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Original Research Article

Synergistic Effect of Trehalose and Saccharose Pretreatment on Maintenance of Lyophilized Human Red Blood Cell Quality

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Abstract

Purpose: *To investigate the synergistic effect of trehalose and saccharose pretreatment on maintenance of lyophilized human red blood cell (RBC) quality.*
Methods: *RBCs were pre-treated with trehalose and saccharose, and then lyophilized and re-hydrated. Prior to lyophilization and after re-hydration, RBC parameters, RBC counts, total hemoglobin concentration, mean corpuscular hemoglobin (MCH), mean corpuscular volume (MCV), comprehensive deformation index, hemolysis ratio and phosphatidylserine (PS) expression, were determined using a hematology analyzer, an RBC deformation instrument, a spectrophotometer and a flow cytometer, respectively. Superoxide dismutase (SOD), glucose-6-phosphate dehydrogenase (G-6-PD), and adenosine triphosphatase (ATPase) activities were determined using kits for SOD, ATPase, and G-6-PD assay, respectively.*
Results: *After lyophilization-rehydration, RBC counts and total hemoglobin recovery rates, deformability, and RBC SOD, ATPase, and G-6-PD activities were significantly decreased by 47.24 – 74.65 % ($p < 0.01$), compared with the normal group. RBC osmotic fragility and PS expression on the outer surface of the RBC membrane were significantly increased by 168.53 and 629.30 % ($p < 0.01$), respectively, compared with the normal group. RBC MCH and MCV values were not significantly affected by lyophilization-rehydration ($p > 0.05$). Trehalose and saccharose pretreatment significantly reversed the effects of lyophilization-rehydration on these RBC parameters by approximately 13.16 – 211.11 % ($p < 0.01$), compared with the control group. The combined effects were synergistic.*
Conclusion: *Trehalose and saccharose pretreatment synergistically enhances maintenance of lyophilized RBC quality.*

Keywords: *Trehalose, Saccharose, Lyophilization, Red blood cell, Hematological parameters*

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INTRODUCTION

Human red blood cell (RBC) preservation methods include short-term preservation at 4 °C for 35 – 42 days and long-term preservation at -80 or -196 °C for approximately 10 years. Because the two preservation methods require specialized preservation devices and are expensive to perform [1], they do not satisfy clinical and emergency use requirements for RBC. Lyophilization is a new RBC preservation method. Compared with the short- and long-term methods, lyophilization advantages include long-term room temperature preservation, convenient transportation, and simple storage conditions [2,3]. However, despite these advantages,

problems such as membrane damage, hemoglobin leakage, and high RBC hemolysis ratio have been reported [4]. In recent decades, lyophilization protectants have been developed to resolve these problems.

Trehalose and saccharose are two intracellular protectants that can reduce the intracellular water content and increase the concentration of cytoplasm components [1]. When cells are freeze-dried, the hydroxyl functional groups of trehalose and saccharose can combine with cell proteins and lipids via hydrogen bonds; this change maintains the stability of cell membranes and proteins [5]. Trehalose and saccharose have been used as lyophilization protectants in many fields [6-8], but the synergistic effects of trehalose and saccharose pretreatment on the maintenance of lyophilized RBC quality are unknown. This study was designed to investigate whether trehalose and saccharose pretreatment can synergistically reverse the effects of lyophilization-rehydration on the maintenance of lyophilized RBC quality.

EXPERIMENTAL

Chemicals and reagents

Acid citrate-dextrose (ACD) anticoagulant and PBS buffer solution were obtained from Sigma-Aldrich (St. Louis, MO, USA). The lyophilization buffer solution consisted of 15 % polyvinylpyrrolidone, 15 % bovine serum albumin, 10 % sodium citrate and 20 % trehalose. The re-hydration buffer solution consisted of 111 mmol/L glucose, 41 mmol/L mannitol, 2 mmol/L adenine, and NaCl (1.0, 0.5, or 0.14 mol/L). Annexin V-FITC, superoxide dismutase (SOD), glucose-6-phosphate dehydrogenase (G-6-PD) and adenosine triphosphatase (ATPase) assay kits were obtained from Beyotime Biotechnology (Shanghai, China).

