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HYPERTONIC CRYOHEMOLYSIS IN BOVINE, EQUINE, CANINE AND HUMAN ERYTHROCYTES: OSMOTIC AND TEMPERATURE PECULIARITIES

N. M. Shpakova, S. S. Ershov, N. V. Orlova

Institute for Problems of Cryobiology and Cryomedicine of the National Academy of Sciences of Ukraine, Kharkov

Hypertonic cryohemolysis in human and animal (bovine, equine and canine) erythrocytes has been compared. Cryohemolysis decrease with time in human, equine and canine erythrocytes is observed in electrolyte medium (1.2 mol/l NaCl) in contrast to a non-electrolyte (0.86 mol/l sucrose) one. This reduction is associated with a probable flux of Na^+ extracellular ions into cells and as the consequence with osmotic gradient diminishing on a membrane at the moment of temperature shift from 37 to 0°C. Decrease with time in hypertonic cryohemolysis for bovine erythrocytes was revealed when salt concentration in medium increased up to 2.1 mol/l NaCl. There were demonstrated the features of mammalian species in erythrocyte responses when varying temperature of incubation medium (37, 25, 15°C) and with following cell cooling down to 0°C. High resistance of bovine erythrocytes to hypertonic cryohemolysis (in comparison with the studied cells of other mammals) can be associated with high content in membrane of cholesterol and sphingomyelin, capable of suppress cug a defect formation in erythrocyte membranes.

Keywords: MAMMALIAN ERYTHROCYTES, HYPERTONIC CRYOHEMOLYSIS, MEDIUM TEMPERATURE, MEDIUM OSMOLARITY

The problem of long-term storage for biological material under low temperature has been under study for a long time [1]. Cell damaging factors under freezing are as follows: medium hyperosmolarity, pH change, temperature shift, ice crystal formation, etc. The model experiments are used to determine the contribution of any of above mentioned damaging factors in general process of biological material low temperature storage. In particular, cold shock as a model of freezing enables the evaluation of cooling effect on cells within positive temperature range. In contrast to many biological objects, which lyse under cooling in isotonic medium, hypertonic conditions are indispensable for human erythrocyte hemolysis [1]. That is why the term of hypertonic cryohemolysis has been proposed for human erythrocytes. The mentioned phenomenon is well studied for human erythrocytes: its developmental peculiarities and regularities in different media, time and temperature dependencies were established [2].

Recently, due to a need arisen in using blood and its components in veterinary practice the interest for a long-term storage of animal erythrocytes in a frozen state has augmented [3]. At the same time, the effect of cryodamaging factors on cells of different animal species has been poorly studied. Therefore basing on the mentioned above of interest was to study the peculiarities of hypertonic cryohemolysis development for animal erythrocytes with certain differences either in plasmatic membrane composition or cytoplasm [4–6] and to compare with those for human erythrocyte hypertonic cryohemolysis.

The aim of this study was to define the time dependencies of hypertonic cryohemolysis of mammalian (human, bovine, equine and canine) erythrocytes in media with different compositions and temperature variations of cell incubation.

Materials and methods

Erythrocytes were derived from bovine, canine, equine and human blood, procured with preservative «Glygicir». After plasm removal the erythromass was twice washed-out by centrifugation at 1,500 g for 3 min in a 10-fold volume of physiological solution (0,15 mol/l NaCl, 0,01 mol/l phosphate buffer, pH 7,4) and stored as a dense sediment not longer than two hours at 0 °C. All the media used in the research were prepared with 0,01 mol/l phosphate buffer, pH 7,4.

NaCl and sucrose concentrations in solutions were controlled with osmolarity measurement (OMKA 1C-01 osmometer, Ukraine).

The standard procedure of hypertonic cryohemolysis we used in the research consisted in a long-term (1–120 min) erythrocyte incubation in hypertonic medium at 37 °C, afterwards the cell aliquots were transferred into hypertonic solution of similar tonicity, cooled down to 0 °C and incubated under the same temperature for 10 min. Final hematocrit was 0,4 %. In experiments on studying temperature and time dependencies of hypertonic cryohemolysis the cells were cooled down to 0°C after a long-term incubation (1–120 min) at 37, 25 and 15 °C.

Hemoglobin amount in supernatant was determined spectrophotometrically (λ=543 nm) and calculated as percentage in respect of 100 % erythrocyte hemolysis at Triton X-100 (0,1 %) presence.

