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ROLE OF POLYVINYL ALCOHOL IN CRYOPROTECTIVE MEDIA: EVALUATING EFFICIENCY AND LIMITATIONS IN ERYTHROCYTE FREEZING

The paper substantiates the feasibility and determines the peculiarities of using polyvinyl alcohol in multicomponent media based on penetrating cryoprotectants during rapid cooling of human erythrocytes. Polyvinyl alcohol is recommended to be used only as a part of multicomponent media based on penetrating cryoprotectants with a low glass transition temperature Tg = –100 ... –150 °C (glycerol, 1,2-propanediol). A cryoprotective effect of combined media containing polyvinyl alcohol of different molecular weights in combination with 20 % glycerol or 1,2-propanediol during freezing of human erythrocytes was comparatively assessed. Based on the results of erythrocyte preservation, the solution containing 20 % glycerol and 0.5 % polyvinyl alcohol with a molecular weight of 9 kDa was found to have the most optimal cryoprotective effect of all the studied combined media.

Key words: human erythrocytes, polyvinyl alcohol, penetrating cryoprotectants, glass transition temperature.

One of the most important tasks of nowadays is to create strategic reserves of donor's blood erythrocytes by low-temperature preservation. Cryopreservation of erythrocytes will ensure their long-term storage, infectious safety and uninterrupted supply to clinical institutions during emergencies.

According to the existing understanding [27, 35, 38], during cryopreservation, erythrocytes are subjected to mechanical, osmotic and biochemical damages, which can significantly affect the viability of thawed cells. Therefore, cryoprotectants are used to protect erythrocytes against the effects of negative factors that arise during cryopreservation. Their role is not only to reduce the temperature of the "water-ice" phase transition, but also to control the mass of ice formed, the morphological structure of its crystals, recrystallization, *etc.*

Currently, the only substance approved for clinical freezing of human red blood cells is the penetrating cryoprotectant glycerol [14]. However, to achieve satisfactory cell preservation, it must be used at concentrations ranging from 30 to 57 %, depending on the cooling method [24, 31, 34]. These concentrations of glycerol can cause intravascular hemolysis of red blood cells, so it must be removed before transfusion. This is a complex and time-consuming procedure, which makes it difficult to use thawed cells, especially in emergency situations. Researchers also use another penetrating cryoprotectant, 1,2-propanediol (1,2-PD) [36]. Compared with glycerol, 1,2-PD is removed from cells more quickly. This is due to the lower molecular weight of this substance and the high permeability coefficient through the erythrocyte

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membrane [17]. However, the effectiveness of 1,2-PD is manifested under the conditions of its use at a concentration of 37 % and two-stage cooling of erythrocytes [37]. In this regard, to increase the efficiency of erythrocyte cryopreservation protocols, it is relevant to search for new non-toxic cryopreservatives that contain cryoprotectants in low concentrations, which are easily removed from cells and do not require complicated cooling.

One of the known approaches to the search for new cryoprotective media is the use of combinations of traditional cryoprotectants with substances capable of exhibiting a cryoprotective effect. Some researchers consider it advisable to reduce the concentration of glycerol by replacing it with carbohydrates [21], antioxidants [1], amino acids [2, 11, 18, 22]. Also, as an additional component, a synthetic water-soluble polymer, polyvinyl alcohol (PVA), is actively being investigated [23, 30, 32, 33]. The interest in this substance is stipulated by the fact that the PVA molecule is characterized by low toxicity [6, 10], high biocompatibility [8], and the ability to exhibit a cryoprotective effect in micromolar concentrations during freezing of various biological objects and some proteins [25]. Thus, R. Deller [9] reported that the addition of 0.5—5 mg / ml of 9 kDa PVA to erythrocytes frozen in a hydroxyethyl starch solution significantly increases their preservation. The principle of the cryoprotective effect of PVA is explained by its ability to inhibit ice recrystallization during cell thawing [5, 12, 15, 19, 20]. Indeed, in model experiments it was shown that the PVA molecule effectively inhibited ice recrystallization in the concentration range from 0.05 to 1 mg/ml [7]. However, this mechanism of protection of biological objects during freezing is a subject of debate. Thus, in some reports [13, 26] it was found that 1 mg/ml of PVA, on the contrary, was able to initiate ice nucleation. The ability of PVA to initiate crystallization is determined by its molecular weight, as well as concentration. In this regard, the search for optimal cryoprotective media for cryopreservation of biological objects containing PVA is a difficult task.

