Original Article

The Influence of cytoskeleton-membrane complex of human erythrocytes by osmotic sensitivity under dehydration-rehydration environment

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Abstract

Aims: The aim of this work to investigate the influence of membrane and cytoskeleton by anion transport inhibitors and medium composition on the sensitivity of erythrocytes to posthypertonic lysis (PHL). Method: This work investigates the response of human erythrocytes to transport isotonic medium from hypertonic media depending on the anion composition of rehydrated medium, affected by anion transport inhibitors [4,4′-Diisothiocyano-2,2′-stilbenedisulfonic acid (DIDS)] and dipyridamole.

Results: We found that redistribution of ions in the cell during incubation in hypertonic media is the factor contributing to higher PHL. Block of effluent chloride anions through the anion transport inhibitor DIDS and inclusion of sodium ions in the cell at dehydration phase during nigericin treatment is accompanied by increased PHL level. Ionophore nigericin, enhancing transmembrane transport of sodium and potassium ions, has a multidirectional effect on erythrocyte's sensitivity to hypertonic cryohemolysis and post-hypertonic lysis. In case of hypertonic cryohemolysis, nigericin desensitizes cells, while in case of post-hypertonic lysis it increases cell sensitivity. We found that the level of cell hemolysis decreases with increased time of erythrocyte incubation in a hypertonic solution. Whereas using of ionophore (Nigricin) it will be reducing hemolysis level practically to 0 within 10 minutes of pre-incubation.

Conclusion: The effect of pH change on osmotic sensitivity of erythrocytes depends on the temperature and osmotic effect. Under conditions of hypertonic cryohemolysis, decrease of pH to acidic values leads to a reduction in the sensitivity of erythrocytes to cooling. In turn, the sensitivity of erythrocytes to post-hypertonic hemolysis increases with pH shift to acidic values. We revealed that hypotonic hemolysis is characterized by a pronounced pH-dependence with maximum hemolysis at pH 5.8. Increase in the osmolarity of pre-incubation medium results in increased level of hemolysis at all pH values in the experiment, and sensitivity of erythrocytes to hypotension increases mainly at acidic pH, although pH-dependence is retained. Change in pH (5.8, 6.6, 7.4) allows to modulate chloride distribution between the cell and its environment.

Keywords: cytoskeleton-membrane, human erythrocytes, osmotic sensitivity, dehydration-rehydration environment

Introduction

In the absence of external flow, red blood cells (RBCs) maintain a biconcave disk shape of about 8 μm in diameter. They must strongly deform to pass through the smallest capillaries of the microcirculation of 3 μm in diameter or the

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Access this article online

Quick Response Code:

Website: www.pnrjournal.com

DOI: DOI:10.47750/pnr.2022.13.S08.268

0.5-μm thick endothelial slits in the red pulp of the spleen [1]. The human erythrocyte (red blood cell, RBC) demonstrates extraordinary ability to undergo reversible large deformation and fluidity [2]. On human erythrocytes 60 Pa for 200 s was the minimum combination to result in significant deformability deterioration. By increasing the magnitude and duration of the applied mechanical stress to 100 Pa shear stress for 300 seconds, the human erythrocytes showed the largest deformability impairment [3]. Such mechanical response cannot be consistently rationalized on the basis of fixed connectivity of the cell cytoskeleton that comprises the spectrin molecular network connected to a phospholipid membrane [4]. On the otherhand, ability to control cell volume is pivotal for cell function and any disturbance in the cell volume will lead to protective measures (e.g., cytoskeletal rearrangement) and adaptive measures (e.g., altered expression of osmolyte transporters and heat shock proteins) and, in most cases, activation of volume regulatory osmolyte transport [5]. In hypertonic solutions, water leaves the cell, decreasing the cellular volume until the balance between the extracellular and intracellular osmotic pressures is reached. In hypotonic solutions, the reverse thing happens. The cell receives fluid from the extracellular region causing an increase in the cellular volume until, just as in the previous case, the osmotic pressures balance [6]. Over recent years, a lot of attention has been paid to the phenomenon of posthypertonic lysis of erythrocytes (PHL), which results in cell destruction during transfer from hypertonic salt and non-electrolyte solutions into media of normal physiological tonicity. showed that posthypertonic lysis of mammalian erythrocytes and the level of damage to mammalian erythrocytes under post-hypertonic shock depend on the concentration of sodium chloride (NaCl) in the dehydration medium. Cytometry studies revealed significant changes in the histograms of the distribution of erythrocytes of all mammalian species with increasing salt concentration in the dehydration medium [7]. The level of posthypertonic Lysis of erythrocytes did not depend on osmolality of dehydra-tion medium, and the presence of a salt component in medium affected the permeability of erythrocytes membranes for glycerol [8]. The primary phase of cell state modification includes water and ion redistribution which, in turn, is accompanied by a decrease in cell volume. At the same time, subsequent return of cells into isotonic conditions increases their volume ("volume shift") and causes structural instability and cell lysis. In an in vitro study done by [9], the cells showed greatly restricted volume changes at osmolarities between 200-700 mOsm. At osmolarities outside this limit, the cells showed nonrestricted volume changes following essentially the predictions of an ideal osmometer with small RBCs are more resistant to hyperosmotic milieu than large ones [10] reported that the level of erythrocytes' post-hypertonic damage is determined by penetrating anions entering in a cell at the moment of rehydration and noted that cytoskeleton participated in the regulation of erythrocytes resistance to PHL [11]. It has been proven that volume change is accompanied by a complex set of structural rearrangements and changes in functional state of the cell. Depending on the depth of volume changes, cell may exhibit either passive response to external stimuli, which can be considered as a factor violating its structure, or active response, which can be considered as adaptation. Ion transport systems are the main systems forming active cell response, so it is very important to study their role in the phenomenon of PHL. In turn, passive cell response is largely determined by the cytoskeleton state, so it is important to study of the role of cytoskeletal proteins in response of dehydrated cells to subsequent rehydration. The main PHL feature consists of the fact that cell lysis is developed during restoration of cell volume from dehydrated state, where there are no manifestations of hypertensive damage associated with an increase of medium osmolarity and decrease in cell volume at the stage of osmotic dehydration. But the fact of PHL development suggests that changes during osmotic dehydration (manifested in a volume shift) sensitize the cell to return to physiological environment. At this point, it is unclear which changes in cytoskeleton and membrane structure and transport system may be considered as factors of sensitizing cell to subsequent rehydration from hypertonic media. Hypertonic incubation activates electrolytes delivery into the cell [12]. Cell volume increase phase consists in redistribution of potassium and chlorine ions from the cell into the extracellular environment [12] reported that after acute swelling, cell volume is regulated by the process of regulatory volume decrease (RVD), which involves the activation of KCl cotransport and of channels mediating K, Cl, and taurine efflux [5]. Conversely, after acute shrinkage, cell volume is regulated by the process of regulatory volume increase (RVI), which is mediated primarily by Na/H exchange, Na-K-2Cl cotransport, and Na channels. In the study of Garcia and Ardila, the highest cellular contraction observed was about 35% which means that, the cellular volume fell to 65% of its base line volume. This happened when 5.85% NaCl was used [6]. It was suggested that the cell became permeable to sodium in hypertonic conditions leading to a loading of sodium during the hypertonic exposure, which caused the cell to swell during resuspension in isotonic media (post-hypertonic lysis) [13]. Violation of volume-regulating mechanisms of the cell may result in secondary osmotic response to rehydration manifested in cell volume increased to a critical rate, membrane rupture and cell lysis. Under these conditions, adverse factors accompanying volume shift are mainly aimed at cytoskeleton-membrane complex, changing not only individual components, but also the entire structure, with appearing structural defects. This means that PHL development is largely dependent on the state of ion transport systems, media composition and modification of the structural membrane and cytoskeleton state. Cytoskeleton consists of several proteins that form a filamentous network composed of spectrin, ankyrin, actin, and protein under the lipid bilayer [14]. In view of the above, this work was aimed at studying the affected of cytoskeleton-

membrane complex on osmotic sensitivity of human erythrocytes under dehydration-rehydration and temperature shift. We study the influence of "electrolyte-nonelectrolyte" ratio in the rehydration medium on the level of posthypertonic lysis of erythrocytes incubated in hypertonic saline medium (1.5, 3.0 M/l NaCl). Also we investigate the influence of ionophore nigericin enhancing the efficiency of transmembrane transport of sodium and potassium ions on erythrocyte sensitivity to hypertonic cryohemolysis and post-hypertonic Lysis and we assess the Effect of the pH of rehydration medium and anion transport inhibitors (DIDS and dipyridamole) on the level of erythrocyte PHL.

Materials and Methods

Human samples

Human blood was obtained from healthy male blood Group II volunteers, in order to unify the object. Erythrocytes were obtained from freshly preserved donor blood prepared on glugicir preservative at Prince Hamzah Hospital blood transfusion center- Jordan, after obtaining all approvals from the Jordan Ministry of Health and hospital administration. After plasma removal, erythromass was washed three times by centrifugation at 1500 g for 3 minutes in a 10-fold volume of saline (0.15 mol/l sodium chloride, 0.01 mol/l tris-buffer, pH 7.4). Buffy coat and supernatant were removed by aspiration. Erythromass residue contained an average of 80% cells. Erythrocytes in the form of a dense residue were stored at 4 ° C and used within 4 hours.