«Chemically pure» and «pure for analysis» graded reagents of Ukrainian production were used in the work.

Results and discussion

It was reported [7] that for human erythrocytes a prolonged cell incubation in hypertonic saline and non-electrolyte solutions at 37 °C affected differently the extent of erythrocyte hemolysis during their cooling down to 0 °C. If there was a decrease in cryohemolysis with time in electrolyte medium, its augmentation was

observed in non-electrolyte one. Cells were suspended in hypertonic saline or sucrose solution to evaluate the medium composition effect on hypertonic cryohemolysis development in bovine, canine, equine and human erythrocytes. After incubating within different time periods at 37 °C the cells were rapidly cooled down by transferring under 0 °C.

Fig. 1 represents dependency of mammalian erythrocyte hypertonic cryohemolysis on incubation duration (1–120 min) at 37 °C following cooling down to 0 °C. As incubation medium we used 1,2 mol/l NaCl since our previous researches [8] demonstrated the maximum or significant erythrocyte damage for many mammals as occurring exactly in this medium after cooling from 37 down to 0 °C.

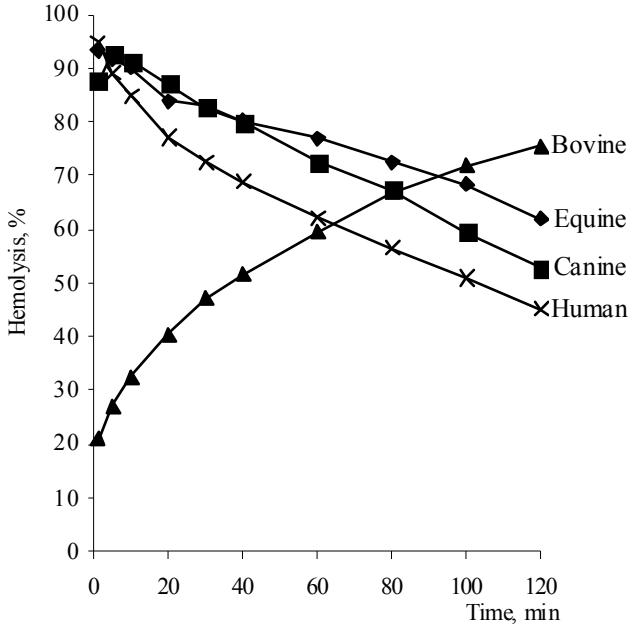

Fig. 1. Effect of incubation duration (37 ˚C) of different mammalian erythrocytes on cell hypertonic cryohemolysis in 1,2 mol/l NaCl-containing mediu (n=6).

The data presented (Fig. 1) demonstrate a decrease with time in hemolytic damage of human, canine and equine erythrocytes. If an initial damage of these cells makes 90–95 %, the cryohemolysis in canine and human erythrocytes reduces down to 45–50 % and 60 % for equine ones to the end of incubation period (120 min). Thus, in this period the extent of hypertonic cryohemolysis of canine, human and equine erythrocytes decrease by 50, 40 and 35 %, correspondingly. At the same time under quite a low initial cell damage rate of about 20 % for bovine erythrocytes we observe an opposite dependence. It is hypertonic cryohemolysis rise for all studied time intervals.

When replacing saline for sucrose medium (0,86 mol/l), quite a low extent of initial hypertonic cryohemolysis in all cells (about 10 %) with following increase with time is observed (Fig. 2). The fact that erythrocyte cryohemolysis develops in both NaCl and sucrose hypertonic solutions enables suggesting rather an increased osmolarity, than medium ion strength as playing an important role in cell cold damage induction. Analysis of time dependency curves for canine, human and equine erythrocyte hypertonic cryohemolysis in non-electrolyte medium allows to emphasise their two peculiarities: an increase with time in erythrocyte hemolysis values at the first stage and a plateau at the second one. Canine erythrocytes are maximally damaged in 20 min and human and equine ones in 60 min. However, if the extent of maximum hemolysis of canine and human erythrocytes makes 95-100 %, it is 60 % for equine ones. The peculiarity of time dependency curve of hypertonic cryohemolysis for bovine erythrocytes is no plateau and a gradual development of hemolytic process within the whole range.