The paper analysed the possibility of effective use of PVA in the composition of combined cryoprotective media and investigated them as potential substitutes for penetrating cryoprotectants (glycerol, 1,2-PD) when freezing human erythrocytes.

The purpose of the research was to analyse the features of using polyvinyl alcohol of different molecular weights in the composition of media with a reduced concentration of glycerol or 1,2-PD and to study their cryoprotective effect during rapid cooling of human erythrocytes.

MATERIALS AND METHODS

The material of the study was erythroconcentrate obtained from male donor blood of group A (II). Donor blood was prepared with hemopreservative "CFDA" in the Kharkiv Regional Center of Blood Service and stored for no longer than 48 hours at a temperature of 4 ± 2 °C. The erythroconcentrate was obtained by centrifugation of preserved donor blood at 2,500 rpm for 20 min.

For experimental studies, cryoprotective media were prepared based on phosphate-buffered saline (0.9 mmol/L Na2HPO4, 0.13 mmol/L NaH2PO4, 150 mmol/L NaCl, pH 7.4) and used after 24-hour exposure at a temperature of 20 ± 2 °C. Cryoprotective solutions were prepared in mass-volume concentrations.

As cryoprotectants, glycerol (Sigma-Aldrich, Germany), 1,2-PD (Sigma-Aldrich) and PVA with a molecular weight of 9 and 31 kDa (PVA 9 kDa, PVA 31 kDa) and a hydrolysis degree of 88 % (Sigma-Aldrich) were used. The composition of multicomponent cryoprotective media included 20 % glycerol or 1,2-PD in combination with 0.1 and 0.5 % PVA 9 or 31 kDa. The control was erythrocytes cryopreserved under the protection of 20 % glycerol or 1,2-PD. The exposure time of erythrocytes in cryoprotective solutions was 15 min at 20 °C. The ratio of the volumes of erythrocytes and cryoprotective solutions was 1:1. The volume of frozen cell suspension was 1.8 ml. The studied samples were frozen in polyethylene ampoules "Nunc" by immersion in liquid nitrogen (–196 °C) and stored from 1 to 3 weeks. The samples were warmed in a water bath at 40 °C with constant shaking of the ampoules until the liquid phase appeared.

The concentration of free hemoglobin in the supernatant and total hemoglobin of the cell suspension was determined by the hemoglobin cyanide method using a set of reagents "Filist-Diagnostics" (Ukraine), the hematocrit — by centrifugation in a centrifuge "CM-70" at 7000 rpm for 5 min (Elmi, Latvia). The percentage of hemolysis of erythrocytes was calculated by the formula:

$$\text{Hemolysis} = \frac{Hb_{free} \times 100 \times (1 - Ht)}{Hb},$$

where Hb_{free} — concentration of free hemoglobin in the supernatant, g/l; *Hb* — concentration of total hemoglobin in the cell suspension, g/l; *Ht* — hematocrit, %.

The number of preserved cells (%) was expressed by the formula:

Preserved erythrocytes = 100 % – Hemolysis (%).

Aqueous solutions of glycerol, 1,2-PD and their combinations with 9 kDa PVA were studied by the thermoplastic deformation. The samples were cooled at a rate of 4 °C/min to –140 °C, stabilized for 10 min at the final temperature. To determine the glass transition temperatures Tg of aqueous solutions of cryoprotectants, a shear deformation stress $\sigma = 4 \times 10^5$ kg / m^2 was applied to the studied samples [28]. Thermoplastic curves of samples were recorded when heating at a constant rate of 1 deg / min.

Statistical analysis of experimental data was performed using the software "Excel" (Microsoft, USA). Experimental data were evaluated using the Mann-Whitney test and expressed as median and percentile. Results were considered drastically different at a significance level of $p \leq 0.01$.

