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DEVELOPMENT OF A MODEL TO INVESTIGATE RED BLOOD CELL SURFACE CHARACTERISTICS AFTER CRYOPRESERVATION

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Abstract

BACKGROUND: Maintaining cell surface properties after freezing and thawing, characterized in particular by the surface potential and associated with it cell ability to intercellular adhesion, could be used as a characteristic of successful cryopreservation. **OBJECTIVE:** This study was conducted to research applying different erythrocytes freezing modes and analyses the regimes cryopreservation effect on the cell surface charge and adhesion to microorganisms. **MATERIALS AND METHODS:** Human erythrocytes frozen by three modes. In order to determine adhesion index was used dried bacterial cells of *S. thermophilus*. The surface charge of erythrocytes was evaluated using Alcian blue cationic dye. **RESULTS:** The results showed the significant decrease in the lactobacillus adhesion to erythrocytes frozen glycerol and 1,2-propanediol. After erythrocytes were freezen with glycerol and 1,2-propanediol, the cationic dye binding to erythrocytes significantly reduced. AB binding to erythrocytes frozen with PEG-1500 does not differ from control data. **CONCLUSION:** Erythrocytes frozen with PEG-1500 mantained surface properties after thawing better, compared to erythrocytes cryopreserved by other methods.

Keywords: Erythrocytes, S. thermophilus, cryopreservation, adhesion, surface charge.

INTRODUCTION

Untill now, the percentage of surviving cells, membrane integrity of the remaining cells not normally permeable to dyes, and biochemical parameters of cellular activity have been the main indicators of cell cryopreservation success, particularly in erythrocytes. Little attention has been paid to the preservation of the surface cellular structures, such as glycocalyx. Mammalian cells are covered by a dense glycocalyx consisting of glycolipids, glycoproteins, and proteoglycans. Many of these glycoconjugates carry glycan chains terminating with sialic acids. Sialic acids are recognized and used as attachment sites by a large number of widely varied microbial cells. Many sialic acid-binding proteins of different pathogens have been well characterized and generally show a high degree of specificity for different types of sialic acids and/or their linkage to the underlying glycan chains (5). Adhesin receptors represent hydrocarbons of surface exposed glycoproteins or glycolipids, attached to membrane proteins of epithelial cells in the target tissue or organ. These

hydrocarbons are found in the same glycocompounds, proteins or sialoglycoproteins of cell membranes in other places apart from the target tissue or organ. For instance, red blood cells exhibit a huge variety of complex glycoproteins, glycosphingolipids and gangliosides which are identical or similar to adhesin receptors on epithelial cells (3).

It was shown in several animal species that all erythrocyte sialic acids are present on membrane surfaces (4). Erythrocytes of most species have negative surface charge, primarily due to the carboxyl group of sialic acids. Human erythrocytes contain the largest amount of sialic acids, which is about twice the amount present in chicken, horse, and calf cells. It is well established for human erythrocytes that the charge density shows little variation over a wide range of ionic strength, and for a given pH and ionic strength the mobility is invariant in many types of buffers. It was concluded from these data that the surface charge is mainly due to fixed anionic components of the plasma membrane rather than anion adsorption.

Scarce data on changes of cells surface potential after freezing and thawing are available (12, 17). For example, the ability of lactobacilli *Lactobacillus acidophilus* to repair cryoinjury, restore growth and form colonis after cryopreservation was shown to depend on their surface potential (17). The surface potential of thawed cells serves as a measure of reversibility of cell damage, and reflects other properties that viability test does not provide.

Maintaining cell surface properties after freezing and thawing, characterized in particular by the surface potential and associated with it cell ability to intercellular adhesion, could be used as a characteristic of successful cryopreservation. Based on this assumption we conducted a study applying different modes of freezing to human erythrocytes and analysed the effect of these cryopreservation regimes on the cell surface charge and adhesion to microorganisms *Streptococcus thermophilus*.