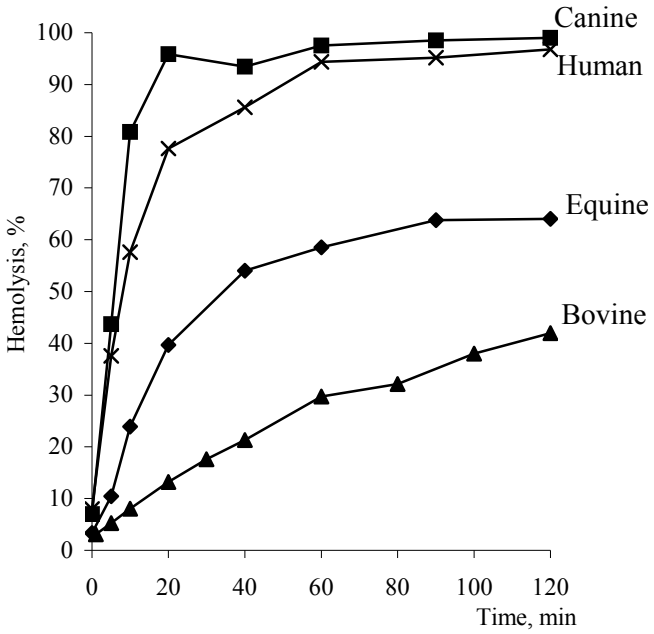

Fig. 2. Effect of incubation duration (37˚C) of different mammalian erythrocytes on cell hypertonic cryohemolysis in 0,86 mol/l sucrose-containing medium (n=6).

Comparing the results detailed in Fig. 1 and 2 of a sharp change (reversion) in hypertonic cryohemolysis curve shape for human, canine and equine erythrocytes is noted when replacing electrolyte medium for non-electrolyte one. At the same time when the character of time dependency of bovine erythrocyte hypertonic cryohemolysis is kept in both media (electrolyte and non-electrolyte), the extent of cell damage is more manifested in electrolyte one.

A special behaviour of bovine erythrocytes under hypertonic cryohemolysis unlike the cells of other mammals (Fig. 1 and 2) stipulated the interest to more thorough study of these cells. Taking into account the fact, that the damage extent of bovine cells is not high under cooling in 1,2 M (Fig. 1), we augmented saline concentration in the medium up to 2,4 mol/l NaCl (isothermal lysis under mentioned conditions is not developed [9]).

The data summarised in Fig. 3 demonstrate changes of time-dependent character of bovine erythrocyte hypertonic cryohemolysis with an increase of salt concentration in medium, if an increase in hypertonic cryohemolysis extent with time from 20 up to 75 % is observed in 1,2 mol/l NaCl-containing medium, time dependency is almost absent in 2,0 M NaCl, but under 2,1 mol/l and 2,4 mol/l NaCl a decrease in cell damage rate within the first 20 min and curve approaching to plateau are noted.

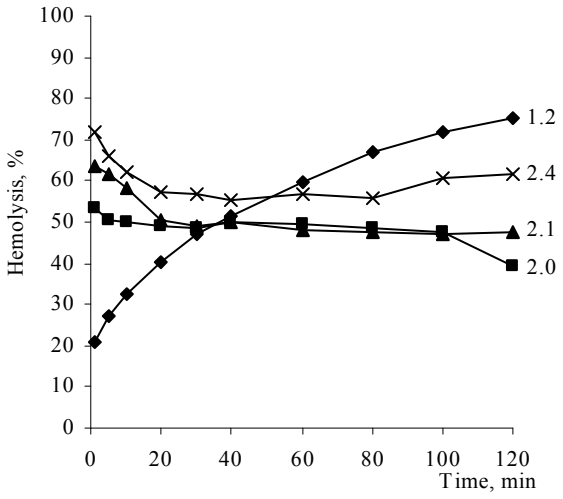

Fig. 3. Effect of incubation duration (37˚C) of bovine erythrocytes on cell cryohemolysis

in the medium containing different NaCl concentrations (mol/l) (n=6)

Thus, a decrease in hypertonic cryohemolysis with time for bovine erythrocytes is observed in the media with higher osmolarity if compared with other mammalian cells (Fig. 1).

To study the role of temperature factor in hypertonic cryohemolysis development for human, canine and bovine erythrocytes, we changed the temperature of incubation medium, when cells were pre-incubated before cooling down to 0 °C. As incubation medium for human and canine erythrocytes we used 1,2 mol/l NaCl and 2,1 mol/l NaCl for bovine ones. The obtained time dependencies of human, canine and bovine erythrocyte hypothermic cryohemolysis, recorded at different temperatures are shown in Fig. 4.

