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Addition of oligosaccharide decreases the freezing lesions on human red blood cell membrane in the presence of dextran and glucose ☆

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ABSTRACT

Although incubation with glucose before freezing can increase the recovery of human red blood cells frozen with polymer, this method can also result in membrane lesions. This study will evaluate whether addition of oligosaccharide (trehalose, sucrose, maltose, or raffinose) can improve the quality of red blood cell membrane after freezing in the presence of glucose and dextran. Following incubation with glucose or the combinations of glucose and oligosaccharides for 3 h in a 37 °C water bath, red blood cells were frozen in liquid nitrogen for 24 h using 40% dextran (W/V) as the extracellular protective solution. The postthaw quality was assessed by percent hemolysis, osmotic fragility, mean corpuscle volume (MCV), distribution of phosphatidylserine, the postthaw 4 °C stability, and the integrity of membrane. The results indicated the loading efficiency of glucose or oligosaccharide was dependent on their concentrations. Moreover, addition of trehalose or sucrose could efficiently decrease osmotic fragility of red blood cells caused by incubation with glucose before freezing. The percentage of damaged cell following incubation with glucose was 38.04 ± 21.68% and significantly more than that of the unfrozen cells (0.95 ± 0.28%, $P < 0.01$). However, with the increase of the concentrations of trehalose, the percentages of damaged cells were decreased steadily. When the concentration of trehalose was 400 mM, the percentage of damaged cells was 1.97 ± 0.73% and similar to that of the unfrozen cells ($P > 0.05$). Moreover, similar to trehalose, raffinose can also efficiently prevent the osmotic injury caused by incubation with glucose. The microscopy results also indicated addition of trehalose could efficiently decrease the formation of ghosts caused by incubation with glucose. In addition, the gradient hemolysis study showed addition of oligosaccharide could significantly decrease the osmotic fragility of red blood cells caused by incubation with glucose. After freezing and thawing, when both glucose and trehalose, sucrose, or maltose were on the both sides of membrane, with increase of the concentrations of sugar, the percent hemolysis of frozen red blood cells was firstly decreased and then increased. When the total concentration of sugars was 400 mM, the percent hemolysis was significantly less than that of cells frozen in the presence of dextran and in the absence of glucose and various oligosaccharides ($P < 0.01$). However, when both glucose and trehalose were only on the outer side of membrane, with increase of the concentrations of sugars, the percent hemolysis was increased steadily. Furthermore, addition of oligosaccharides can efficiently decrease the osmotic fragility and exposure of phosphatidylserine of red blood cells frozen with glucose and dextran. In addition, trehalose or raffinose can also efficiently mitigate the malignant effect of glucose on the postthaw 4 °C stability of red blood cells frozen in the presence of dextran. Finally, addition of trehalose can efficiently protect the integrity of red blood cell membrane following freezing with dextran and glucose. In conclusion, addition of oligosaccharide can efficiently reduce lesions of freezing on red blood cell membrane in the presence of glucose and dextran.

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Introduction

In clinical therapy, freezing with glycerol at −80 or −196 °C is a main approach for long-term storage of human red blood cells. This method can greatly prolong the storage time of red blood cells to over 10 years [28]. However, freezing with glycerol has some limitations which may influence the activeness of people to use frozen red blood cells in clinical therapy. For example, the thawed cells require complicated washing process to remove glycerol. In addition, addition and removal of glycerol can lead to serious osmotic damages on red blood cells [20].

The limitations of the present freezing method tightly correlated with the permeability of glycerol. In order to simplify the

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complicated washing procedure after thawing, some polymers, such as hydroxyethyl starch (HES), polyvinyl pyrrolidone, and dextran, have been used as substitutes of glycerol [11,15,20,24,25,30,32]. Polymer cannot penetrate the cell membrane and only exist on the outer side of membrane, so red blood cells frozen with polymer do not require the complicated washing procedure after thawing. Moreover, some polymer, such as dextran or HES, has been used as a plasma expander in clinical therapy [20]. But transfusion of red blood cells frozen with polymer may cause in vivo hemolysis which may limit their application in the clinical therapy.

Some sugars can efficiently stabilize frozen or dried cells [1,6,7,9,10,17,18,20,24,31,33]. So sugar, especially trehalose, may be used to improve the quality of human red blood cells frozen with polymer. But in order to obtain the optimal protective effect, trehalose need to be loaded into the cytoplasm [4,33]. At present, some methods, including genetic engineering [6,9], microinjection [7], fluid phase endocytosis [33], and so on may not be suitable for introduction of trehalose into red blood cells. Although the combination of osmotic imbalance and membrane lipid phase transition can introduce trehalose into red blood cells [28], this method can also result in osmotic and oxidative lesions which can influence the final freezing effect of red blood cells [13,21–23]. Based on these studies, we tried to freeze human red blood cells using various sugars including monosaccharides and oligosaccharides in the previous study [24]. But our previous results indicated the cryoprotective effect of glucose might be better than that of trehalose because sugar loading process causes more cell injuries in case of trehalose compared with glucose, and these injuries in turn manifest themselves during subsequent freezing and thawing [21,24].