Standards and Chemicals

Posthypertonic lysis of erythrocytes was modeled as follows. Packed erythrocytes were diluted with hypertonic solution containing 1.5 or 3.0 mol/l sodium chloride (1:10 and 5mcl of final suspension were transferred into 2 ml isotonic medium of the following composition (pH 7.4):

- 0.15 mol/l sodium chloride;
- 0.125 mol/l sodium chloride + 0.05 mol/l sucrose;
- 0.1 mol/l sodium chloride + 0.1 mol/l sucrose;
- 0.05 mol/l sodium chloride + 0.2 mol/l sucrose;
- 0.025 mol/l sodium chloride + 0.25 mol/l sucrose;
- 0.01 mol/l sodium chloride + 0.28 mol/l sucrose;
- 0.3 mol/l sucrose;

Cell exposure time in a medium containing 1.5 mol/l sodium chloride was 45 minutes, 3 mol/l for 8-10 seconds, in isotonic medium 3 minutes, temperature of incubation in hypertension and isotonic was 22°C. The level of hemolysis was determined spectrophotometrically.

Hypotonic hemolysis

Erythrocytes were diluted with saline in a ratio of 1:10, and 5mcl of final suspension was transferred to a hypotonic medium with different pH: 5.8; 6.6; 7.4, Osmolarity of hypotonic medium for each experiment was adjusted so that at pH 7.4 hypotonic injuries was 50%. At the average, it amounted to 0.067 mol/l sodium chloride.

Hypertonic hemolysis

To study hypertonic cryohemolysis, erythrocytes with final hematocrit of 2% were added to the solution containing 0.86 mol/l sucrose, 1.2 mol/l sodium chloride (in the presence and absence of 50 μmol/l nigericin), incubated 0-60 min at 37°C and cooled in an ice bath for 5 minutes. Cooled tube containing erythrocyte suspension was centrifuged at room temperature for 3 minutes, 1500g. Cells were spun down by centrifugation for 3 min at 1500 g. The content of hemoglobin released in the supernatant was determined spectrophotometrically with the help of SF-4A and a flow-cell at a wavelength of 543 nm. Absorption of a sample added with X-100 Triton at a concentration of 0.1% was taken as 100%.

Level and dynamics

To determine the level and dynamics of erythrocyte hemolysis we used the device for measuring light scattering of cell suspensions (divergence of the light beam - 3°) created on the basis of SF-4A monochromator [15]. The level of erythrocyte hemolysis was registered by light scattering method; and the level of hemolysis was determined by registering the change in optical density (OD) of erythrocyte suspension at a wavelength of 720 nm. The concentration of cells in the cuvette was adjusted so that the level of optical density in the suspension was 0.25 - 0.3 units of optical density, corresponding to (1.7 - 3.5) x 106 cells / ml. Decrease in the intensity of light transmitted through the sample cuvette is caused by suspension scattering at small angles determined by the amount of hemoglobin - containing cells that retain a significant difference in refractive index inside and outside the erythrocyte (Ar). Release of hemoglobin from a single cell is usually complete in 5-10 seconds and in the scale of a minute experiment (Ar) for single cell changes abruptly. Light scattering of shades formed during erythrocyte lysis, and light absorption by hemoglobin at a said wavelength is negligible [16].

Specific Density

The level of erythrocyte hemolysis as a time function was determined by registering the change in optical density of erythrocyte suspension over time (wavelength 720 nm). The concentration of erythrocyte suspension in the cuvette was (1.7 - 3.5) x 106 cells. / ml. In this concentration range optical density of cell suspension was directly proportional to the number of intact cells.

Cell treatment

Cell treated with nigericin was performed in parallel to hypertensive incubation in a solution containing 1.5 mol/l sodium chloride. Cells were incubated in hypertension in the presence of nigericin (50 µmol/l) for 45 minutes at 22 ° C (PHL) and at 37 ° C (hypertonic cryohemolysis).

Statistical Analysis:

The results were mean ± SD of different parallel measurements. All statistical comparisons and reliability were processed by Student-Fischer method criterion to determine the standard deviation with a level of significance of 95%. Statistical analysis of the results of the chemical experiment" (2015). GraphPad Prism software, version 8, was used to evaluate significant differences between experimental groups using one-way ANOVA, followed by Tukey's post hoc test for multiple comparisons. A $p < 0.05$ difference was considered statistically significant. Also we use origin Lab corporation software (Origin PRo 2022b) for graphing.