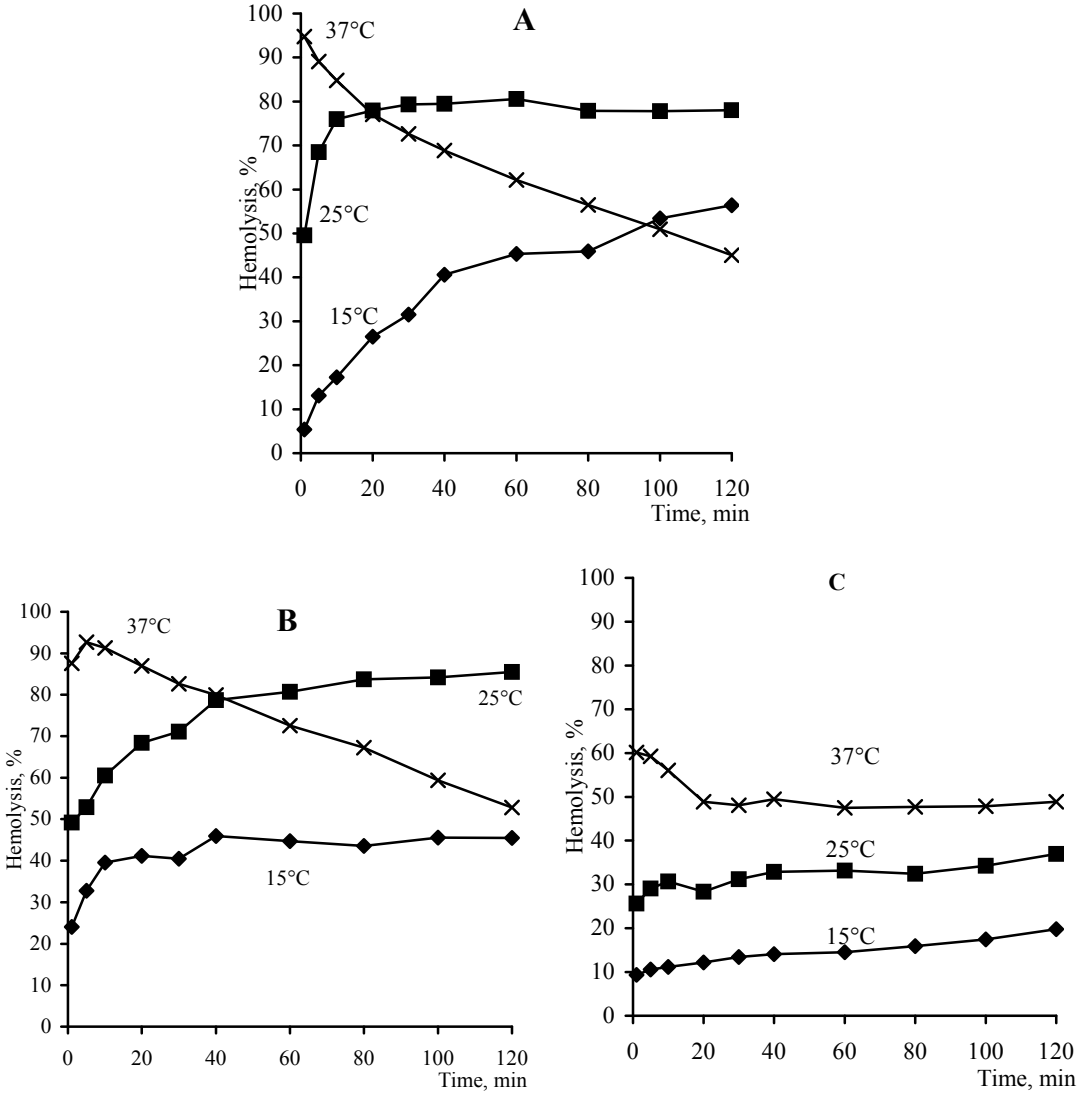

*Fig.*4. Hypertonic cryohemolysis time dependence for human (A), canine (B) and bovine (C) erythrocytes incubated at different temperatures and cooled down to 0 ˚C. Incubation medium for human and canine erythrocytes is 1,2 mol/l and 2,1 mol/l NaCl for bovine ones (n=6).

