

APPLICATION OF ALKYL SULFATES AND HEAT TREATED ERYTHROCYTES IN HYPERTONIC CRYOHEMOLYSIS

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The research aim was to study the application efficiency of alkyl sulfates and heat treatment of erythrocytes of mammals (human, horse, bull and rabbit) in order to increase their resistance to hypertonic cryohemolysis. The hemolysis rate for erythrocytes was recorded by spectrophotometry; the efficiency of alkyl sulfates was assessed on the values of maximum antihemolytic activity and the ones of effective concentrations; mammalian erythrocytes were morphologically analyzed by light microscopy. It has been found that anionic amphiphiles (sodium decyl and dodecyl sulfates) exhibit an antihemolytic activity in hypertonic cryohemolysis of erythrocytes. There was shown the transformation of erythrocytes on the "discocyte-echinocyte" type in the presence of alkyl sulfates, indicating to the distribution of amphiphilic molecules in an outer monolayer of erythrocyte membrane bilayer. It has been revealed that pre-incubation of the cells at 49 °C increases the resistance to hypertonic cryohemolysis effect for human and equine erythrocytes and as well as it reduces the one for bovine and rabbit cells.

Sodium decyl and sodium dodecyl sulfates exhibit an antihemolytic activity under hypertonic cryohemolysis of heat treated mammalian erythrocytes, but it is lower if compared with the control cells. The findings about distribution of alkyl sulfates in membranes and their antihemolytic activity under conditions of hypertonic cryohemolysis of heat treated erythrocytes testify to the perspective of using these substances as the tool for assessing the state of erythrocyte membranes when changing the temperature-osmotic environment.

Key words: hypertonic cryohemolysis of erythrocytes, sodium decyl sulfate, sodium dodecyl sulfate.

The plasma membrane is primarily damaged when deep freezing of erythrocytes [1]. The main factors negatively affecting the cells during freezing are reduced temperature, extracellular solution concentrating associated with freezing out of a free water, ice crystal formation, changes in pH, etc. [1]. To study the effect of various factors of cryodamage on the erythrocytes there are used the model approaches. In particular, to investigate the effect of reduced temperature and high tonicity of the medium on mammalian erythrocytes hypertonic cryohemolysis is applied (HC). This term is used both to directly refer to the procedure itself, i.e. cell cooling from 37 down to 0 °C in hyperconcentrated media, and to describe the phenomenon of hemolytic damage of cells under these conditions. Mammalian

erythrocytes are characterized with various sensitivity to cooling in hypertonic media [2], which is related to the peculiarities of lipid composition of their plasma membranes. A statistically significant strong correlation between the HC indices of erythrocytes of different mammalian species and contents of membrane lipids has been established [3]. More resistant to HC effect are mammalian erythrocytes, the membranes of those are characterized by a high content of cholesterol, phosphatidylethanolamine and low one of phosphatidylcholines. The mechanism of injury of erythrocytes under HC conditions is associated with the appearance of transmembrane pores, the formation of which depends on the state of cytoskeletal-membrane complex of the cells. Heat treatment of the

erythrocytes influences the protein and lipid components of membrane, in particular the incubation of human erythrocytes at 49 °C leads to denaturation of the main cytoskeletal protein, spectrin [4]. In addition, heat treatment of cells is accompanied by their transformation [5] and an impaired ability to deformation [6].

The application of amphiphilic substances is now expanding both in cryobiological and other biological/medical studies [7–9]. The use of different amphiphilic compounds at micromolar concentrations makes it possible to adjust the sensitivity of erythrocytes to HC [3, 10, 11]. Protective impact of these compounds is due to their ability to be incorporated into membrane and modify it [12]. Thus, antihemolytic activity of amphiphilic compounds on the one hand can be determined by their physical-chemical properties and on another by the composition and state of erythrocyte membranes. Based on the above mentioned in the research there were used the alkyl sulfates referred to anionic amphiphiles; erythrocytes of different mammalian species, featured by various cytoskeletal-membrane compositions and heat treatment of the cells (at 49 °C) as the modifier of the state of erythrocyte membranes.

