Part 2. Biotechnology

UDC 619:591.111.1:57.086.13:612.014.43:636.7 **DOI 10.36016/JVMBBS-2024-10-4-3**

EVALUATION OF MECHANICAL STABILITY OF DOG ERYTHROCYTES UNDER THE INFLUENCE OF CRYOPROTECTANTS

Denysova O. M.

State Biotechnological University, Kharkiv, Ukraine, e-mail: denysova78@gmail.com

Summary. The mechanical stability of erythrocytes is a critical factor in ensuring their effective functioning during storage, transportation, and cryopreservation. The objective of this study was to ascertain the impact of diverse cryoprotectants, including glycerol, sucrose, dimethyl sulfoxide (DMSO), polyethylene glycol-1500 (PEG-1500), and hydroxyethyl starch (HES), on hemolytic damage to dog erythrocytes subjected to mechanical stress. For this purpose, dog erythrocytes were incubated in varying concentrations of cryoprotectants and NaCl. The cells were subjected to mechanical stress by stirring the suspension in a container filled with plastic beads at room temperature. The resulting hemolysis was evaluated spectrophotometrically. The results demonstrated that the most pronounced stabilization of erythrocyte membranes was observed during incubation with PEG-1500 and HES, while high glycerol concentrations caused membrane destabilization. Sucrose demonstrated a dual effect: at low concentrations, it exhibited protective properties for cellular membranes, while at higher concentrations, enhanced membrane vulnerability to stress. The results demonstrated that DMSO at all studied concentrations did not significantly change the mechanical stability of erythrocytes compared to the control group. Our findings indicate that an increase in salt concentration in the extracellular medium is associated with a reduction in the mechanical stability of dog erythrocytes. The effect of cryoprotectants on the mechanical stability of erythrocytes was found to be closely related to their physicochemical properties. This highlights the importance of precise selection of cryoprotectant concentrations to improve the results of red blood cell storage and transportation. The conclusions of this study are important for further optimization of technologies for the long-term storage of canine erythrocytes, in particular in cryobanks

Keywords: mechanical stress, cryopreservation

Introduction. The mechanical stability of erythrocytes is an important parameter that determines their ability to withstand physical and osmotic stress during storage, transportation, and cryopreservation (Baskurt and Meiselman, 2013; Ugurel et al., 2017). Erythrocytes, whose main function is to transport oxygen throughout the body, must have high mechanical stability to ensure this function even after prolonged storage or freezing (Tarasev, Chakraborty and Alfano, 2015). Erythrocyte cryopreservation is one of the most effective methods of long-term blood storage, but it is accompanied by numerous negative factors: crystal formation during the transition of liquid to solid state, increase in salt concentration and osmotic pressure in the cooled liquid, dehydration of macromolecules, changes in the location and composition of membrane lipids (Gao and Critser, 2000), increased lipid peroxidation due to a decrease in superoxide dismutase activity (Alvarez and Storey, 1992), and ionic and electrical effects associated with the integration of ions into ice crystals. Osmotic stress caused by changes in the concentration of ions in the medium during the crystallization and thawing of water during cryopreservation is one of the factors that affect the mechanical stability of erythrocytes. During cryopreservation, cells are exposed to extreme temperatures, which leads to the formation of ice crystals and dehydration (Gao and Critser, 2000). To prevent these processes, penetrating and non-penetrating cryoprotectants are used to protect cells from intracellular ice formation and minimize osmotic stress (Elliott, Wang and Fuller, 2017). However, the choice of the optimal concentration of cryoprotectants is critical, as excessive concentrations can cause additional osmotic stress and damage the cell membrane. In addition, during cryopreservation, the volume of cells changes, which leads to mechanical stress that can affect their structure and function. This can be caused by the formation of extracellular ice crystals or by the interaction of cells with each other and the container walls (Ishiguro and Rubinsky, 1994; Saragusty et al., 2009). Thus, the choice of the right cryopreservation conditions, including the concentration of cryoprotectants and NaCl, is key to ensuring high mechanical stability of erythrocytes after long-term low-temperature cell storage. The optimal concentrations of these substances allow to preserve the integrity of the cell membrane, reduce the level of hemolysis, and maintain the functionality of erythrocytes during a long storage period.