Of note are the features of hemolysis time dependencies in all studied cells (Fig. 4). Firstly, with a decrease in experimental temperature there is observed a shift in initial extent of cell damage towards lower values. Secondly, the profiles of time dependency curves of mammalian erythrocyte hypertonic cryohemolysis are determined by the temperature, which was used for cells incubation prior to their cooling down to 0 °C

Significant decrease in the extent of erythrocyte hypertonic cryohemolysis with time is observed for human erythrocytes (Fig. 4, A) at 37 °C. Under lower temperature (25 °C) the augmentation of erythrocyte damage extent to the 10^{th} min is noted with further curve approaching to plateau. But at 15 °C a gradual augmentation of erythrocyte hypertonic cryohemolysis extent up to 120 min is observed. Thus a change in curve shape is noted with temperature decrease.

For canine erythrocytes (Fig. 4. B) the cryohemolysis decrease at a long-term cell incubation at 37°C is characteristic. An increase in hypertonic cryohemolysis within 40 min is observed at 25 and 15 °C, at lower temperature all cryohemolysis values reduce approximately by 20–40 % .

Time dependencies of bovine erythrocyte hypertonic cryohemolysis in 2,1 M NaCl, recorded under various temperatures are summarised in Fig. 4, C. A decrease in hypertonic cryohemolysis extent within 20 min with following curve approaching to the plateau is observed at 37 °C, meanwhile at 25 and 15 °C we can state a certain cryohemolysis increase with time (by 10–15 %). We should note that a time dependency of bovine erythrocyte hypertonic cryohemolysis at 37 °C is similar with those for human and canine erythrocytes, procured at 25 °C.

Thus, for human, canine and bovine erythrocyte the cryohemolysis decrease is observed at 37°C, but there is an increase in cell damage extent with time under lower temperatures.

The research data testify to the fact, that erythrocyte damage under hypertonic cryohemolysis is determined by combining two parameters: temperature and medium tonicity. Based on the example of bovine erythrocytes, an equal effect might be obtained by changing either temperature when cells were incubated before cooling down to 0 °C or salt concentration in incubation medium.

In contrast to many biological objects, lysis under cooling in isotonic medium, the hypertonic conditions are necessary for human and animal erythrocyte cryohemolysis [1, 7]. In this case hypertonic medium acts as the factor, sensibilising cells to following cooling.

One believes that the necessity for erythrocyte hypertonic treatment prior to cooling is stipulated by a high cholesterol content in mammalian erythrocyte membranes [1]. Cholesterol is capable of modulating physical and chemical properties of cell membranes [9]. A change in enthalpy and cooperativity of gel-liquid crystal transition is observed at cholesterol presence, but an increase in cholesterol content in lipid bilayer results in elimination of mentioned phase transition [11–13]. Cholesterol increases the degree of orientation order and reduces the movement rate of phospholipid hydrocarbon chains [12], causing a condensation of lateral packing of the lipids in the membrane. This will rise a mechanical stability and reduce membrane permeability [15, 16].

The data, summarised in our research about a decrease in the extent of hypertonic cryohemolysis of human erythrocytes with a time increase of cell exposure in hypertonic electrolyte medium (1,2 M NaCl) at 37 °C in contrast to non-electrolyte conditions (0,86 M sucrose) correlate well with the results, described in the papers [7]. For equine and canine erythrocytes the analogous dependencies of hypertonic cryohemolysis under long-term cell incubation at 37 °C are the similar to human ones, i.e. there are a decrease in cryohemolysis extent with time in saline medium and increase in cell damage in non-electrolyte solution (Fig. 1 and 2). The base for this behaviour of human and animal erythrocytes is the possibility of extracellular sodium entering (in contrast to sucrose, whose molecules are characterised with bigger size) into the cells, that will accompany with a decrease in osmotic gradient on a membrane at the moment of temperature shift from 37 towards 0 °C.

Bovine and canine erythrocytes are referred to low-potassium cells, meanwhile equine and human ones to high-potassium ones [6]. Assuming as a main damaging power of hypertonic cryohemolysis in mammalian erythrocytes is the osmotic gradient, stipulated by high sodium chloride concentration in extracellular medium, then human and equine cells should be the most sensitive under all other equal conditions. In addition, a similar time dependency should be expected for them. However no correlation between erythrocyte sensitivity of different species to hypertonic cryohemolysis and dominating intracellular cell cation was revealed.