Results And Discussion

Incubation of erythrocytes in hypertonic solution and their transfer into isotonic solution leads to cell lysis – posthypertonic lysis (PHL). At this stage of PHL study it is known that the barrier function of erythrocyte membrane is disrupted at the initial rehydration of cells transferred from hypertonic to isotonic media [17], when a sharp increase in water flow into the cell causes an increase in cell volume, which in turn can induce defects in the membrane structure and hemolytic pores. Pore formation, which is energetically advantageous, under these conditions is caused by membrane stretching [12]. Pore formation may be affected by certain substances, such as divalent calcium and zinc cations, sucrose, pH, etc. [18]. PHL development is also strongly dependent on the temperature and time of cell incubation in rehydrated and dehydrating media, osmolarity and ionic strength of the medium [18]. Equilibration of erythrocytes in hyper concentrated electrolyte solutions leads to a number of consequences at the stage of hypertensive incubation. Observed erythrocyte dehydration results in a concentration of intracellular contents and, as a consequence, increase of its ionic strength, which is aggravated by the influx of cations due to disruption of barrier membrane properties. Hypertension affects ion cell homeostasis [19], leading to a change in cytosol pH and hemoglobin charge, as well as to cytoskeleton reduction according to the principle of shielding the negatively charged spectrin groups [20]. Due to the fact that erythrocyte lysis during PHL occurs at the recovery of extracellular osmolarity from hypertension to isotonic, rehydration medium composition can have a significant impact on the post-hypertonic erythrocyte sensitivity.

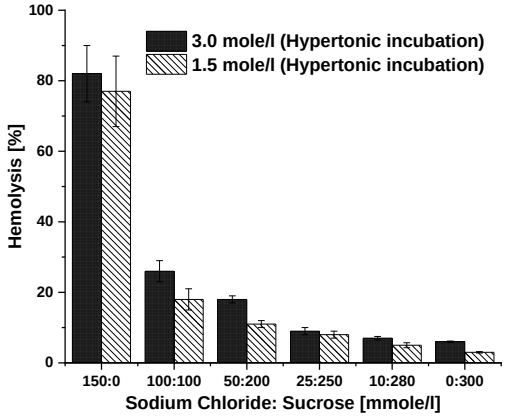

Figure 1. Level of PHL erythrocytes in rehydrated media of different sodium chloride and sucrose ratios at pH 7.4; Data are presented as mean ± SD; n= 5

Figure (1) shows the level of posthypertonic lysis of erythrocytes in isotonic rehydrated media containing various amounts of sucrose and sodium chloride (pH 7.4). It can be seen with increasing non-electrolyte concentration hemolysis level falls from 80 to 3-4%. At the same time, there are no significant differences in erythrocyte response to rehydration after incubation to hypertonic sodium chloride solutions of different concentrations: 1.5 mol/l (45 minutes) and 3.0 mol/l (8-10 seconds). There is no reason to use concentrations of NaCl higher than 5.85%. Higher concentrations do not induce more contraction of cellular volume, but they do provoke cellular damage [6]. During osmotic compression cell accumulates various ions, in particular, this may occur through activation of Na+-K+-2Cl- co-transporter, Na+-H+ exchanger [12] and HCO3-/Cl-

exchange mechanism [12]. It is known that during osmotic swelling cells release chlorine and potassium ions due to K+ - Cl- co-transport activation in order to prevent further water ingress and consequent volume growth. Furthermore, a parallel activation of K+-H+ exchanger and HCO3-/Cl- exchange may occur [12]. At the time of rehydration, erythrocyte begins to increase its volume during water

admission accompanying the redistribution of extracellular solution components. Consequently, we can assume that the presence of chloride anions in the rehydration medium may serve as a regulator of post-hypertonic erythrocyte sensitivity. Osmotic erythrocyte sensitivity is controlled, first of all, by transport processes [21]. Erythrocytes "in vitro" are in nonequilibrium state which is maintained by metabolic and transport processes, such as active transport of sodium and potassium. This state is regarded as stationary [22]. Experimental manipulation of erythrocytes in vitro may be accompanied by a change in environmental parameters such as osmotic pressure, electrolyte composition, pH, etc. In many cases, such changes result in Ma/K-ATPase activation. If membrane permeability is not interrupted by ionophores or other factors, the intracellular content of sodium and potassium ions can be maintained for hours or even days. Erythrocyte transfer to hypotonic solution with changed electrolyte composition activates a number of transport processes induced sequentially over time [22].