Preparation of RBCs and grouping

Whole blood that was derived from venous blood of healthy volunteers was obtained from the Department of Transfusion of the Central Hospital of Zhumadian City (Henan Province, China). After pretreatment with ACD anticoagulant, whole blood was centrifuged at 3000 rpm for 10 min at 4 °C and the supernatant and buffy coat were discarded to obtain the concentrated RBCs. RBCs were then washed thrice with isotonic PBS and centrifuged at 3000 rpm for 10 min at 4 °C to obtain standby RBCs. The standby RBCs were suspended in isotonic PBS buffer solution to provide the 45 % hematocrit RBCs. These RBCs were stored at a temperature of 4 °C until they were used for the experiment.

The 45 % hematocrit RBCs were randomly divided into 5 groups of 20 mL each (n = 10 replicates per group). The groups were the normal RBC group (NG, fresh RBCs, no lyophilization-rehydration), the control group (CG, no pretreatment with lyophilization-rehydration), the trehalose group (TG, 800 mmol/L trehalose pretreatment with lyophilization-rehydration), the saccharose group (SG, 800 mmol/L saccharose pretreatment with lyophilization-rehydration), and the trehalose and saccharose group (TSG, 400 mmol/L trehalose and 400 mmol/L saccharose pretreatment with lyophilization-rehydration).

Trehalose and saccharose pretreatment

The 20 mL 45 % hematocrit RBCs samples were separately pre-treated with different concentrations of trehalose and saccharose as described above. For example, for one pretreatment (800 mmol/L trehalose), 25 mL 1.6 mol/L trehalose and 15 mL isotonic PBS were slowly added to 20 mL 45 % hematocrit RBCs. After a 5-min equilibration period, an additional 25 mL 1.6 mol/L trehalose and 15 mL isotonic PBS were slowly added to the sample. The final samples were incubated at 37 °C for 7 h. The RBCs were then washed thrice with isotonic PBS and isolated using centrifugation at 3000 rpm for 10 min at 4 °C.

Pre-freezing and lyophilization

Each sample of pre-treated RBCs (0.3 mL) was re-suspended in 0.7 mL lyophilization buffer and then transferred into a freeze dry vial and equilibrated for 30 min at 4 °C. The sample was then cooled to -70 °C at a cooling rate of -5 °C/min in a programmable freezer and was held for 2 h. The vial was then placed on the shelf of a vacuum freeze-drying apparatus (Advantage EL, Virtis, USA) at 150 mbar vacuum pressure. The lyophilization process included two steps (i.e., primary, and secondary drying), which is presented in Table 1.

Re-hydration

Re-hydration was performed by directly adding pre-heated re-hydration buffer (37 °C) to each lyophilized RBC sample. The sample was completely dissolved using gentle shaking. To decrease osmotic damage of lyophilized RBCs during the lyophilization to re-hydration process, the lyophilized RBCs were washed with gradient

saline solutions. Each solution contained the re-hydration buffer and 1.0, 0.5, or 0.14 mol/L NaCl. The RBCs were then isolated using centrifugation at 3000 rpm for 10 min at 4 °C. The final volume of each sample was 1 mL.

Table 1: Lyophilization process of pre-treated RBCs

Drying process	Shelf temp (°C)	Hold time (h)
Primary	-70	1
	-60	2
	-50	2
	-45	2
	-40	2
	-35	2
Secondary	-30	2
	-20	2
	-10	1
	0	1

Conventional RBC indicators

Conventional RBC indicators, including RBC counts, total hemoglobin concentration, mean corpuscular hemoglobin (MCH), and mean corpuscular volume (MCV)] were determined using a hematology analyzer (Sysmex XE-2100, Tokyo, Japan). The RBC counts and total hemoglobin recovery rates were calculated as in Eqs 1 and 2.

RBC counts recovery rate (%) = $(N_1/N_2)100$ ……. (1)

N_2 and N_1 represented RBC counts before lyophilization and after re-hydration, respectively.

Total hemoglobin recovery rate (%) = $(Hb_1/Hb_2)100$ ….. (2)

Hb_2 and Hb_1 were total hemoglobin concentrations before lyophilization and after re-hydration, respectively.

Evaluation of RBC deformability

RBC deformation capability was determined using an RBC deformation instrument (LBY-BX, China) according to a reported method [9]. An RBC deformation index was determined and a comprehensive RBC deformation index was calculated for each sample.

