Golde (1976) have reported that lipid exchange between rat RBCs and lipid vesicles is a temperature dependent process that only becomes significant at temperatures higher than 20 °C, which explains why we observed a difference in hemolysis immediately after incubation with liposomes at 37 °C, but was no longer detectable after the storage period at 4 °C.

The concentration of MPs was comparable between the two treatment groups when fresh and after the 6-week storage period. However, some slight differences in the PS profile of those MPs lead us to argue whether they would have the same biological activity. The percentage of PS (+) MPs decreased in control samples and remained stable in liposome-treated samples over time, with overall values being comparable between the groups for both time points evaluated (fresh and 6 weeks). However, even though the percentage of PS (+) MPs is comparable in both groups after 6 weeks, MPs from liposome-treated group exposed less PS per MP compared with control (as shown by MP-MFI/MP-PS% values; Table 1). Percentage of PS (+) RBCs increased over time in both groups, with a more prominent increase in the liposome-treated group. On one hand, PS exposure is often associated

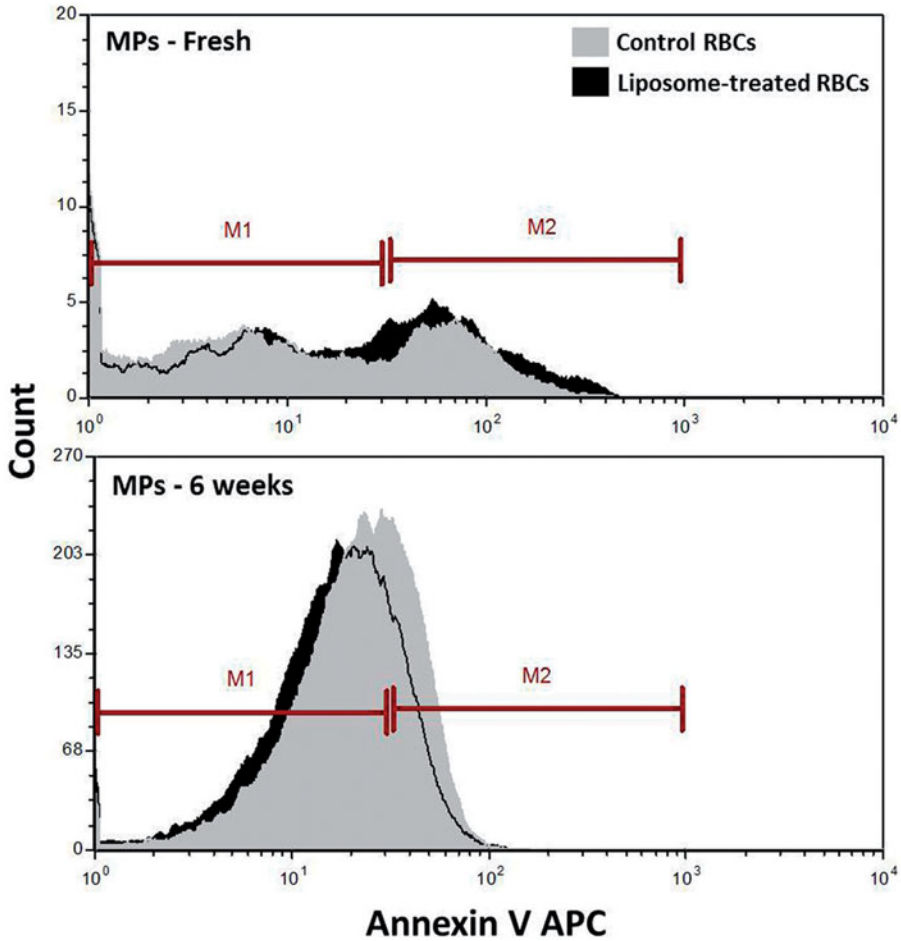

Figure 3. Percentage of phosphatidylserine (PS) exposure by MPs in fresh control and liposome-treated rat RBCs and after 6 weeks of storage. Histogram: region M1 is negative for PS exposure while, region M2 is positive.

with increased inflammation, coagulation, and endothelial injury (Kriebardis et al., 2012; Tissot et al., 2013) so it would be fair to assume that less PS exposure on MPs could result in less adverse events. On the other hand, more PS exposure is also present in senescent cells and correlates with increased *in vivo* uptake by the reticuloendothelial system (Schroit et al., 1985) which would potentially result in faster removal of those MPs from the circulation. Further investigation is needed to establish the clinical relevance of these findings and understand whether the two types of MPs are equivalent in terms of bioactivity.