RESULTS AND DISCUSSION

One of the main factors affecting the preservation of cells during cryopreservation is the mass of ice that forms inside the cells during cooling and the change in their volume due to osmotic pressure differences between the cytoplasm and the extracellular medium [16]. These changes occur within the temperature range of –10... –30 °C, which corresponds to a sharp decrease in the plasticity of cell membranes. That is why a change in cell volume above some critical values leads to irreversible disorders of the permeability of their membranes. At the same time, according to the available experimental data [16], partial dehydration of cells can be effectively used to optimize cryopreservation technologies. This is facilitated by the fact that cell damage during compression (volume reduction) is less than during stretching (volume rise). Therefore, to implement the partial dehydration of cells, it is necessary that during their cooling an extracellular crystallization begins as early as possible before intracellular one, *i. e.*, maximum supercooling of the cytoplasm is achieved. Under such conditions, it is possible to initiate cell dehydration, which is controlled by the cooling rate and the permeability coefficient of membranes to water molecules. This phenomenon is of particular importance when using penetrating cryoprotectants. In this case, partial dehydration of the cell leads to an increase in the effective concentration of the cryoprotectant in the cytoplasm and a sharp decrease in both the temperature of the transition of the remaining free water to ice and the mass of ice formed inside the cell. The thermodynamic scheme of this process is presented in Fig. 1.

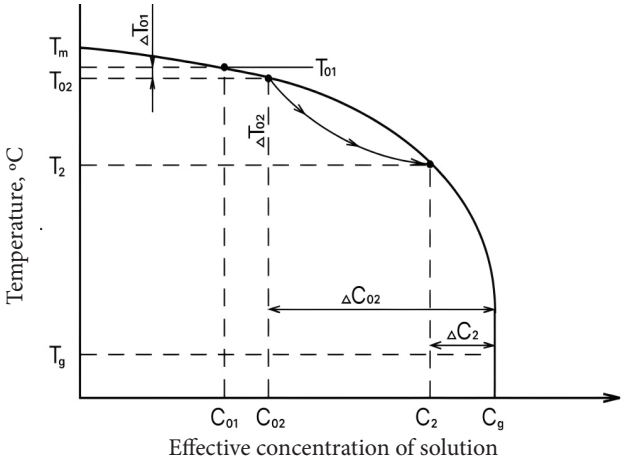


Fig. 1. State diagram of extracellular solution in the presence of cryoprotectant

As Fig. 1 demonstrates, the differences in the effective concentrations of the extracellular medium C_{01} and the cytoplasm C_{02} provide a relative supercooling of the cytoplasm by the value ΔT01. This leads to the fact that during the cooling of the cell suspension, the formation of ice crystals begins in the extracellular medium at a temperature of T_{01}. This disorders the osmotic equilibrium, and as a result, the outflow of water from the cells begins and the further increase in the effective concentration of substances in the cytoplasm from the value of C_{02} to C_2. Accordingly, the temperature of intracellular crystallization decreases, as shown in Fig. 1. A decreased temperature of crystallization of the cytoplasm to the values of T_2 leads to a significant decrease in the mass of intracellular ice by the value:

$$\Delta m = K_1 \Delta C_{02} - K_2 \Delta C_2, \quad (1)$$

where K_1, K_2 are coefficients proportional to the length of the cathodes, which are drawn on the state diagram at temperatures T_2 and T_{02}, respectively [4].

It is for this reason that the cell dehydration increases their preservation during cryopreservation. It should be noted that it is very difficult to implement the schematically presented process at the stages of cryopreservation, especially in the presence of cryoprotectants. Even when using penetrating cryoprotectants, which are characterized by a high ability to enter the cell membrane, their extracellular concentration will be higher than the intracellular one. This completely eliminates the effect of the difference in concentrations of C_{01} and C_{02} and requires special approaches to initiate intracellular crystallization.