Osmotic fragility assay

The RBC osmotic fragility assay was performed according to a reported method [10]. After re-hydration, RBC subsamples (20 µL) were separately added to varying concentrations of a 4.5 mL NaCl solution (2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, or 7 g/L). After 30 min, each sample was centrifuged at 2000 rpm for 20 min. The absorbance (A) of the supernatant was measured at a 540 nm wavelength using a Shimadzu spectrophotometer (UV-9100, Kyoto, Japan). Before lyophilization, each 20 µL RBC sample was added to 0.9 % 4.5 mL NaCl solution; the absorbance (A_0) value for the supernatant was used as the value for the blank control. Before lyophilization, each 20 µL RBC was added to distilled water and the absorbance (A_{100}) value of the supernatant was used as the hemolysis control value. The RBC hemolysis ratio was calculated as in Eq 3.

Hemolysis ratio (%) = $\{(A-A0)/(A_{100}-A_0)\}100$ ... (3)

Determination of membrane phosphatidyl-serine (PS) expression

After re-hydration, the RBC sample was re-suspended in PBS to obtaine a 1×10^6/mL suspension. Each 100 µL cell suspension was stained with Annexin V-FITC according to the manufacturer's instructions. The stained RBCs were analyzed using a FACSCalibur flow cytometer (BD Bioscience, Franklin Lakes, NJ, USA).

SOD, ATPase, and G-6-PD assays

SOD, ATPase, and G-6-PD activities were determined using corresponding kits according to the manufacturer's instructions. The SOD activity (U/gHb) was determined using a xanthine oxidase method [11]. The ATPase activity (U/10^7 RBCs) was determined based on ATP-ATPase reaction that generates inorganic phosphate [12]. The G-6-PD activity (U/gHb) was determined based on $NADP^+$-G-6-PD reaction that generates NADPH [13].

Statistical analysis

All results are presented as mean ± standard error of the mean (SEM, n = 10). Between-group differences were analyzed using one-way ANOVA (LSD test) on SPSS 21.0 software, IBM Corporation, Armonk, NY, USA. Differences were considered statistically significant at $p < 0.05$ or 0.01.

RESULTS

Effects of trehalose and saccharose pretreatment on conventional lyophilized RBC indicators

After lyophilization-rehydration, the RBC counts and total hemoglobin recovery rates in the TG, SG, and TSG were significantly increased by 21.24 - 180.57 % ($p < 0.01$), compared with the

CG (Fig. 1A). RBC and total hemoglobin recoveries among the TG, SG, and TSG were also significantly different (TSG > SG > TG; $p < 0.01$). The differences in MCH and MCV values among the NG, CG, TG, SG, and TSG were not statistically significant (Fig. 1B).

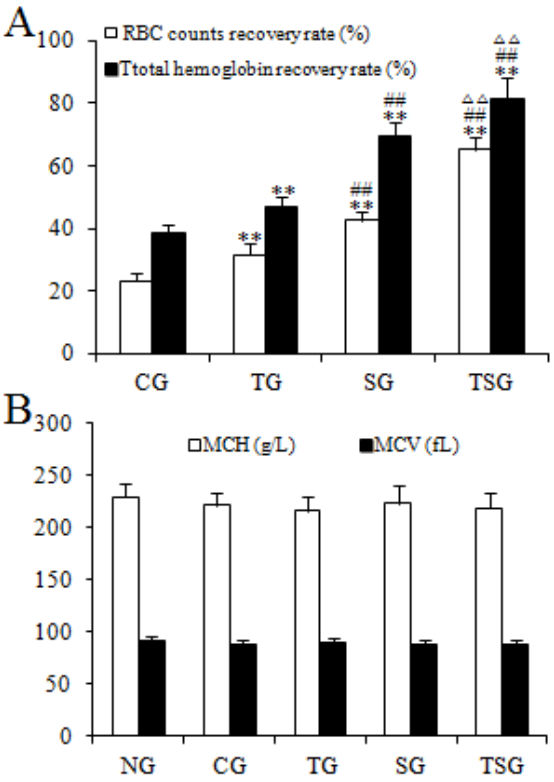

Figure 1: Effects of trehalose and saccharose pretreatment on conventional lyophilized RBC indicators including RBC counts and total hemoglobin recovery rates, MCH and MCV; $^{**}p < 0.01$, compared with the CG; $^{\#\#}p < 0.01$, compared with the TG; $^{\triangle\triangle}p < 0.01$, compared with the SG

Effects of trehalose and saccharose pretreatment on comprehensive lyophilized RBC deformation index

The lyophilized RBC comprehensive deformation index in the CG was significantly decreased by 56.25 % ($p < 0.01$), compared with the NG (Fig. 2). After trehalose and saccharose pretreatment, the comprehensive lyophilized RBC deformation indices in the TG, SG, and TSG were significantly increased by 38.10, 71.43 and 100.00 % ($p < 0.01$), respectively, compared with the CG. The comprehensive lyophilized RBC deformation indices among the TG, SG, and TSG were also significantly different (TSG > SG > TG; $p < 0.01$).