The research aim was to study the efficiency of alkyl sulfates (sodium decyl and dodecyl sulfates) to improve the stability of heat-treated (49 °C) mammalian erythrocytes cells (human, equine, bovine and rabbit) to the HC effect.

Materials and Methods

For the study there were used the erythrocytes derived from human, bovine, equine and rabbit blood procured with hemopreservative “Glyugitsir”. The experiments were carried out in accordance with the “General Principles of Experiments in Animals” approved by the 5th National Congress in Bioethics (Kyiv, 2013) and consistent with the statements of European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (Strasbourg, 1985). Erythrocytes were isolated according to the standard procedures [13].

Hypertonic cryohemolysis of erythrocytes was performed by placing the erythrocytes in the solution of 1.2 mol/l NaCl at 37 °C for 10 min, followed by transferring an aliquot to a solution of the same tonicity cooled down to 0 °C for 10 min. The final hematocrit was

0.4%. Sodium decyl (C10) and sodium dodecyl sulfates (C12) were added to hypertonic medium at 0 °C before introducing the cells into it [13]. Mammalian erythrocytes (25% hematocrit) were incubated at 49 °C (thermostat U4, Germany) for 10 min [14], afterwards the cells were subjected to HC.

Hemoglobin content in the supernatant was spectrophotometrically determined at 543 nm wavelength. The sample absorption, whereto the Triton X-100 (0.1%) was added, was assumed as 100%.

The plateau sizes and the values of effective concentrations ($C_{AG_{max}}$); calculated values of maximum antihemolytic activity (AG_{max}) of substances (C10 and C12) were examined on the base of the obtained dependency of hypertonic cryohemolysis of mammalian erythrocytes on the concentration of a substance in the medium to characterize the efficiency effect of amphiphilic compounds.

The concentration range of amphiphilic compound, within which there is observed a minimum level of erythrocytes hemolysis is the plateau the center of which is an effective concentration of the substance ($C_{AG_{max}}$).

The maximum antihemolytic activity (AG_{max}) of an amphiphilic compound was calculated by the formula:

$$AG_{max} = \frac{k - a}{k} \times 100 \%,$$

where k is the value of erythrocyte hemolysis in the absence of an amphiphilic substance; and a is minimum value of erythrocyte hemolysis in the presence of an amphiphilic substance.

Mammalian erythrocytes placed into autologous plasma were morphologically analyzed by light microscopy with microscope STUDAR E (Poland) using photo recording of blood morphology with digital camera CANON PowerShot A510. The response of the cells to the introduced amphiphilic compounds was evaluated in the drop, which was placed between a slide and cover slip and evenly spread with a thin layer. To assess the morphological features of erythrocytes' shape there was used the standard classification [15].

We used sodium decyl and sodium dodecyl sulfates (SintezPav, Russia) and the reagents of domestic production of “chemically pure” and “pure for analysis”.

Statistical analysis was performed using ANOVA test and the Mann-Whitney [StatgraphWin]. Differences between groups were considered as statistically significant at $P < 0.05$.

Results and Discussion

Human, bovine, equine and rabbit erythrocytes, which differed in compositions of the cytoplasm, ability to deform, activity of transport pathways, phospholipid and protein composition of membrane [16–20], are shown in Fig. 1. It is evident that all the investigated erythrocytes being in autologous plasma are of discocyte shape. The comparative analysis demonstrates a pronounced to a different extent ability of cells of human, horse and rabbit cells to form the “rouleaus” while for bovine erythrocytes the aggregation is not characteristic: they are represented by single discocytes.

To implement the HC the erythrocytes are firstly incubated in a hypertonic medium at 37 °C, then an aliquot is transferred to the medium of same tonicity but at 0 °C. The HC dependence for the erythrocytes of different species of mammals on salt concentration in the medium has a different character [2]. If for bovine and rabbit erythrocytes the hemolysis dependence on salt concentration in incubation medium when cooled from 37 to 0 °C is characterized with monotony, then human and equine cells is done with a gradual increase in an injury rate, followed by more or less pronounced decrease. In the latter case, a high injury rate (about 90%) was observed in the medium containing 1.2 mol/l NaCl, which was chosen for further experimental studies.