1- Distribution of water on the osmotic gradient in less than 1 sec. (w-state)
2- Redistribution of inorganic anions (mainly chloride and carbonate) within several minutes in accordance with extracellular pH (C-state).
3- During pump inactivation or abnormal permeability under the action of ionophores cells reach full Donnan equilibrium for all inorganic anions (D-state).

Redistribution of anions from cells into the extracellular medium during rehydration is a factor that prevents an increase in erythrocyte volume to the critical hemolytic

volume. In this case, a rapid release of chlorine anions acts as an element of a mechanism protecting cells from swelling [12]. Potassium in this mechanism is not involved in volume regulation, since its release requires much more time compared to the rehydration time. Under the conditions of hypertonic incubation, erythrocytes in sodium chloride solutions with increased osmolarity are in osmotic state, which differs from the physiological range, including by ionic equilibrium [23]. In connection with the foregoing, it is important to study the sensitivity of erythrocytes to hypertonic cryohemolysis when cooled from 37°C to 0°C in hypertonic media, as its development depends on osmotic membrane gradient, which in turn is a factor controlling the osmotic and cold sensitivity of erythrocytes. Early studies of hypertensive cryohemolysis showed that the sensitivity to cooling in hypertonic sodium chloride solutions decreases in time with increased duration of cell incubation at 37°C before cooling. Reduced sensitivity to cryohemolysis was explained by decrease of osmotic membrane gradient due to penetration of sodium cations into the cell. To test this hypothesis, in our experiments we used ionophore nigericin, which form channels for sodium and potassium cations when embedded in a membrane, ensuring their movement along concentration gradients. Figure. 2 shows time dynamics of erythrocyte cryohemolysis in a hypertonic solution containing 1.5 mol/l sodium chloride (Control). It can be seen that the level of cell hemolysis decreases with increased time of erythrocyte incubation in a hypertonic solution (Figure. 2, curve 1). The use of ionophore (Nigricin) reduces hemolysis level practically to 0 within 10 minutes of pre-incubation (Figure 2, curve 2).

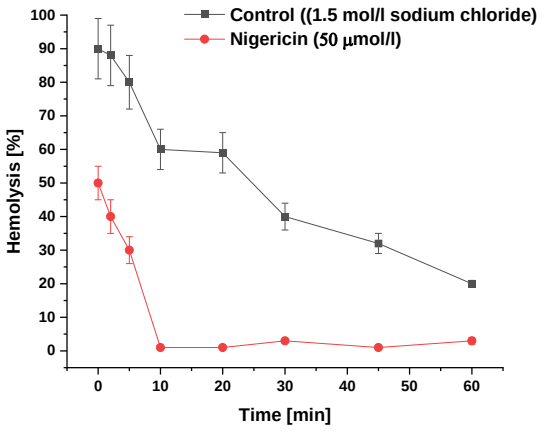

Figure 2. Level of hypertonic cryohemolysis of erythrocytes (cooled to 0 °C after incubation at 37°C) in a solution containing 1.5 mol/l sodium chloride: control (1.5 mol/l sodium chloride) and nigericin (50 µmol/l). Data are presented as mean ± SD, n= 5

Ionophore Nigricin, enhancing transmembrane transport of sodium and potassium ions, has a multidirectional effect on erythrocyte's sensitivity to hypertonic cryohemolysis and post-hypertonic lysis. In case of hypertonic cryohemolysis, nigericin desensitizes cells, while in case of post-hypertonic lysis it increases cell sensitivity, whereas without Ionophore the level of hypertonic cryohemolysis reduces, though does not reach zero point even after 60 minutes of incubation.

Formation of nigericin channels provides penetration of sodium cations into the cell, which removes the osmotic gradient across the membrane. As a result, erythrocytes lose their sensitivity to hypertonic cryohemolysis. Therefore, sodium cations flow into cells at the hypertension pre-incubation stage is insufficient to prevent cell lysis. During hypertonic cryohemolysis violation occurs character Association membrane skeleton proteins with each other due to dehydration and deformation of cells under the action of hypertonic stress these disorders develop when the minimum amount of cells [24]. In terms PHL contrary, an increase in cell volume due to rehydration, respectively, one can assume that the inclusion of sodium cations into erythrocytes hypertensive incubation step will increase their sensitivity to the PHL, unlike cryohemolysis hypertonic. Figure (3) presents data on the post-hypertonic sensitivity of erythrocytes in sucrose and citrate media after cell incubation in solutions containing 1.5 mol/l sodium chloride for 45 minutes, in the presence and absence of ionophore nigericin. It can be seen that hemolysis level for erythrocytes rehydrated after hypertonic incubation in sodium chloride solution in sucrose and citrate media is equal, amounting to approximately 5%. The presence of nigericin in a hypertonic medium causes increased erythrocyte sensitivity to rehydration compared to the control: the level of hemolysis in this case is from 35 to 45%. It should be noted that the level of post-hypertonic hemolysis in both cases is higher as compared with the control (without nigericin). This indicates that the flow of sodium cations inside cells sensitizes erythrocytes to PHL. Corresponding anion effects in the modulation of erythrocyte's osmotic sensitivity may