MATERIALS AND METHODS

Erythrocytes were isolated from human donor blood, received from Kharkiv Regional blood service centre, and frozen by three different modes:

Mode No1 – freezing by Babiychuk's method (2) - protection with PEG -1500. Cooled 40% PEG-1500 aqueous solutions was added by drops to erythromass pre-cooled to 0ºC in a final ratio1:1. The suspension was placed in 2 ml containers and frozen by immersion in liquid nitrogen. It was thawed in a water bath at 37ºC. Defrosted suspension was centrifuged at 700 g for 10 min. Erythrocyte pellet was resuspended in 0.1 M phosphate buffer saline (PBS), pH 7.4. PBS composition: 11.5 g/l Na_2HPO_4 + 2.28 g/l NaH_2PO_4 + 2.92 g/l NaCl.

Mode No2 – freezing by the method developed at the Institute of Hematology and Blood Transfusion (Moscow) (9). Preservation solution, containing 300 ml glycerol, 40 g mannitol, 7 g sodium chloride in 1000 ml of solution, was slowly added to erythromass in 1:1 ratio and kept for 15-20 min at room temperature . The suspension was placed in 2 ml containers and frozen by immersion in liquid nitrogen. It was thawed in a water bath at 37ºC. Cells were washed in three steps using 3 washing solutions, containing 160, 50 or 25 g mannitol and 7 g sodium chloride in 1000 ml of solution, according to the protocol (9). After third wash erythrocytes were resuspended in 0.1 M PBS pH 7.4.

Mode No3 – freezing by Vorotilin's method (13) - protection with the 1,2-propanediol. Erythromass was mixed in 1:1 ratio with a cryoprotectant solution "Propandiosaharol": 370 g 1,2-propanediol, 32 g sucrose, 6 g sodium chloride in 1000 ml of solution. Erythrocyte suspension was kept for 5 min at room temperature , placed in 2 ml containers and frozen at 12-14°C/min. It was warmed in a water bath at 37ºC. Defrosted suspension was centrifuged at 700 g for 10 min. Erythrocyte pellet was resuspended in 0.1 M PBS, pH 7.4.

Percentage of hemolysis was calculated based on spectrophotometrical measurements of the hemoglobin optical density at 577 nm in the supernatant after freezing-thawing and after complete hemolysis in the control sample.

Streptococcus thermophilus bacterial cells were purchased from yogurt ferment VIVO (Kyiv, Ukraine). All lactobacilli were taken from the same batch. In order to determine adhesion index, dried bacterial cells of *S. thermophilus* were suspended in PBS with the addition of 5% glucose and incubated at 37ºC for 30 minutes, washed in 0.1 M PBS and harvested by centrifugation at 4000 g for 10 min . The cells were resuspended in 1:2 ratio in PBS, pH 7.4. Erythrocytes and lactobacilli suspensions were mixed in 1:1 ratio, incubated at 37 C for 30 min with shaking every 5 min. Adhesion of bacterial cells to human erythrocytes was observed by means of the Axio Observer Z1 microscope (oil-immersion lens x63). The number of adherent bacteria was calculated in five fixed microscope fields after mechanical shaking of the sample to take into account only adherent lactobacilli. The number of bacteria adherent to each erythrocyte was counted in each microscopic field and the average number of adherent bacteria per erythrocyte (adhesion index) was calculated.

The surface charge of erythrocytes was evaluated using Alcian blue cationic dye (Alcian blue 8GX) (AB) (50 mg/ml in 100% ethanol) (19). Dye solution was filtered through a filter paper and diluted 100 times in PBS to obtain a final concentration of 1% ethanol. To determine the actual concentration of AB in the final solution after filtration, its losses on the walls of the sample tube and the losses of AB and the solvent (alcohol) were taken into account by weighing the sample tube and the filter before preparation and after filtration of the solution as well as the filter after drying. Absorbance of the AB solution was measured with a spectrophotometer (Pye Unicam SP 8000, UK) at 650 nm immeadiatly before use. We used the same concentration of cells, as in (6) (1.25×10^9 cells / ml) for experimental evaluation of the surface charge of erythrocytes. 0.1 ml of the erythrocyte suspension (1.25×10^9 cells / ml) were mixed with 2 ml of the final AB solution. The mixture was incubated for 30 min at 37 °C. After cells were removed by centrifugation the residual amount of AB was measured by its absorbance at 650 nm. The amount of bound AB per cell was calculated by the difference in absorbances of the initial AB solution and the supernatant and expressed in nanograms per 10^6 cells. Experiments were performed in five repetitions.