In Fig. 2 there are presented the results of hemolytic damage of human and animal cells in 1.2 mol/l NaCl when cooling from 37 to 0 °C. In contrast to human and equine erythrocytes, bovine and rabbit cells are characterized by a higher resistance to HC.

When pre-incubating the mammalian cells at 49 °C their response to the following action of HC is changed. There was observed a reduced hemolysis of human and equine erythrocytes, whereas for bovine and rabbit cells hemolysis rate was increased (Fig. 2).

Erythrocytes state of different mammalian species when changing the temperature range from 20 to 70 °C was studied by impedance spectroscopy [4]. There was determined the critical temperature value, which the authors regard as the one of spectrin denaturation, a main component of cytoskeleton, by rapid change in resistance and capacitance of erythrocyte suspension. Thus, according to the data, spectrin denaturation temperature for equine erythrocytes made 48.5 °C, for human erythrocyte it was 49.5 °C, for rabbit — 50 °C, for bovine — 52 °C. In mammalian erythrocytes the denaturation spectrin temperature was lower (equine, human), there was observed a decrease of HC level after preincubation at 49 °C, while in rabbit and bovine cells, with higher denaturation temperature there was done the increased HC (Fig. 2). It is possible that in the latter case, the state of spectrin denaturation was not achieved. Considering that the differences may occur at the level of cell membranes, it is of interest to study their barrier properties compared to hemoglobin at a combined effect of HC and 49 °C with the involvement of amphiphilic substances. These compounds are able to protect cells from hemolytic damage under the effect of HC conditions [2]. The protective effect of amphiphiles is based on the ability of the given compounds to perturbate the membrane when

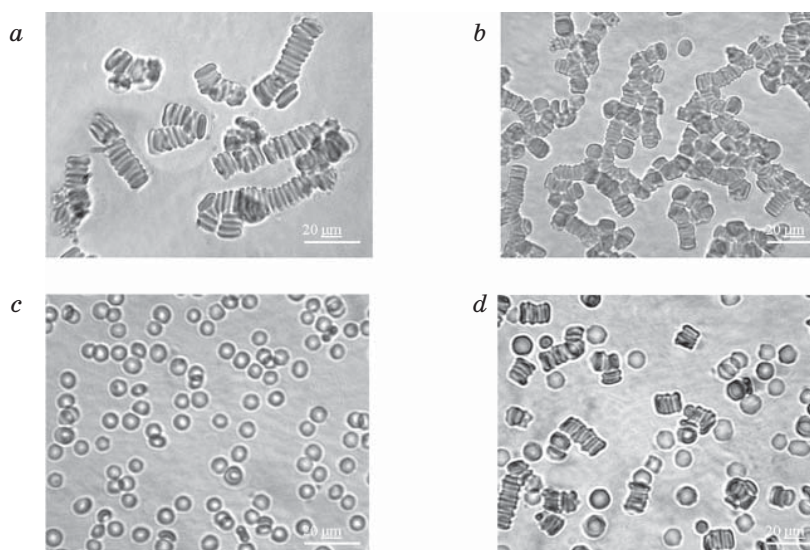


Fig. 1. Morphology of mammalian erythrocytes in autologous plasma: a — human; b — equine; c — bovine; d — rabbit

incorporating into it and thereby prevent the formation of transmembrane pores. Thus, the state of erythrocyte membrane under stress conditions could be judged by efficiency of the amphiphilic compounds, represented by a maximum antihemolytic activity.