This study **aimed** to evaluate the effect of cryoprotectants such as glycerol, sucrose, dimethyl sulfoxide (DMSO), polyethylene glycol-1500 (PEG-1500), and hydroxyethyl starch m. m. 200 (HES), on the development of hemolytic damage to erythrocytes under mechanical stress.

Materials and methods. The object of the study was dog erythrocytes. All animals were clinically healthy, sexually mature males of an unspecified breed, aged 2 to 10 years. Manipulations with animals were performed by veterinarians following the recommendations of the 'European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes' (CE, 1986) and Council Directive 2010/63/EU (CEC, 2010), and in accordance with Art. 26 of the Law of Ukraine No. 3447-IV of 21.02.2006 'About protection of animals from cruel treatment' (VRU, 2006) and basic bioethical principles (Simmonds, 2017).

Blood was taken by venipuncture from the brachial vein following current ethical standards. No animals were harmed during the experiment.

The blood was collected in a glucose-citrate preservative and stored at 5°C for no more than 48 h before the experiments commenced. Red blood cells were isolated by centrifuging the whole blood at 750 g for five minutes, after which the plasma and leuko-platelet layer were removed. The red blood cells were then washed three times in a solution of four times the volume of isotonic saline (150 mM NaCl, 10 mM phosphate buffer, pH 7.4).

The resistance of erythrocytes to mechanical stress was assessed by the degree of hemolysis caused by the action of small beads moving in suspension (Shpakova, Orlova and Alexandrova, 2010). The washed erythrocytes (1 ml) were mixed with cryoprotectant solutions (5 ml) in 20 ml plastic cups, with a final hematocrit of approximately 15%. Solutions of glycerol, sucrose, DMSO, PEG-1500, and HES m. m. 200 were prepared based on isotonic saline at 5–20% concentrations. Then 50 plastic beads (5 mm in diameter, 1.5 g) and a magnetic stirrer were carefully added. The erythrocytes and plastic beads suspension was stirred on a magnetic stirrer MM-5 at 1,200 rpm. To evaluate hemolytic cell damage, aliquots of the erythrocyte suspension were taken, centrifuged at 1,200 g, and the supernatant was collected. Hemolysis was determined by the spectrophotometric method (device SF-46 LOMO, Russia) in a flow-through cuvette at $\lambda = 543$ nm to determine the amount of hemoglobin released from damaged cells. The level of hemolysis was expressed as a percentage relative to 100% hemolysis of erythrocytes in the presence of 0.1% Triton X-100 detergent.

Statistical results were processed using the Statgraphics software package (Manugistic Inc.; STATistical GRAPHICs system, USA). The data were presented in the format M ± SE (mean ± standard error). Differences between the experimental and control groups were evaluated using a nonparametric method, in particular, Fisher's multiple range test using the procedure of grouping samples by the least significant difference. Each series of experiments was performed at least six times.

Results and discussion. Hemolytic damage to dog erythrocytes under the influence of mechanical stress was studied for 60 min in the presence of various cryoprotectants (Fig. 1). The cryoprotectant solutions, balanced by ionic strength and pH to physiological values, had different effects on the stability of dog erythrocyte membranes. The greatest destabilization was observed when using glycerol, except for its 5% concentration, and sucrose at 20% concentration.