depend on several factors, but the most important among them is, apparently, membrane permeability to given anion and anion influence on the pH, the composition of intracellular environment. It can be also assumed that the level of PHL in changing anion composition of the medium depends on how the volume changes of cytoskeleton on one side are consistent with volume changes of the membrane on the other side during osmolality change. Less consistent is the process of restoring the volume of membrane-cytoskeleton complex when during hypertonic dehydration the minimum amount of cells and the maximum concentration of intracellular proteins with a concomitant increase in the number of connections between them is achieved [25]. The amplitude of volume changes may be the key factor in these conditions. The greater the amplitude, the more pronounced the breach of membrane association to cytoskeleton, and the lower cell resistance to PHL. This means that anion effect during PHL development may ultimately be reduced to control of cell volume changes during osmotic dehydration [9]. Accordingly, we can expect that penetrating anions included into the rehydration medium will manifest in increased PHL level due to low ability to prevent or retard cell volume increase during rehydration. This means that in media with penetrating anions a membrane having carriers to remove anions from cells can cause cell swelling to a volume greater than the normal. This can also be expected, given that under conditions of hypertonic incubation cell receives a portion of hypertonic solution which increases the tonicity of intracellular environment at the rehydration stage. In this case, swelling is the final effect.

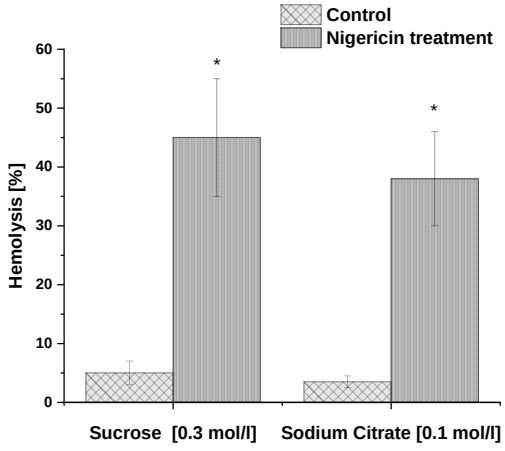

Figure 3. Sensitivity of erythrocytes control and nigericin-treated (50 μmol/l) erythrocytes dehydrated in a medium containing 1.5 mol/l sodium chloride to rehydration. Data are presented as mean ± SD, n= 5; SEM; *=p<0.05 indicates a significant difference between concentrations

The results obtained indicate that the level of post-hypertonic erythrocyte injury is determined by intracellular ionic composition, which in turn affects transport processes at rehydration stage. A reasoned and important factor affecting the post-hypertonic sensitivity of erythrocytes consists of the ability of erythrocyte membrane to control the redistribution of anions from extracellular medium into the cell and vice versa. Cytoskeleton-membrane complex is characterized by a specific component integration. Factors such as increasing ionic strength, osmolarity medium, pH

shift, change in temperature, which play an important role in the process of freezing-thawing, affect erythrocyte cytoskeleton. Presence of negatively charged groups on phosphatidylserine and spectrin molecules sensibilizes membrane erythrocyte complex to changes in medium pH and ionic strength. As a result of changes in osmotic and temperature conditions of the medium these factors may play an important role in disorders of membrane barrier function. Existing data suggest that the degree of erythrocyte injury under conditions of hypertonic cryohemolysis depends on the rate of cell membrane isotropic stretching, developed as a result of thermotropic structural transformations of membrane lipids at 13-18°C [26]. The main factor that can change cation permeability when erythrocytes are exposed to hyperosmotic stress is the mechanical stress developed on the membrane. At the same time, it should be noted that in this case passive permeability to cations may be limited by chloride transfer [27]. It means that the level of erythrocyte sensitization to cooling in hyperosmotic solutions may depend on medium pH - the main factor controlling chloride flows. It is proved that the change in medium pH has a significant influence on membrane deformability, its distribution in the plane of integral proteins and the structure of its surface [28]. The level of hypertonic cryohemolysis of erythrocytes in hypertonic salt and non-electrolyte solutions also depends on the pH of incubation medium [24]. Work shows that at pH 5.0 during cooling hemolysis is practically absent, whereas increasing the pH to 9.0 results in maximum hemolysis [24]. This indicates the connection between processes of chlorine anion redistribution through the membrane and the processes underlying post-hypertonic