In the research the negatively charged amphiphilic compounds (alkyl sulfates) with the alkyl chain length of 10 or 12 carbon atoms were used. Effective concentrations and plateau sizes of these substances of mammalian erythrocytes under HC are presented in the Table. It follows that plateau sizes are wider, and the values of effective concentration of C10 is higher than the ones of C12 under erythrocyte HC of different mammalian

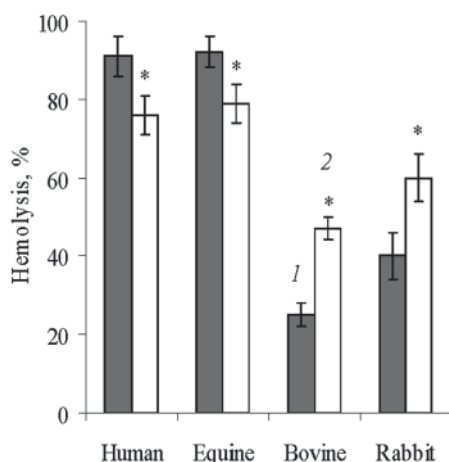


Fig. 2. Hemolysis of mammalian erythrocytes when cooling from 37 down to 0 °C in the medium containing 1.2 mol/l NaCl

Here in after: 1 — control erythrocytes;
2 — erythrocytes, modified at 49 °C;
* — significant differences if compared with the control: $P < 0.05$

Values of effective concentrations (C_{AHmax}) and sizes of concentration plateau of alkyl sulfates under hypertonic cryohemolysis of mammalian erythrocytes in the medium containing 1.2 mol/l NaCl ($M \pm m, n = 7$)

Mammalian erythrocytes	Sodium decyl sulfate			Sodium dodecyl sulfate		
	C_{AHmax} , $\mu\text{mol/l}$	Plateau size, $\mu\text{mol/l}$		C_{AHmax} , $\mu\text{mol/l}$	Plateau size, $\mu\text{mol/l}$	
		Lower boundary	Upper boundary		Lower boundary	Upper boundary
Human	105±5	50±18	160±20	2,5±2 ^Δ	2,5±1 [#]	5±2 [©]
Equine	170±10	60±15	280±30	20±7 ^Δ	10±7 [#]	30±10 [©]
Bovine	180±9	80±13	280±30	25±1 ^Δ	10±2 [#]	40±2 [©]
Rabbit	215±10	30±7	400±7	25±8 ^Δ	10±3 [#]	40±16 [©]

Note: * — concentrations range of amphiphilic compound, within which there is observed a minimum level of erythrocyte hemolysis; ^Δ, [#], [©] — $P < 0.05$ relative to corresponding values of effective concentrations and sizes of concentration plateau of sodium decyl sulfate, assumed as a control.

species. In the following experiments C10 and C12 were used at effective concentrations.

Antihemolytic effect of alkyl sulfates under HC action is more expressed for animals' erythrocytes than human cells (Fig. 3, 4). In addition, short-chain alkyl sulfate homologue (C10; Fig.3) exhibits a higher antihemolytic activity if compared with a long chain one (C12; Fig. 4).

When the cells were initially heated up to 49 °C, C10 and C12 manifested antihemolytic effect under erythrocyte HC, but it was less expressed as compared to the control cells. Antihemolytic activity of C10 decreases in equine and rabbit erythrocytes twice, and human and bovine ones it reduces almost thrice (Fig. 3). Antihemolytic activity of C12 decreases by 2–3 times for human, equine and bovine cells, while for rabbit ones it reduces almost in 5 times (Fig. 4). It should be noted that both for the control cells and mammalian erythrocytes modified at 49 °C, the protective effect at HC of C10 is higher if compared with C12 (Fig. 3, 4), that is probably associated with different ability of these substances having different alkyl chain length to perturbate the membrane.

Fig. 5 presents the changes in mammalian erythrocyte shape (for example, human and rabbit) in response to introduced anionic amphiphilic compounds in concentrations within which there is observed a significant cell transformation.

The findings suggest that the same changes in shape of rabbit erythrocytes, and human one occur with alkyl sulfates. Under the action of negatively charged C10 and C12 a pronounced erythrocytes' echinocytosis develops (Fig. 5). The character of changes in equine and bovine cell shape when introducing C10 and C12 is similar to the data presented in Fig. 5.