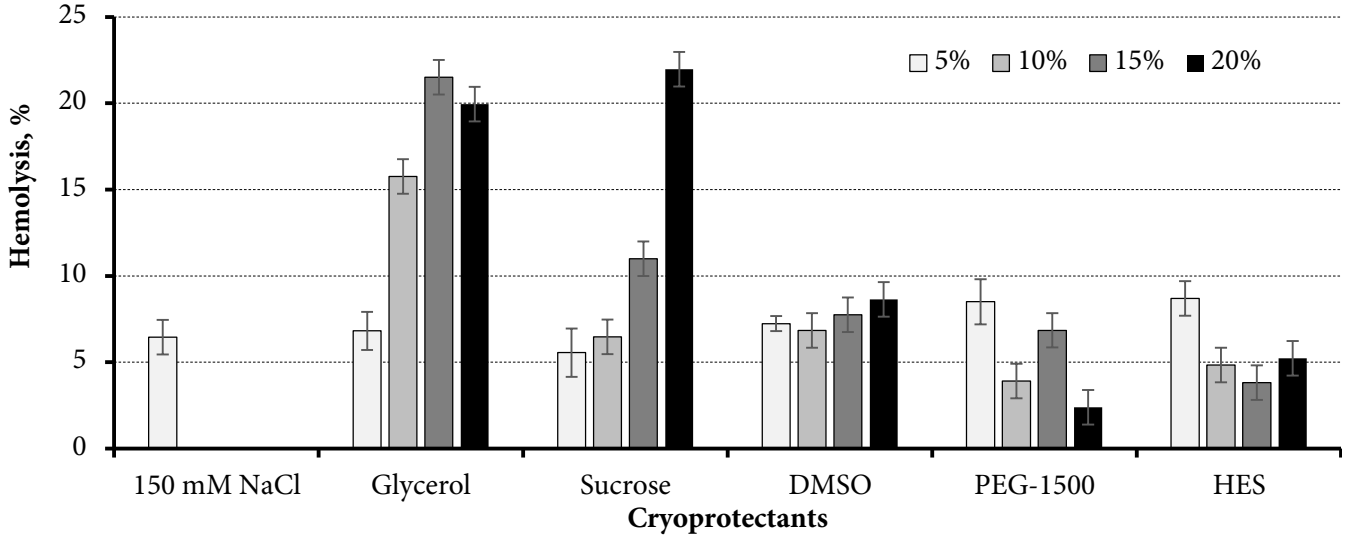

Figure 1. Hemolytic damage of dog erythrocytes under mechanical stress for 60 min in the presence of 5–20% cryoprotectants. * — decrease in hemolysis compared to control (150 mM NaCl, 10 mM phosphate buffer, pH 7.4). $P < 0.05$.

Other cryoprotectants, such as DMSO, PEG-1500, and HES, had variable effects on the mechanical stability of erythrocytes, depending on the concentration. PEG-1500 at concentrations of 10% and 20%, as well as HES at concentrations of 10%, 15%, and 20% demonstrated the ability to stabilize cells during mechanical stress. In contrast, DMSO at all studied concentrations did not demonstrate significant differences from the control group (150 mM NaCl, 10 mM phosphate buffer, pH 7.4).

The mechanical stability of erythrocytes is significantly affected by the concentration of NaCl in the medium. In a hypertonic medium, for instance, at concentrations of 400 mM or 600 mM NaCl, cells undergo dehydration, which reduces their volume and increases the risk of mechanical damage to the membrane. In contrast, a hypotonic medium, where the NaCl concentration is much lower than physiological, can result in cell swelling and, ultimately, hemolysis. Studying the mechanical stability of erythrocytes in different concentrations of cryoprotectants and NaCl allows us to gain a deeper understanding of how these factors interact with each other and affect cell integrity. For instance, the use of an optimal cryoprotectant concentration can minimize the adverse effects of osmotic stress caused by high NaCl concentration when the temperature is lowered, while an inadequate cryoprotectant concentration can exacerbate membrane damage due to osmotic stress.

Increasing the NaCl concentration to 400 mM resulted in a 6-fold increase in hemolytic damage after 30 min and a 7-fold increase after 60 min compared to the control (Fig. 2). At a NaCl concentration of 600 mM, these values increased to 8 and 9 times, respectively.

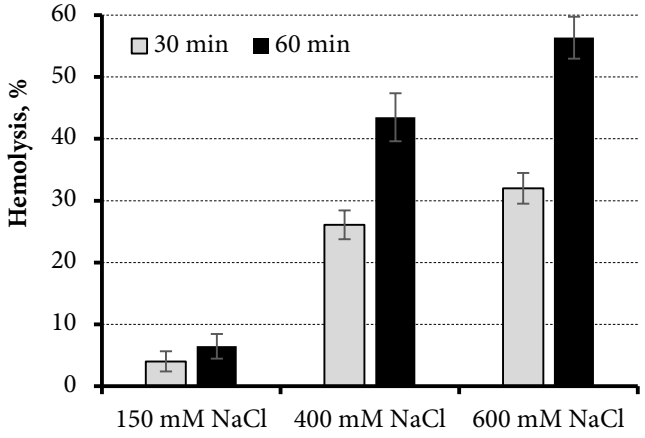

Figure 2. Hemolytic damage of dog erythrocytes under mechanical stress in NaCl solutions of different concentrations. $P < 0.05$.

The effect of cryoprotectants on the mechanical stability of dog erythrocytes during osmotic stress is closely related to their physicochemical properties and mechanism of action.