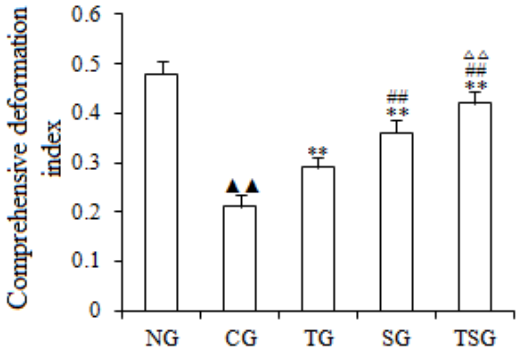

Figure 2: Effects of trehalose and saccharose pretreatment on comprehensive lyophilized RBC deformation index; $^{\blacktriangle\blacktriangle}p < 0.01$, compared with the NG; $^{**}p < 0.01$, compared with the CG; $^{\#\#}p < 0.01$, compared with the TG; $^{\triangle\triangle}p < 0.01$, compared with the SG

Effects of trehalose and saccharose pretreatment on lyophilized RBC hemolysis ratio

The hemolysis ratio versus NaCl curve in the CG was shifted to the right, compared with the NG (Fig. 3A), indicating that the hemolysis ratios were increased in the CG. After trehalose and saccharose pretreatment, the hemolysis ratio versus NaCl curves in the TG, SG, and TSG were shifted to the left, compared with the CG, suggesting that the hemolysis ratios were decreased in these groups. The statistical analysis of the hemolysis ratios at 4.5 g/L NaCl indicated that the lyophilized RBC hemolysis ratio in the CG was significantly increased by 168.53 % ($p < 0.01$), compared with the NG (Fig. 3B). After trehalose and saccharose pretreatment, the lyophilized RBC hemolysis ratios in the TG, SG, and TSG were significantly decreased by 13.16, 26.81 and 54.57 % ($p < 0.01$), respectively, compared with the CG. The lyophilized RBC hemolysis ratios among the TG, SG, and TSG were also significantly different (TSG < SG < TG; $p < 0.01$).

Effects of trehalose and saccharose pretreatment on PS expression in lyophilized RBC

PS expression on the outer surface of lyophilized RBC membrane in the CG was significantly increased by 629.30 % ($p < 0.01$), compared with the NG (Fig. 4). After trehalose and saccharose pretreatment, PS expression in the TG, SG, and TSG were significantly decreased by 44.58, 52.49 and 70.54 % ($p < 0.01$), respectively, compared with the CG. PS expressions among

the TG, SG, and TSG were also significantly different (TSG < SG < TG; $p < 0.01$).