Stored RBCs show a progressive decrease in deformability and other rheological impairments that can affect perfusion and oxygen delivery especially in critically ill patients (Berezina et al., 2002; Zimring, 2015). In this study, both treatment groups presented an equivalent decrease in EI_{max} over the 6 weeks of storage. The decrease in deformability might be explained by membrane loss in the form of MPs that was not overcome by the liposome formulation used. The K_{EI} from liposome-treated RBCs increased significantly immediately after treatment and remained stable during storage, while in control RBCs, it significantly increased over time and to a greater extent. Lipid transfer or fusion of liposomes with RBCs in the treatment group could alter the association of the membrane with the cytoskeleton, changing the rigidity at first but then maintaining it constant throughout storage. In terms of aggregation parameters, there was no significant difference between the two groups. However, fewer changes were observed in the liposome-treated group over time suggesting a stabilization effect of liposomes on RBCs.

The elements of rat RBC HSL are similar to human, except that the rat RBC storage lesion of 7 d is equivalent to the human RBC storage lesion of 29 d (D'Almeida et al., 2000). This animal model has been used in the past to evaluate effects of hypothermic storage lesion and leukoreduction (Hu et al., 2010, 2012; Xenocostas et al., 2010). By using sensitive measures of specific organ injury and myocardial infarct size, we have been able to quantitate the effects of anemia, as well as differentiate between fresh and stored blood transfusion. In the presence of pre-existing or experimentally induced coronary occlusion, a modest drop in hemoglobin can lead to further ischemic injury while raising the Hgb by 20 g/L improves cardiac function. The liposome formulation used in the *in vivo* study did not show superior effects in terms of the outcomes of I/R in anemic rats, despite having increased Hgb levels by 18.4 g/L. The present study was limited to examining the acute effects of transfusing liposome-treated RBCs in a relatively small number of animals per group. Even so, because the *in vivo* study did not prompt any immediate safety concerns, we feel encouraged to continue investigating the beneficial effects of liposome treatment on stored human RBCs *in vitro*.

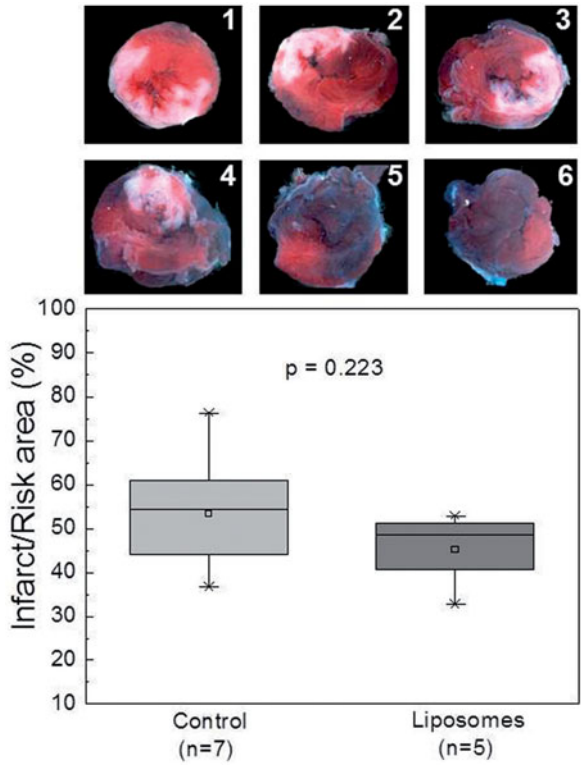

Figure 4. Effect of liposome treated red blood cells on infarct size after ischemia and reperfusion in anemic rats. Transverse sections of the heart from apex to base (1–6) were stained with 2% triphenyltetrazolium chloride (TTC). Blue area, non-ischemic, red area: at risk area; white area, infarct area. Shown in the box plots are 99% percentile (higher ×), 1% percentile (lower ×), and mean (□). B&W print: Transverse sections of the heart (1–3), dark area, at risk area; white area, infarct area. Sections (4–6) dark area, non-ischemic plus at risk area; white area, infarct area.

Conclusions

In conclusion, of the formulations tested unsaturated-uncharged liposomes (DOPC) were the most beneficial for rat RBCs and were, therefore, used to further evaluate effects on hemorheology and in vivo studies. DOPC liposome treatment resulted in an overall improvement in rat RBC hemorheology upon storage. However, in vivo study results suggested that the changes observed with liposome treatment in vitro were not sufficient to improve the outcomes of myocardial I/R in anemic rats transfused with liposome-treated RBCs.

Declaration of interest

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