A prolonged bovine erythrocyte exposure in 1,2 M NaCl-containing medium enabled to reveal significant peculiarities of these cells in their response to a long-term incubation at 37 °C: cryohemolysis rise for all time interval both in electrolyte and non-electrolyte media (Fig. 1 and 2). This is apparently stipulated by composition peculiarities in bovine erythrocyte membrane. Firstly, bovine erythrocyte membranes are characterized with higher cholesterol content if to compare with human, canine and equine cell membranes [4]. In addition, they comprise a big amount of sphingomyelin (2–4 times higher than human, equine and canine ones) at the background of quite a complete phosphatidylcholine absence [5].

A preferential interaction of sphingomyelin with cholesterol [17] is considered as a central event during forming in membranes of ordered domains and rafts [18], which are characterized with a high resistance to non-ion detergents [19].

Up today some authors believe [1] that the development of erythrocyte cryohemolysis is associated to membrane microdefects formation within the cholesterol-deficient area (at the stage of hypertonic incubation under physiological temperature). Further cell cooling will result in evolution of these defects up hemolytic pore size. The fact, that bovine erythrocyte membranes contain a big amount of cholesterol and sphingomyelin, allows believing that the revealed resistance for these cells to hypertonic cryohemsolysis may be stipulated with

cholesterol and sphingomyelin capability to suppress the formation of structure defects in erythrocyte membranes [20].

Н. М. Шпакова, С. С. Єршов, Н. В. Орлова

ГІПЕРТОНІЧНИЙ КРІОГЕМОЛІЗ ЕРИТРОЦИТІВ БИКА, КОНЯ, СОБАКИ І ЛЮДИНИ: ОСМОТИЧНІ І ТЕМПЕРАТУРНІ ОСОБЛИВОСТІ

Р е з ю м е

Проведено порівняльне дослідження гіпертонічного кріогемолізу еритроцитів тварин (бик, кінь і собака) і людини. В електролітному середовищі (1,2 моль/л NaCl), на відміну від неелектролітного (0,86 моль/л сахароза), спостерігається зниження з часом кріогемолізу еритроцитів людини, коня і собаки. Це пов'язують з можливим входом позаклітинних іонів Na^+ у клітини, що супроводжується зниженням осмотичного градієнту на мембрані у момент зсуву температури від 37 до 0 °С. Для еритроцитів бика зниження гіпертонічного кріогемолізу з часом виявлено у тому випадку, коли концентрація солі у середовищі підвищується до 2,1 моль/л NaCl. Виявлені видові особливості реакції еритроцитів тварин і людини при варіюванні температури середовища інкубації (37, 25, 15 °С) та охолодженні клітин до 0 °С. Високу стійкість еритроцитів бика до гіпертонічного кріогемолізу (у порівнянні з клітинами інших ссавців) пов'язують з високим вмістом холестерину і сфінгомієліну у мембрані, які здатні утруднювати утворення дефектів в еритроцитарних мембранах.

Н. М. Шпакова, С. С. Ершов, Н. В. Орлова

ГИПЕРТОНИЧЕСКИЙ КРИОГЕМОЛИЗ ЭРИТРОЦИТОВ БЫКА, ЛОШАДИ, СОБАКИ И ЧЕЛОВЕКА: ОСМОТИЧЕСКИЕ И ТЕМПЕРАТУРНЫЕ ОСОБЕННОСТИ

А н н о т а ц и я

Проведено сравнительное исследование гипертонического криогемолиза эритроцитов животных (бык, лошадь и собака) и человека. В электролитной среде (1,2 моль/л NaCl), в отличие от неэлектролитной (0,86 моль/л сахароза), наблюдается снижение со временем криогемолиза эритроцитов человека, лошади и собаки, что связывают с возможным входом внеклеточных ионов Na^+ в клетки, сопровождающееся снижением осмотического градиента на мембране в момент сдвига температуры от 37 до 0 °С. Для эритроцитов быка понижение гипертонического криогемолиза со временем выявлено в том случае, когда концентрация соли в среде повышается до 2,1 моль/л NaCl. Показаны видовые особенности реакции эритроцитов животных и человека при варьировании температуры среды инкубации (37, 25, 15 °С) и охлаждении клеток до 0 °С. Высокую устойчивость эритроцитов быка к гипертоническому криогемолизу (по сравнению с исследуемыми клетками других млекопитающих) связывают с высоким содержанием холестерина и сфингомиелина в мембране, способных подавлять образование дефектов в эритроцитарных мембранах.

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Рецензент: професор, доктор біологічних наук, член-кореспондент НАН України, Інститут біології клітини НАН України Р. С. Стойка.