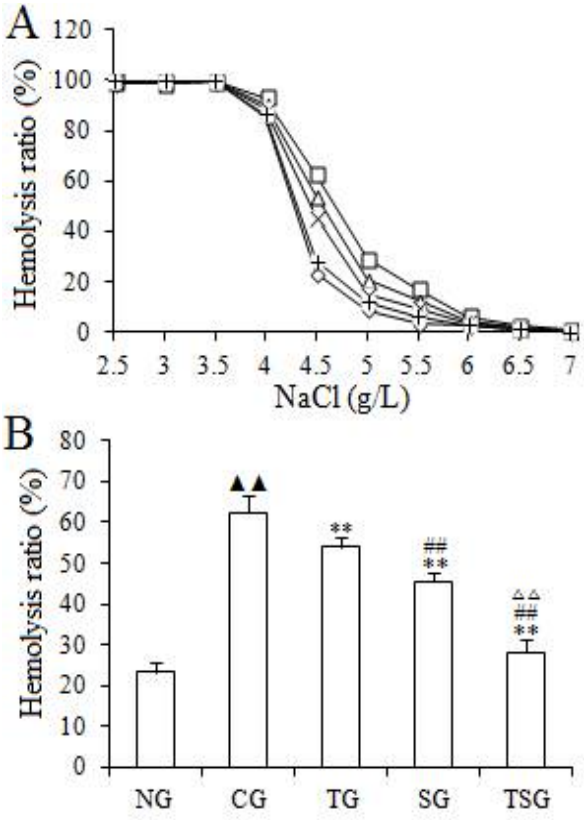

Figure 3: Effects of trehalose and saccharose pretreatment on lyophilized RBC hemolysis ratio; A: Lyophilized RBC hemolysis ratio in the NG (◇), CG (□), TG (△), SG (×) and TSG (+) when the concentrations of NaCl were 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5 and 7 g/L, respectively; B: Lyophilized RBC hemolysis ratio when the concentration of NaCl was 4.5 g/L; $^{▲▲}p < 0.01$, compared with the NG; $^{**}p < 0.01$, compared with the CG; $^{\#\#}p < 0.01$, compared with the TG; $^{△△}p < 0.01$, compared with the SG

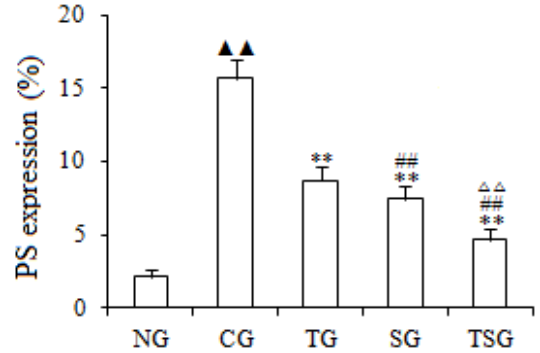

Figure 4: Trehalose and saccharose pretreatment decreased PS expression on the outer surface of lyophilized RBC membrane; $^{▲▲}p < 0.01$, compared with the NG; $^{**}p < 0.01$, compared with the CG; $^{\#\#}p < 0.01$, compared with the TG; $^{△△}p < 0.01$, compared with the SG

Effects of trehalose and saccharose pretreatment on SOD, ATPase, and G-6-PD activities of lyophilized RBC

The SOD, ATPase, and G-6-PD activities of lyophilized RBC in the CG were significantly decreased by 47.27, 49.46 and 74.65 % ($p < 0.01$), respectively, compared with the NG (Fig. 5). After trehalose and saccharose pretreatment, the SOD, ATPase and G-6-PD activities in the TG, SG, and TSG were significantly increased by 20.21 – 211.11 % ($p < 0.01$), compared with the CG. The SOD, ATPase, and G-6-PD activities among the TG, SG, and TSG were also significantly different (TSG > SG > TG; $p < 0.01$).

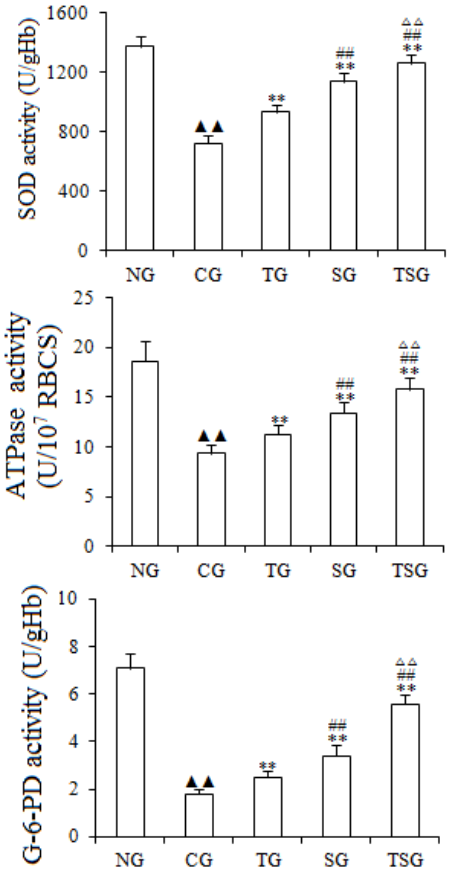

Figure 5: Effects of trehalose and saccharose pretreatment on SOD, ATPase and G-6-PD activities of lyophilized RBC; $^{▲▲}p < 0.01$, compared with the NG; $^{**}p < 0.01$, compared with the CG; $^{\#\#}p < 0.01$, compared with the TG; $^{△△}p < 0.01$, compared with the SG