The results of the analysis indicate that to increase the preservation of cells during cryopreservation, it is necessary to use substances that are capable of initiating extracellular crystallization. According to the experimental data of S. Ogawa et *al.* [26], such a substance may be PVA. Cell-impermeable PVA molecules adsorb water molecules in the extracellular environment. Due to this, the kinetic motion of water molecules slows down, and their local concentration increases near individual fragments of the PVA molecule. This accelerates the ice nucleation. In fact, PVA acts as a catalyst for crystallization, ensuring osmotic dehydration of cells at sufficiently high cooling rates. At the same time, aqueous solutions of PVA solidify at sufficiently high temperatures (– 20… –25 °C), which sharply increases the likelihood of mechanical damage to cryopreserved cells due to plastic relaxation of thermoelastic stresses.

The amplitudes of internal thermoelastic stresses of the 1st kind σ1, which are associated with temperature gradients in a cooled (heated) solid-phase sample, are estimated using the formula:

$$\Delta\sigma_1 = <\alpha> E \cdot l_1 \partial T/\partial l, \quad (2)$$

where <α> is the average coefficient of thermal linear expansion of the solid-phase matrix created below the temperature T_g; E is the effective modulus of elasticity of this matrix; ∂T/∂l is the temperature gradient that appears in samples that are cooled (heated) along the directions of maximum temperature change.

Since the yield strength of the ice matrix σflu, which practically coincides with its strength limit, does not exceed the values of $6 \cdot 10^6$ N / m^2, it is possible to estimate the permissible temperature gradients during the development of cooling-heating modes below the temperature T_g. According to condition (2) we have:

$$\partial T / \partial l \le \sigma_{flu} / <\alpha> \cdot E \cdot l_1, \quad (3)$$

With the characteristic values of the quantities included in condition (3), $<\alpha> = 5 \cdot 10^{-5}$ deg^{-1}; $E = 10_{11}$ N/m^2; $l_1 = 10^{-2}$ m, we have:

$$\partial T / \partial l \le 10^2 \text{ deg / m}, \quad (4)$$

If condition 4 is not met, then the sample inevitably experiences the plastic relaxation of stresses σ1, which lead to significant plastic displacements in the ice matrix and formation of cracks in amorphous fractions. As a result of these processes, mechanical damage to cryopreserved biological objects is formed. To avoid these damages, it is necessary to use holders of various designs. With their help, it is possible to achieve the fulfillment of condition (4).

However, a more complex problem arises when reducing damage to cryopreserved objects due to thermoelastic stresses of the 2nd kind σ_2. The magnitude of these stresses can be estimated using the expression:

$$\sigma_2 = E (\alpha_{max} - \alpha_{min}) \Delta T_2, \quad (5)$$

where $\alpha_{max} - \alpha_{min} = \Delta\alpha$ is the difference between the coefficients characterizing the thermal linear expansion of the fractions that make up the solid-phase matrix of the cooled cryoprotective solution or the biosystem frozen with it as a whole. ΔT is the change in temperature of the cryoprotective solution that is cooled (heated) by the biosystem after their transition to the solid-phase state. Usually, the value of ΔT is determined by the difference between the glass transition temperature Tg of the cryoprotective solution and the boiling point of liquid nitrogen TN = −196 °C. It should be noted here that the maximum value of Δα can be achieved not only between the crystalline and amorphous fractions, but also between the thermal linear expansion of ice crystals of different crystalline mixtures. Expression (5) allows us to determine the permissible values of the value ΔT_2 as:

$$\Delta T_{cr} \le \sigma_{flu}/E\Delta\alpha, \quad (6)$$

Under the conditions of using the values of the quantities characteristic of the ice matrix included in expression (6), we obtain ΔT ≈ 10 °C. If this value is exceeded, thermoelastic stresses will arise in the frozen biosystem, which will lead to plastic relaxation and crack formation, and as a result, damage to these biosystems.