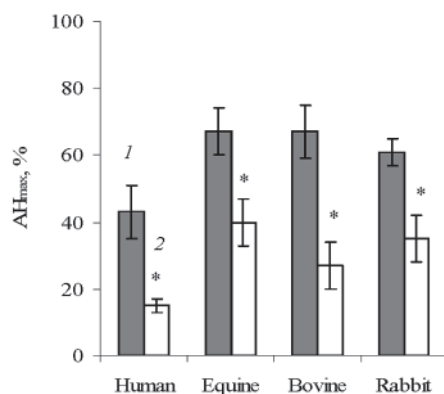


Fig. 3. Maximum antihemolytic activity (AH_{max}) of sodium decyl sulfate when cooling mammalian erythrocytes from 37 down to 0 °C in the medium containing 1.2 mol/l NaCl

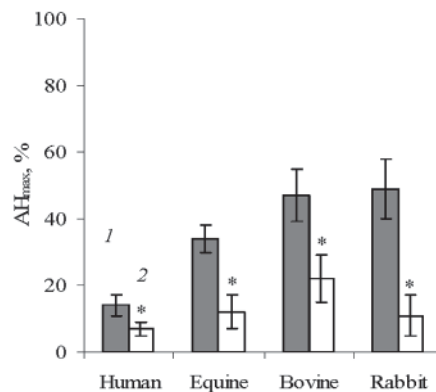


Fig. 4. Maximum antihemolytic activity (AH_{max}) of sodium dodecyl sulfate when cooling mammalian erythrocytes from 37 down to 0 °C in the medium containing 1.2 mol/l NaCl

It should be noted that amphiphilic substances in morphological studies were used in higher concentrations (Fig. 5) than antihemolytic ones presented in the Table. This is due to, on the one hand the ability of plasma proteins to bind amphiphilic molecules, and on another hand by adsorption of plasma components on cell surface, which may limit an accessibility to erythrocyte membrane of substance molecules and, as a result, prevent their distribution in it.

The presence of both hydrophobic and hydrophilic parts is common for amphiphilic molecules. It is the very this parameter that allows them to penetrate into the membrane and to be distributed therein. Morphological analysis of the cells treated with C10 and C12 concludes about intramembrane localization of the studied compounds or rather, their transmembrane distribution (mainly in the outer monolayer of lipid bilayer), based on the hypothesis of coupled monolayers of bilayer [21].

The amphiphilic substances are capable of protecting the erythrocytes from damage caused by the action of various stress factors. Thus, they exhibit antihemolytic activity under the temperature and osmotic stress [2, 3, 10, 11] and lysis caused by the effect of a high hydrostatic pressure, ultrasound and poison [7–9]. The damage of erythrocytes is associated with the formation in membrane pores penetrable for hemoglobin molecules. The protective effect (antihemolytic) of amphiphiles is implemented at the level of erythrocyte membrane. They are incorporated into the membrane, perturbate it and prevent the growth of membrane defect to a hemolytic pore size. Amphiphiles efficiency (values of AH_{max} and C_{AHmax}) depends on the one hand, on amphiphile type, and on the another hand on the state of erythrocyte membrane.

It is shown that the effect on membrane of factors, making it more rigid (low temperature, phenylhydrazine, diamide) [1, 22, 23] results to a reduced antihemolytic activity of amphiphiles under temperature and osmotic stress [3, 13, 23, 24].

The data for all the studied mammals presented in Fig. 3 and 4 testify to the fact that an antihemolytic activity of alkyl sulfates under HC of erythrocytes, modified at 49 °C is reduced. It can be assumed that the decrease in efficiency of amphiphiles under these conditions is due to the fact that it is more difficult for amphiphilic molecules both to be incorporated into the membrane and to disorder it. Therefore, it is possible not only to find the change in a state of erythrocyte membranes at 49 °C, but also to determine the direction of this change. The reduction of antihemolytic activity of C10 and C12 testify to the fact that membrane of heat treated erythrocytes becomes harder. This is confirmed by the results of the research [6], within which it is shown an increase in modulus of shift elasticity and viscosity of human erythrocyte membranes after heat treatment.