cryohemolysis. The significance of processes associated with anion redistribution during sensitization of erythrocytes to changes in temperature and osmotic conditions is proved by the results obtained in our experiments, where chloride transport was inhibited by specific inhibitors of this process. Since chlorine anion is exchanged for hydroxyl anion co-transporting with proton [29], this means that the change of extracellular pH is also a factor that can change the direction of ion flow controlled by band 3 protein. As mentioned above, the development of erythrocyte PHL is strongly dependent on the time of incubation in rehydrated and dehydrated media, as well as on the osmolarity and the ionic strength of the medium [18]. Table (1) presents data on the level of post-hypertonic hemolysis of erythrocytes depending on electrolyte concentration in dehydrated medium. Erythrocytes were dehydrated in sodium chloride solutions of different osmolarity for 10 seconds and rehydrated in normal hypotonic sodium chloride solutions under the effect of transport inhibitors – DIDS and dipyridamole. In this series of experiments hypotonic hemolysis served as control, and its level was modulated by changing the medium pH [30]. Osmolarity of hypotonic medium for each experiment was adjusted so that hemolysis at pH 7.4 was about 50% (65-70 mol/l sodium chloride). This table shows that hypotonic hemolysis is characterized by a pronounced pH-dependence with maximum hemolysis at pH 5.8. Increase in the osmolarity of pre-incubation medium results in increased level of hemolysis at all pH values in the experiment, and sensitivity of erythrocytes to hypotension increases mainly at acidic pH, although pH-dependence is retained. Change in pH (5.8, 6.6, 7.4) allows to modulate chloride distribution between the cell and its environment.

Table 1: Effect of the pH of rehydration medium and anion transport inhibitors (DIDS and dipyridamole) on the level of erythrocyte PHL (hypertonic exposure (8-10 sec.))

Inhibiting Agent	*pH*	*Osmolarity (Sodium chloride) mol/L (hypotonic hemolysis)*			
		0.15	0.60	0.80	1.50
	5.8	80.05±6.85	87.55±0.9	87.55±1.05	89.35±2.95
Control	6.6	58.7±4.9	58.0±3.5	67.15±5.65	80.15±0.65
	7.4	49.4±5.2*	46.5±8*	56.05±9.85*	71.6±10.15
	5.8	60.4±9.3	61.4±11.45	67.5±9.8	83.2±9.95
DIDS	6.6	58.7±4.9	51.65±7.45	67.55±1.65	78.2±1.3
	7.4	65.55±11.8	62.05±6.75	69.75±12.05	82.1±8.8
	5.8	76.95±7.75	71.35±5.9	71.35±5.95	80.5±3.6
Dipyridamole	6.6	46.2±3.85	43.9±1.6	49.9±11.45	74.35±5.15
	7.4	53.5±5.5	46.5±8	63.4±13.45	70.75±2.55

Data are presented as mean ± SD, n= 5; SEM; *=p<0.05 indicates a significant difference between concentrations.

Cell volume is changed depending on the distribution of this anion, which in turn affects the osmotic resistance of erythrocytes. In respect of erythrocytes, we can define two mechanisms that influence chloride distribution: pH-dependent and gradient. In the case of hypotonic media pH-dependent mechanism is involved in controlling chloride distribution across the erythrocyte membrane: at pH 6.6 hemoglobin charge is equal to zero (isoelectric hemoglobin point), so the ratio of Cl in / Cl out ≈ 1 [31]. At pH 7.4 hemoglobin acquires a negative charge which prevents penetration of chloride anions into the cell (Cl in / Cl out <1). At pH 5.8 we can observe a reverse situation: hemoglobin charge becomes positive, and thus the input of negatively

charged chloride will be driven by positively- charged hemoglobin (Cl in / Cl out <1), therefore, at pH 5.8 erythrocyte volume will be greater than at pH 7.4. This will lead to the situation when critical hemolytic amount is achieved more quickly at pH 5.8 compared to pH 7.4, which is manifested in a higher level of hypotonic hemolysis at pH 5.8. DIDS present in hypotonic medium promotes leveling of hemolysis pH dependence: at pH 5.8 its level decreases, while at pH 7.4 the opposite pattern is observed. In the latter case, the increasing osmolality of pre-incubation medium, as is the case of control cells, results in hemolysis increase. Just like DIDS, dipyridamole changes the nature of pH-dependence, but this change is even less pronounced. The action of anionic transport inhibitors of dipyridamole and DIDS can be explained considering the influence of medium pH on the osmotic state of erythrocytes. Increasing the pH above neutral (pH 7.4) causes chloride output from the cell by osmotic gradient and subsequent decrease in the concentration of intracellular chloride comparing to extracellular, which leads to volume reduction and, accordingly, hemolysis slowing. In case of decreasing medium pH (pH 5.8) chloride redistribution leads to increase in its concentration in the cell to a level higher than in extracellular medium, and subsequent increase in cell volume will contribute to hemolysis intensification. DIDS and dipyridamole block chloride anions redistribution, thus eliminating hemolysis pH-dependence. More pronounced DIDS effect in pH-dependence leveling may be explained by dimerization of spectrin [28]. At the same time, it is shown that aggregation of spectrin molecules is responsible for spectrin precipitation in an acidic hypotonic buffer (pH 6.0) to a much greater extent than disulfide crosslinks [32].