RESULTS

Total cell losses after freezing-thawing and washing following three methods presented here are comparable and reach 15%.

We determined adhesion indexes of lactobacilli *S. thermophilus* to human erythrocytes and binding characteristics of AB to erythrocytes under the same conditions before and after cryopreservation by three different methods (Table. 1).

The data obtained shows significant decrease in the lactobacillus *Streptococcus thermophilus* adhesion to erythrocytes frozen by modes No2 (glycerol) and No3 (1,2-propanediol), whereas the adhesion index of lactobacilli to erythrocytes, that were frozen by mode No1 (PEG-1500) is slightly lower, but does not differ significantly from control values. Data on AB binding to erythrocytes correlate with the data on adhesion index with a correlation coefficient $r = 0.9$. After erythrocytes were freezen with glycerol and 1,2-propanediol, the cationic dye binding to erythrocytes significantly reduced, indicating a decrease in negative charge on an erythrocyte surface.

At the same time, while AB binding to erythrocytes frozen with PEG-1500 decreases, it does not differ significantly from that for the control.

DISCUSSION

In this work we compared the effect of three different cryo-preservation methods on the surface properties of erythrocytes. The method of erythrocyte's freezing under glycerol protection has been developed over 60 years ago and with some modifications is still applied in medical practice (10). Multi step washing of erythrocytes to remove glycerol is the main disadvantage of the method. Glycerol is a penetrating cryo-protectant, however the rate of glycerol penetration through erythrocyte membranes is not very high. Characteristic penetration time of glycerol to erythrocytes is 10-20 minutes at room temperature and shortens to 5-7 minutes at 37°C. While characteristic penetration times for such cryo-protectants as DMSO, acetamide or 1,2-propanediol constitute only 1-10 seconds (7,8). Therefore researchers were seeking alternatives to glycerol such as rapidly permeating cryo-protectants, easily removed from the cells, or non-permeating high molecular weight substances such as hydroxyethyl starch, polyvinylpyrrolidone or variety of glycols with different molecular masses (10, 2). It was proposed that removal of such non-permeating substances from thawed RBCs prior to transfusion was not required (25). Yet to date, non-permeating additives have not been licensed or used in clinical practice (11, 22). Recently, combinatorial media containing permeating and non- permeating cryo-protectants are often proposed (18, 20). Thus in our work we compared the cryo-protective effect of glycerol with that of rapidly permeating 1, 2 –propanediol and non-permeating PEG-1500.

An effect of cryopreservation on surface properties of blood cells was investigated long time ago in (12). A significant loss of net negative charge without detectable alteration in hydrophobic membrane properties was observed in cryopreserved and reconstituted human blood phagocytes by a method of cell partitioning in two-phase aqueous polymer systems.

It was previously shown (15), that platelet microparticles (PMPs) used for enhancing engraftment after cord blood transplantation by means of their adhesion to CD34+ cells, adhered less well to CD34+ cells after cord blood was frozen – thawed compared to PMPs from fresh cord blood , and CD34+cells coated with these PMPs had poor adherence to endothelium.

Addition of membrane stabilizer catalase and bio-antioxidant trehalose to the conventional freezing medium was shown to result in better protection of growth factor receptors, adhesion molecules, and functionality of hematopoietic cells (mononuclear cells from cord blood and fetal liver cells), yielding a better graft quality (14, 21). The authors did not observe an effect of these additives on parameters such as viability and nucleated cell recovery and yet they obtained consistently better colony formation in the speciments where

Table 1: Effect of different erythrocyte freezing modes on AB binding and *Streptococcus thermophilus* adhesion index to human erythrocytes.