Therefore, the use of PVA in technologies of low-temperature cryopreservation of biological objects is possible only in the solutions containing substances with a low glass transition temperature Tg (–100… –150 °C): glycerol and 1,2-propanediol. In this case, the choice of optimal concentrations of these substances is important. In fact, in the *Tm* > *T* > *Tg* temperature range, cryoprotective substances should provide structural compositions, the scheme of which is shown in Fig. 2. In this case, the required concentration of these substances should be from 5 to 20 %. It is convenient to determine these concentrations by studying the plastic characteristics of frozen cryoprotective solutions according to the method described by Oleksandr I. Osetsky *et al.* [29]. Fig. 3 shows the concentration dependence of the yield point of aqueous solutions of glycerol, obtained at a temperature of $T_g < T = -70$ °C.

According to the dependence $\sigma_{flu} = f(C)$ at values of C > 7 %, intergranular liquid inclusions begin to appear in the samples, which reduce their effective area: $S_{eff} = \frac{F_{flu}}{\sigma_{flu}}$, where F_{flu} is the load applied to the sample.

At values of C > 20 % by mass of the liquid phase, it is sufficient to form intergranular liquid-phase interlayers, as shown in Fig. 2. From this moment, the plastic flow of the sample is determined by the mixing of ice crystals relative to each other along the hydrophase interlayers. These interlayers provide relaxation of thermoelastic stresses of types I and II. They significantly reduce mechanical damage to cryopreserved bioobjects. In turn, it is at C > 20 % that these interlayers determine the glass transition temperature of the compositions "water / glycerol / PVA" and "water / 1,2-PD / PVA". The thermoplastic curves of these compositions demonstrate the glass transition of these compositions (Fig. 4).

The results of the assessment of the safety of thawed erythrocytes after cryopreservation in the studied combined solutions are presented

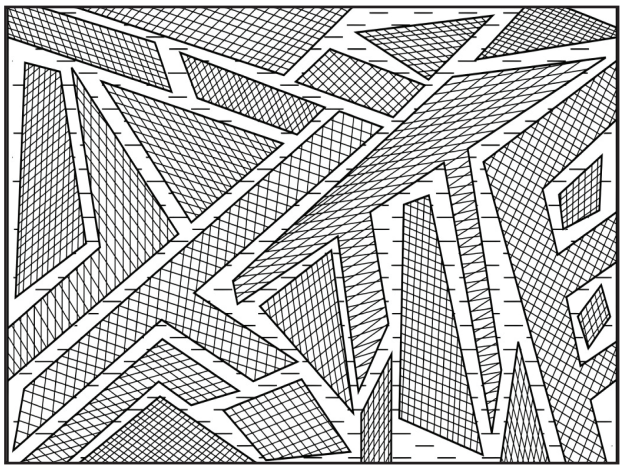


Fig. 2. Scheme of the structure of a cooled solution within temperatures range *Tm* > *T* > *Tg* and concentrations of cryoprotectant 10% < C < 30%

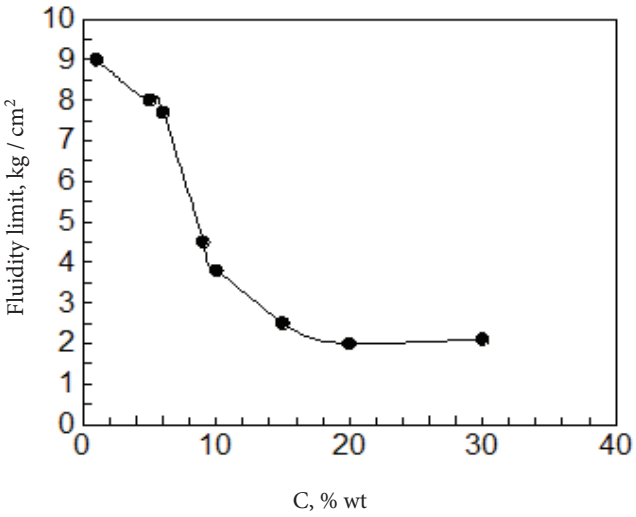
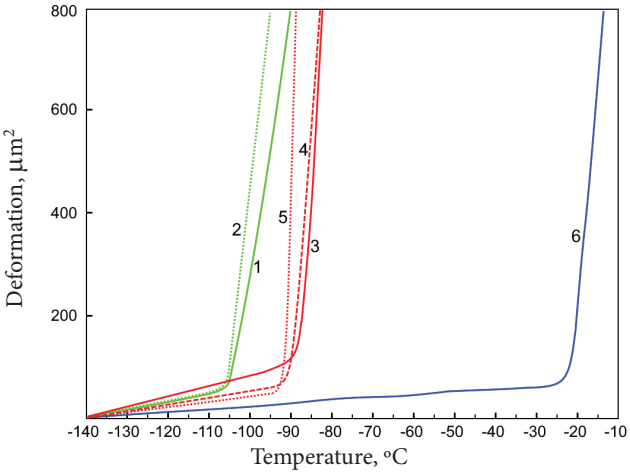