DISCUSSION

Lyophilization has been successfully used to preserve RBC. RBC membrane damage, hemoglobin leakage, high hemolysis ratio, and reduction of ATPase, G-6-PD and SOD activities are common problems that result from RBC

lyophilization [4,14]. The damages result from the water loss that occurs during lyophilization [15]. Fortunately, these problems can be resolved using lyophilization protectants. Trehalose and saccharose are two commonly used lyophilization protectants [15,16], but the synergistic effects of trehalose and saccharose pretreatment on the maintenance of lyophilized RBC quality are unknown. We studied the synergistic effects of trehalose and saccharose pretreatment on the maintenance of lyophilized RBC quality by determining RBC counts recovery rate, total hemoglobin concentration recovery rate, MCH, MCV, comprehensive deformation index, hemolysis ratio, PS expression, and SOD, ATPase, and G-6-PD activities.

The cell is the basic structure and function unit of human metabolism. Hemoglobin, the main RBC protein, has an important role in carrying oxygen and carbon dioxide [17] and is an NO storage library; hemoglobin is an antioxidant and participates in homeostasis and platelet aggregation [18]. Therefore, the RBC counts and total hemoglobin recovery rates are two acceptable indices used to evaluate the effects of trehalose and saccharose pretreatment on maintenance of lyophilized RBC quality [19]. Trehalose and saccharose pretreatment synergistically decreased lyophilization-rehydration-induced damage on RBC counts and total hemoglobin by increasing the RBC counts and total hemoglobin recovery rates (Fig. 1A). MCH and MCV are two conventional indicators used to evaluate RBC function [20,21]. MCH and MCV were not affected by lyophilization-rehydration.

RBC deformability has an important role in the apparent viscosity of blood, microcirculation perfusion, and lifespan of RBCs [22]. Trehalose and saccharose pretreatment synergistically decreased lyophilization-rehydration-induced damage on RBC deformability by increasing the comprehensive deformation index (Fig. 2). Osmotic fragility is an important factor in the maintenance of RBC integrity and normal functions [23]. Trehalose and saccharose pretreatment synergistically decreased lyophilization-rehydration-induced damage on RBC osmotic fragility by reducing the osmotic fragility (Fig. 3). PS is only distributed in the internal side of the lipid double-membrane in the normal cell. When the cell undergoes apoptosis or death, PS is transferred from the inside to the outside of the lipid double-membrane. When that occurs, PS can be evaluated using Annexin V-FITC stain [24]. Trehalose and saccharose pretreatment synergistically decreased lyophilization-rehydration-induced damage on RBC membrane by decreasing the PS expression on the outside of the RBC membrane.

Adenosine triphosphate (ATP) is an important product of energy metabolism in RBC. ATP has an important role in the maintenance of the morphology and the RBC rheological characteristics [25]. The pre-condition of the release of ATP energy is that ATP is hydrolyzed by ATPase [26]. SOD is a kind of metal enzyme and is distributed throughout the organism; it has important roles in maintaining the balance between oxidation and anti-oxidation and in scavenging free-radicals of RBC [22,27]. RBC G-6-PD can catalyze glucose-6-phosphate to generate NAD-PH, which is the coenzyme of glutathione reductase. The reduced glutathione is necessary for the maintenance of hemoglobin stability and RBC membrane integrity [28]. Trehalose and saccharose pretreatment synergistically decreased lyophilization-rehydration-induced damage on the SOD, ATPase and G-6-PD activities of RBCs by increasing their activities (Fig. 5).

CONCLUSION

Trehalose and saccharose pretreatment synergistically improves the maintenance of lyophilized RBC quality by reversing lyophilization-rehydration-induced damage on RBC parameters. Therefore, trehalose and saccharose may be considered to be a combined lyophilization protectant in the lyophilization of RBC. However, studies on further protective mechanisms of trehalose and saccharose in the lyophilization of RBC need to be investigated in future work.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest associated with this work.

CONTRIBUTION OF AUTHORS

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Yan-Qiong Li conceived and designed the study. Yan-Qiong Li, Rui Hu, Li-Hui Zhong, Qian Sun and You-Ping Yan performed the experiments, collected and analyzed the data.

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