Sample	Adhesion index	Quantity of bound AB, ng/10^6 er.	Cell loss after freezing-thawing/after washing, %
Blank test	1.91±0.96	219±4.5	–
Erythrocytes, freezing with PEG -1500	1.67 ±0.92	215±7.5	2±0.5/13.2±3.2
Erythrocytes, freezing with glycerol	1.38±0.67*	203.5±6.5$^+$	4.5±1.3/9.5±2.8
Erythrocytes, freezing with 1,2-propanediol	1.31±0.87*	189±13$^+$	5.7±0.8/7.9±2.5

* - data significantly differ from the data for a blank test, $p < 0.01$; $^+$ - data significantly differ from the data for a blank test, $p < 0.05$; correlation coefficient between adhesion index and binding r=0.9.

the additives were used in the freezing mixture. Increased expression of adhesion molecules in cryopreserved cells was also observed. Moreover, increased adhesion on vascular endothelial cell surfaces due to increased expression of adhesion molecules was shown after freezing and thawing (16). However it is clear that in this case increased adhesion is a result of induction of cellular recovery mechanisms after freezing-thawing stress and does not reflect the damaging effect of freezing and thawing on the surface properties of the cells.

Most studies on the effect of freezing on cell surface properties and their adhesion ability relate to bacteria. For example in (17) it was shown that after freezing and thawing of lactobacilli *Lactobacillus acidophilus* without cryoprotectant the bacterial population consisted of cells in three states: intact or normal cells; irreversibly damaged or dead cells; and reversibly damaged cells. After freezing and thawing, cell walls have irregular and heterogeneous structure and reduced zeta potential -32 mV compared to -45 mV in non-frozen cells in a stationary phase. Untreated culture showed a smooth increase in zeta potential over time, whereas after freezing and thawing a sharp increase in zeta potential from -32 to -38 mV has been observed. According to the authors, zeta potential evolution to more negative values under conditions when cells do not grow may be associated with restoration of damaged cells during first two hours. The authors showed that cells partially repaired their damage during this period. More cells survived freezing-thawing when protected with glycerol. The number of survived cells reached a constant value when glycerol concentration exceeded 1 M. However, for retaining zeta potential, comparable to the potential of not frozen cells, bacteria had to be frozen- thawed in 2 M glycerol. These results indicate that, although the number of cells survived in 1 M glycerol is as high as in 2 M glycerol, the cell surface properties differ considerably. In other words, zeta potential reflects properties other than viability test. The value of zeta potential correlates better with a proportion of the damaged cells. Changes in zeta potential indicate the cell wall damage which can affect properties such as adhesion.

The effects of freezing-thawing cycles on bacterial viability and survival strategies (namely motility and biofilm formation) were studied in a model pathogen *Bacillus subtilis* (1). The authors showed that although freezing and thawing had not significantly affected bacterial growth rate, at the same time the biofilm formation of *B.subtilis* reduced significantly, as well as the bonding between *B. subtilis* and the quartz surface.

According to (23, 24), surface hydrophobicity, being a generalized characteristic of cell surfaces, is an important parameter for the prediction of intact probiotic bacteria ability to endure extreme environments including freezing. A certain balance of cell components, which can be characterized by the reduced cell surface hydrophobicity, apparently helps to ensure resistance, improved viability and hence the overall probiotic properties of bacteria. It was suggested (19) that correlation between physico-chemical properties and adhesion likely demonstrates the role of the external layer composition, in particular proteins play more significant role in adhesion abilities than hydrophobicity. For example D41 strain was shown to contain proteins with NH2 and NCO groups. This strain was found to adhere to stainless steel, glass, or Teflon surfaces at a much higher efficiency (2 orders of magnitude) than the two other strains DA and D01 enriched in COOH or sulfates, which makes them more hydrophilic and less adherent to all substrates.

Thus, retaining cell surface properties after freezing-thawing, in particular surface potential, and the resulting cell's ability to intercellular adhesion may serve as indicators of successful cryopreservation. Therefore we suggest these parameters are feasible for evaluating cell quality after freezing. Our results demonstrate that

erythrocytes frozen with PEG-1500 mantained surface properties after thawing better, compared to erythrocytes cryopreserved by two other methods. This may be a consequence of a high surface activity of PEG-1500 adsorbed on the surface of cells. Therefore, PEG-1500 can provide additional protection mechanisms to the cell surface layers against damaging factors during freeze-thawing. This mechanism is likely to be the cause of increased cell viability after freezing in the combined medium containing both penetrating and non-penetrating cryoprotectants (18, 20).

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