Fig. 3. Dependency of the fluidity limit of glycerol frozen aqueous solutions on cryoprotectant concentration

in Fig. 5. The findings indicate that the use of 20 % glycerol or 1,2-PD in combination with 0.1 % PVA 9 kDa did not lead to a significant increase in the preservation of thawed erythrocytes relative to 20 % glycerol and 1,2-PD solutions. In the case of using 0.5 % PVA 9 kDa with 20 % glycerol or 1,2-PD, the preservation of cells was much higher than in 20 % glycerol and 1,2-PD solutions, but not significantly. At the same time, the preservation of erythrocytes after freezing in solutions containing a combination of glycerol with 9 kDa PVA was higher than in a combination with 1,2-PD.

The use of 31 kDa PVA with 20 % glycerol or 1,2-PD, on the contrary, decreased the preservation rate of erythrocytes compared to control solutions (Fig. 6). Thus, with an increased concentra-



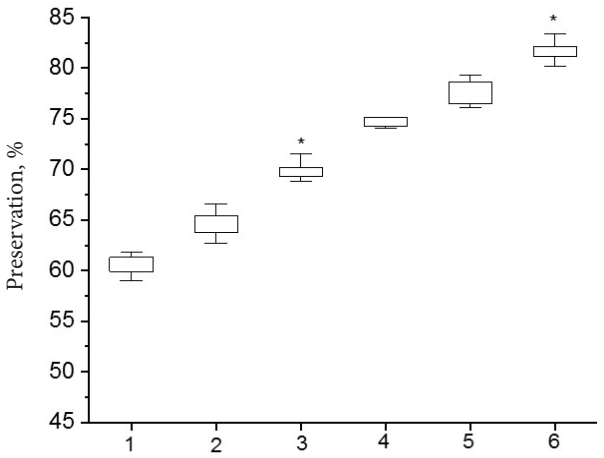


Fig. 4. Thermoplastic carves of frozen aqueous solutions: 1 — 20% glycerol; 2 — 20% glycerol + 0.5% PVA 9 kDa; 3 — 20% 1,2-PD; 4 — 20% 1,2-PD + 0.1% PVA 9 kDa; 5 — 20% 1,2-PD + 0.5% PVA 9 kDa; 6 — 2% PVA 9 kDa

Fig. 5. Preservation of human erythrocytes after cryo-preservation in solutions based on glycerol and 1,2-propanediol containing PVA 9 kDa. * — difference is sig-nificant compared to the control, $p < 0.01$

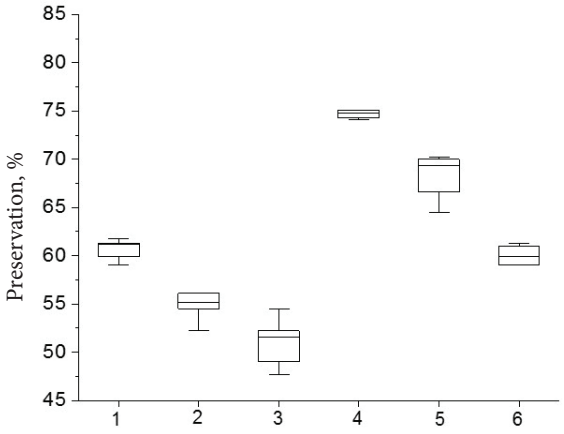


Fig. 6. Preservation of human erythrocytes after cryopreservation in solutions based on glycerol and 1,2-propanediol containing PVA 31 kDa. * — difference is significant compared to the control, $p < 0.01$