Conclusions

Longer erythrocyte incubation in hypertonic medium (45 min. in the medium containing 1.5 mol/l sodium chloride, 37°C) at baseline prior to cooling is followed by declining hypertonic cryohemolysis. Moreover, erythrocyte load with sodium cations (using nigericin) helps to reduce cold damage of erythrocytes. At the same time, erythrocyte load with sodium cations increases the erythrocyte's sensitivity to PHL. We can assume that during hypertonic erythrocyte incubation sodium and chloride ions enter into the cell, sensitizing it to rehydration. This means that the level of post-hypertonic hemolysis of erythrocytes depends primarily on membrane's ability to reduce its volume during rehydration. Therefore, under conditions of hypertonic pre-incubation osmotic gradient may be considered as a factor sensitizing cells to subsequent transfer into isotonic medium when they dehydrate and reach minimum volume. Experiments showed that lysis of erythrocytes cooled in a hypertonic medium, or incubated in hypotonic medium, depends on pH. Anion transport inhibitors DIDS and dipyridamole remove pH dependence of hypotonic lysis and hypertonic cryohemolysis, while the cytoskeleton does not eliminate pH dependency. This indicates that the

development of hypotonic and hypertonic cryohemolysis depends on chloride redistribution between cell and extracellular environment, and is less dependent on the state of cytoskeleton. An important feature of PHL is absence of dependence of cell lysis upon rehydration on medium pH. This feature of PHL development were also unchanged during cell rehydration in the presence of DIDS and dipyridamole. Summarizing the results, we can conclude that the sensitivity of erythrocytes to PHL is defined by consistency between transports processes aimed at adapting cells to changing medium conditions, primarily at the stage of rehydration. The main factor determining the ability of cells to withstand adverse changes in their state under rehydration is the safety of its barrier function, preserving its continuity and lack of breaches in transport systems through which ions are redistributed. This means that during rehydration, just like in other variants of osmotic impact on the cell, such as hypertonic shock and hypertonic cryohemolysis, volume shift acts as a damage factor which violates the consistency of changes in spatial membrane structure on the one hand and cytoskeleton structure on the other. Inclusion of components with low penetrating power into rehydration medium eliminates the factor of volume shift, which makes cell more resistant to stress accompanying changing osmotic conditions of the medium. Under conditions of post-hypertonic hemolysis, the level of erythrocyte lysis is determined by the composition of rehydration medium. Inclusion of sucrose having a low ability to penetrate cells and high ability to hold water and slow down cell swelling into the medium increases the resistance of cells to post-hypertonic lysis. Ionophore nigericin, enhancing transmembrane transport of sodium and potassium ions, has a multidirectional effect on erythrocyte's sensitivity to hypertonic cryohemolysis and post-hypertonic lysis. In case of hypertonic cryohemolysis, nigericin desensitizes cells, while in case of posthypertonic lysis it increases cell sensitivity. The effect of pH change on osmotic sensitivity of erythrocytes depends on the temperature and osmotic effect. Under conditions of hypertonic cryohemolysis, decrease of pH to acidic values leads to a

reduction in the sensitivity of erythrocytes to cooling. In turn, the sensitivity of erythrocytes to post-hypertonic hemolysis increases with pH shift to acidic values. The effect of anion transport inhibitors DIDS and dipyridamole on the temperature and osmotic sensitivity of erythrocytes show a dependence between osmolarity and pH. Under conditions of hypertonic cryohemolysis DIDS manifests higher protective efficacy, while dipyridamole is more efficient under post-hypertonic lysis. During cryohemolysis the effect of Deeds and dipyridamole is more pronounced at increasing pH to alkaline values (pH 8.0-9.0), while in posthypertonic lysis it is more pronounced at lowering osmolarity of hypertonic medium to moderate values (0.60 - 0.80 mol/l).

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