Cell membrane of human, bovine, equine and rabbit erythrocytes is characterized by some specific features. In contrast to human and equine erythrocytes, rabbit and bovine cell membranes having a high content of cholesterol, phosphatidylethanolamine and low one of phosphatidylcholine. Furthermore, in bovine erythrocytes the choline-containing phospholipids are represented mainly by sphingomyelin [25, 26]. The cells of the studied mammals are differed in content of transmembrane proteins as well. Thus, equine and rabbit erythrocytes containing less protein

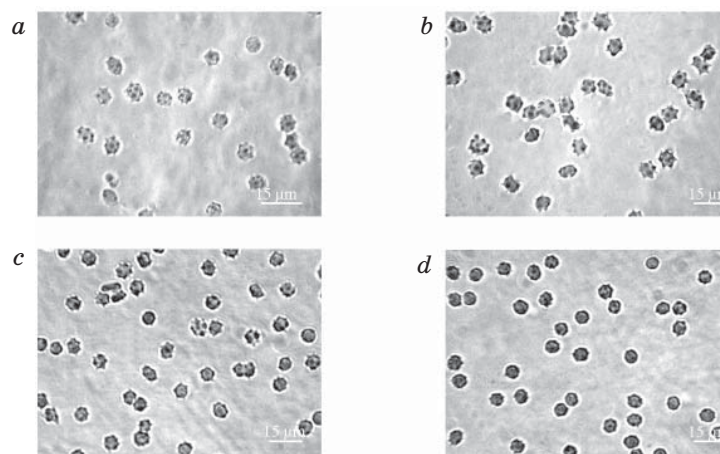


Fig. 5. Morphology of human (a, b) and rabbit (c, d) erythrocytes with alkyl sulfates:
 a and c — sodium decyl sulfate (350 and 215 $\mu\text{mol/l}$, correspondingly);
 b and d — sodium dodecyl sulfate (150 and 75 $\mu\text{mol/l}$, correspondingly)

of band 3 if compared to human cells [19]. Rabbit erythrocytes are free of glycophorin A [27], resulting in a lower ability of their membranes to bind hemoglobin [28]. Protein of band 4.2 responsible for mechanical resistance of cytoskeleton-membrane complex was not found in cytoskeleton of equine erythrocytes compared to other investigated mammalian cells [29].

In spite of the specific peculiarities of cytoskeleton-membrane complex [19, 25–27, 29] and some differences of spectrin denaturation temperature of human, bovine, equine and rabbit erythrocytes [4], reduction of antihemolytic activity of both alkyl sulfates under HC of the heat-treated cells is characteristic for erythrocytes of different mammalian species (Fig. 3, 4). One orientation of the revealed changes suggests the hidden injuries accumulated in erythrocyte membrane at 49 °C. Therefore, the observed decrease in HC level of heat-treated human and equine erythrocytes compared to bovine and rabbit cells (without the addition of alkyl sulfates) (Fig. 2) does not testify to the improved state of their membranes.

Thus, the revealed decrease in damage of human, equine, bovine and rabbit

erythrocytes under HC with sodium decyl and sodium dodecyl sulfates provides the application of alkyl sulfates as effective antihemolytic agents. Transformation of mammalian erythrocytes under the action of sodium decyl sulfate and sodium dodecyl sulfate indicates that the effect of alkyl sulfates is implemented at the plasma membrane. Response of the cells modified at 49 °C of various mammalian species (human, horse, bull, rabbit) to cooling effect in hypertonic medium is different. For human and equine erythrocytes there was observed a decrease in hemolysis rate, while for bovine and rabbit cells the increase was found. When combined using of alkyl sulfates and heat treatment of cells there was established an antihemolytic activity in substances under hypertonic cryohemolysis of erythrocytes, but it is lower if compared with the control cells. The obtained results and analysis of the publications' point to the perspective of alkyl sulfates' usage to assess the state of erythrocytes when changing the temperature and osmotic conditions of the environment.