Glucose can also stabilize the membrane of frozen or dried red blood cells through forming hydrogen bonds with phospholipids and proteins like trehalose [5]. In addition, accumulation of intracellular glucose can increase the vitrification degree of cytoplasm and decrease formation of lethally intracellular ice. Most importantly, human red blood cells can utilize glucose through glycolysis, but cannot metabolize trehalose owing to lack of trehalases which are required for enzymatic hydrolysis of trehalose [28]. So glucose may stabilize the metabolic function of frozen red blood cells [24].

But glucose can also result in osmotic and oxidative lesions on red blood cells during pretreatment and freezing [12,21,23,24]. So how to decrease the malignant effect of glucose on osmotolerance of red blood cells is very critical for application of glucose in the cryopreservation of red blood cells. In this study, oligosaccharide can mitigate the malignant effect of glucose on red blood cell membrane during pretreatment and freezing. So addition of oligosaccharide may further improve the postthaw quality of red blood cell frozen in the presence of polymer and monosaccharide.

Materials and methods

Reagents, solutions, and preparation of red blood cells

Unless otherwise stated, all chemicals were analytical reagent grade. Trehalose, sucrose, maltose, raffinose, and dextran (MW ~ 40,000 Da) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All solutions were prepared in double glass-distilled water. The modified phosphate buffered saline (mPBS) containing 154 mM NaCl, 1.06 mM KH_2PO_4, 5.6 mM Na_2HPO_4 and 2.0 mM adenine (300 mOsm, pH 7.4) was used as the basic buffer. The mPBS containing 40% dextran (W/V) was used as the protective solution.

After collection from different healthy donor volunteers in the Beijing Blood Center, the fresh whole blood was immediately transported to our laboratory. Then the blood was centrifuged at 1000*g* for 10 min at 4 °C to remove the supernatant, including plasma, white blood cells, and platelets [30]. After washing three times using mPBS, the final concentrated red blood cells were used in the study.

Pretreatment, freezing, and thawing

Before freezing, the washed red blood cells were incubated in the mPBS containing 400 mM glucose and 0, 50, 100, 200, or 400 mM oligosaccharides for 3 h in the 37 °C water bath. Then the effect of oligosaccharide on the osmotic fragility caused by incubation with glucose was evaluated in this study.

In order to evaluate the effect of oligosaccharide on frozen red blood cells, following incubation in the mPBS containing both glucose and oligosaccharide (the molar ratio between glucose and oligosaccharide was 3:1) for 3 h, the cell suspensions were centrifuged at 1000*g* for 10 min to remove the supernatant. Then red blood cells were frozen according to the previous procedure [21]. Briefly, the concentrated cells and 40% dextran were mixed in a ratio of 1:1 (V/V) equally. The final cell suspension was divided into the standard 2.0 ml cryogenic tubes (Axygen Scientific, California, USA) and frozen in liquid nitrogen at approximately 150 °C/min. The cooling rate was measured using a thermocouple (EastSun Electronic, Zhejiang, China) placed in the middle of the sample. Measurement of the cooling rate was made between 25 and −50 °C, which represents the linear changing range of the cooling rate. Following storage in liquid nitrogen for 24 h, the frozen red blood cells were thawed for 5 min in the 37 °C water bath.

Finally, in the absence of glucose and various oligosaccharides, red blood cells frozen with dextran were used as the control group following incubation in the isotonic mPBS at 37 °C for 3 h. Moreover, to further elucidate the protective effect of oligosaccharides, red blood cells were frozen with dextran and glucose according to the previous report [21].

The loading efficiency of oligosaccharides or glucose in red blood cells

The process of loading sugar into red blood cells has been described by Satpathy et al. and Quan et al. [28,21–24]. Briefly, following incubation with glucose and various oligosaccharides for 3 h at 37 °C, the red blood cells were washed three times in the isotonic mPBS (300 mOsm, pH 7.4) by centrifugation at 300*g* for 10 min to discard the supernatant. Then the cell pellet was mixed with 5 ml 80% methanol (v/v) and treated in the 85 °C water bath for 60 min. The intracellular concentrations of various oligosaccharides or glucose were determined using the anthrone method and the glucose oxidase method using kits supplied by Zhongsheng Beikong Bio-Technology and Science Inc. (Beijing, China). In this study, the anthrone method can detect both oligosaccharides and glucose accumulated in red blood cells. However, the glucose oxidase method can only detect glucose. So the final intracellular concentrations of various oligosaccharides can be calculated by subtraction of the data of the glucose oxidase method from the data of the anthrone method. Finally, since these methods detect all the sugars that are present in red blood cells, unfrozen red blood cells that had not been loaded with the sugars were treated in parallel.

Hemolysis

The percent hemolysis was determined using the Drabkin's solution (Sigma Diagnostics). After thawing, the cell suspensions were initially mixed with equal volume of mPBS to decrease the viscosity. Then the cell suspension was used to measure the total hemoglobin concentration. The supernatant was used to measure the free hemoglobin concentration following centrifugation.

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