tion of 31 kDa PVA in all the studied cryoprotective media, a tendency to a reduced rate of cell preservation was noted. So, it was shown that combined cryoprotective solutions based on 20 % glycerol or 1,2-PD, which contained 31 kDa PVA, were ineffective for cryopreservation of erythrocytes. According to the results of a comparative assessment of the erythrocyte preservation index in the studied cryoprotective solutions, it was found that the media containing a combination of glycerol and 9 kDa PVA more effectively protected erythrocytes against cryodamage. As Fig. 5 demonstrates, the highest value of erythrocyte preservation was in the samples cryopreserved in 20 % glycerol with 0.5 % PVA 9 kDa.

The results of our studies showed that the use of PVA in combined cryoprotective media based on glycerol had a synergistic effect during cryopreservation of erythrocytes. At the same time, the cryoprotective effect of PVA was determined by its molecular weight and concentration in the cryoprotective medium. Thus, PVA 9 kDa, unlike PVA 31 kDa, had a more pronounced cryoprotective effect - the level of preserved erythrocytes after cryopreservation increased. The low cryoprotective effect of PVA 31 kDa can be explained by the fact that with an increase in the number of acetate groups, the ability of PVA to affect cell membranes increases, which can contribute to their deformation and damage to erythrocytes.

It should also be noted that glycerol-based cryoprotectants in combination with PVA oligomers exhibit better cryoprotective effects than 1,2-PD-based solutions. This is likely due to the fact that 1,2-PD is a less effective cryoprotectant for human erythrocytes during rapid cooling in liquid nitrogen [3].

CONCLUSIONS

Our findings expand the understanding of the features of using PVA in multicomponent cryoprotective media for freezing human erythrocytes. The main advantage of using PVA as a cryoprotectant in cryoprotective media may be its ability to initiate ice crystallization. Due to this

property of PVA, the probability of reducing the mass of intracellular ice increases, which may improve the safety of cryopreserved cells. To develop an effective cryoprotective medium, the PVA should be used only in combination with substances that have a low glass transition temperature Tg (–100… –150 °C) — glycerol or 1,2-propanediol. This will reduce possible mechanical damage, which is caused precisely by the influence of PVA on crystallization. Thus, the use of 0.5 % PVA with a molecular weight of 9 kDa in the composition of media based on 20 % glycerol or 1,2-propanediol led to an enhanced preservation of erythrocytes after cryopreservation compared to monosolutions of these cryoprotectants. The highest preservation rate of thawed erythrocytes was found for a medium containing 20 % glycerol and 0.5 % PVA 9 kDa.

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РОЛЬ ПОЛІВІНІЛОВОГО СПИРТУ В КРІОЗАХИСНИХ СЕРЕДОВИЩАХ:
ОЦІНКА ЕФЕКТИВНОСТІ ТА ОБМЕЖЕНЬ ПРИ ЗАМОРОЖУВАННІ ЕРИТРОЦИТІВ

У роботі обґрунтовано доцільність і визначені особливості використання полівінілового спирту у складі багатокомпонентних середовищ на основі проникаючих кріопротекторів під час швидкого охолодження еритроцитів людини. Рекомендовано використовувати полівініловий спирт тільки у складі багатокомпонентних середовищ на основі проникаючих кріопротекторів, які мають низьку температуру склування Tg = –100 … –150 °C (гліцерин, 1,2-пропандіол). Проведено порівняльну оцінку кріозахисної дії комбінованих середовищ, які містять полівініловий спирт різних молекулярних мас у поєднанні з 20% гліцерином або 1,2-пропандіолом, при заморожуванні еритроцитів людини. На основі результату збереженості еритроцитів встановлено, що найбільш оптимальну кріозахисну дію з усіх досліджених комбінованих середовищ має розчин, який містить 20%-й гліцерин та 0,5%-й полівініловий спирт з молекулярною масою 9 кДа.

***Key words*:** еритроцити людини, полівініловий спирт, проникаючі кріопротектори, температура склування.