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ВИКОРИСТАННЯ АЛКІЛСУЛЬФАТІВ І ТЕРМООБРОБКИ ЕРИТРОЦИТІВ ЗА ГІПЕРТОНІЧНОГО КРІОГЕМОЛІЗУ

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Метою роботи було дослідження ефективності застосування алкілсульфатів і термообробки еритроцитів ссавців (людина, кінь, бик і кролик) з метою підвищення їхньої стійкості до дії гіпертонічного криогемолізу. Рівень гемолізу еритроцитів реєстрували спектрофотометричним методом; ефективність алкілсульфатів оцінювали за величинами максимальної антигемолітичної активності та значеннями ефективних концентрацій; морфологічний аналіз еритроцитів здійснювали методом світлової мікроскопії. Встановлено, що аніонні амфіфіли (децилсульфат і додецилсульфат натрію) виявляють антигемолітичну активність за умов гіпертонічного криогемолізу еритроцитів досліджуваних ссавців. Показано трансформацію еритроцитів за типом «дискоцит–ехіноцит» у присутності алкілсульфатів, яка свідчить про розподіл амфіфільних молекул у зовнішньому моношарі бішару еритроцитарної мембрани. Встановлено, що попереднє інкубування клітин при 49 °С підвищує стійкість еритроцитів людини і коня до дії гіпертонічного криогемолізу, однак знижує стійкість еритроцитів бика і кролика. Децилсульфат і додецилсульфат натрію виявляють антигемолітичну активність за умов гіпертонічного криогемолізу термооброблених еритроцитів ссавців, проте вона нижча, ніж для контрольних клітин. Отримані дані щодо розподілу алкілсульфатів у мембрані та їхньої антигемолітичної активності за умов гіпертонічного криогемолізу термооброблених еритроцитів свідчать про перспективу використання цих речовин як інструменту для оцінювання стану мембран еритроцитів за зміни температурно-осмотичних умов середовища.

Ключові слова: гіпертонічний криогемоліз еритроцитів, децилсульфат натрію, додецилсульфат натрію.

ПРИМЕНЕНИЕ АЛКИЛСУЛЬФАТОВ И ТЕРМООБРАБОТКИ ЭРИТРОЦИТОВ ПРИ ГИПЕРТОНИЧЕСКОМ КРИОГЕМОЛИЗЕ

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Целью работы было исследование эффективности применения алкилсульфатов и термообработки эритроцитов млекопитающих (человек, лошадь, бык и кролик) с целью повышения их устойчивости к действию гипертонического криогемоллиза. Уровень гемоллиза эритроцитов регистрировали спектрофотометрическим методом; эффективность алкилсульфатов оценивали по величинам максимальной антигемолитической активности и значениям эффективных концентраций; морфологический анализ эритроцитов млекопитающих осуществляли методом световой микроскопии. Установлено, что анионные амфилилы (децилсульфат и додецилсульфат натрия) проявляют антигемолитическую активность в условиях гипертонического криогемоллиза эритроцитов. Показана трансформация эритроцитов по типу «дискоцит–эхиноцит» в присутствии алкилсульфатов, что свидетельствует о распределении амфифильных молекул во внешнем монослое бислоя эритроцитарной мембраны. Установлено, что предварительная инкубация клеток при 49 °С повышает устойчивость к действию гипертонического криогемоллиза эритроцитов человека и лошади, однако снижает — быка и кролика. Децилсульфат и додецилсульфат натрия проявляют антигемолитическую активность в условиях гипертонического криогемоллиза термообработанных эритроцитов млекопитающих, однако она ниже по сравнению с контрольными клетками. Полученные данные о распределении алкилсульфатов в мембране и их антигемолитической активности в условиях гипертонического криогемоллиза термообработанных эритроцитов свидетельствуют о перспективе использования этих веществ в качестве инструмента для оценки состояния мембран эритроцитов при изменении температурно-осмотических условий среды.

Ключевые слова: гипертонический криогемоллиз эритроцитов, децилсульфат натрия, додецилсульфат натрия.