The results of the study revealed that glycerol, known for its ability to stabilize human erythrocyte membranes under mechanical stress (Zemlianskykh, 2018), destabilizes dog erythrocyte membranes under mechanical stress. These differences may be related to the different structures of membrane lipids and proteins in these two species. In particular, dog erythrocyte membranes have a higher content of cholesterol and saturated phospholipids, which increases membrane stiffness and reduces its ability to adapt during stress (Zhegunov and Denisova, 2010). It has also been noted (Shpakova et al., 2015) that the higher the content of phosphatidylethanolamine (PEA), the less sensitive the cells are to mechanical stress. The percentage of PEA in human erythrocytes is 27.2% of the total phospholipid content, while in dog erythrocytes it is 22.4%. At the same time, glycerol can cause an increase in osmotic pressure within the cell, which, when combined with a stiffer membrane, leads to an elevated susceptibility to hemolysis. Furthermore, the protein composition of canine erythrocyte membranes differs from that of human erythrocytes (Zemlyanskikh and Denisova, 2009). Dog erythrocyte membranes contain higher levels of spectrin and ankyrin, proteins that support the membrane skeleton. In conditions of elevated osmotic pressure, these proteins can activate mechanisms that lead to a decrease in membrane flexibility, contributing to mechanical damage during stress.

Sucrose demonstrated a twofold effect. At high concentrations (15% and 20%), it increased the sensitivity to mechanical stress, which led to membrane damage. At low concentrations (5% and 10%), however, this cryoprotectant helped to maintain membrane stability. Based on the results of this study, sucrose can be used as a cryopreservative component in low concentrations, as it contributes to the preservation of membrane stability. This makes it a promising addition to cryoprotective solutions where minimizing cell damage during cryopreservation is a priority.

PEG-1500 and HES have been shown to effectively reduce cell sensitivity to mechanical stress. This is due to their ability to form a protective barrier around the cell membrane, which reduces osmotic pressure and increases resistance to mechanical damage (Elliott, Wang and Fuller, 2017). High concentrations of these substances have been shown to have a positive effect on the mechanical stability of cells, which highlights their potential as effective cryoprotectants in the context of mechanical stress. Research (Kameneva et al., 2003) indicates that PEG can alter the physicochemical properties of surfaces through adsorption on beads, enhancing biocompatibility, and through adsorption on cells, reducing membrane sensitivity to mechanical stress. However, this does not fully explain the effectiveness of HES in stabilizing dog erythrocytes under mechanical stress.

In contrast to non-penetrating cryoprotectants such as PEG-1500 and HES, which previous studies (Denysova and Zhegunov, 2021; Zhegunov, Denysova and Zhegunova, 2022) have shown to cause membrane damage after freezing and thawing, this study did not reveal any significant changes in the mechanical stability of erythrocytes when using DMSO. The results demonstrated the effectiveness of this cryoprotectant in maintaining the physicochemical properties of membranes during cryopreservation.

The mechanical stability of erythrocytes was found to be significantly affected by the concentration of NaCl. High concentrations of NaCl, such as 400 mM and 600 mM, were observed to significantly increase hemolytic damage, indicating an increase in the

membranes sensitivity to osmotic stress. This effect can be explained by an increase in osmotic pressure, which leads to cell dehydration and increased mechanical stress on the membrane (Gao and Critser, 2000).

Conclusions. The studied cryoprotectants have a significant effect on the mechanical stability of dog erythrocytes. Stabilization of cell membranes was observed during incubation with PEG-1500 and HES, while glycerol in high concentrations destabilized the membranes. Sucrose showed a dual effect: low concentrations provided protection, while high concentrations increased sensitivity to mechanical stress. Unlike other cryoprotectants, the use of DMSO in all concentrations studied did not lead to significant differences in the mechanical stability of erythrocytes compared to the control group. This indicates the potential safety and efficacy of DMSO as a cryoprotectant for preserving the mechanical stability of erythrocytes after cryopreservation. Changes in the mechanical stability of erythrocytes under the influence of cryoprotectants may be related to their effect on the physicochemical properties of cell membranes, which is important for optimizing the conditions of storage and transportation of erythrocytes.

References

Alvarez, J. G. and Storey, B. T. (1992) 'Evidence for increased lipid peroxidative damage and loss of superoxide dismutase activity as a mode of sublethal cryodamage to human sperm during cryopreservation', *Journal of Andrology*, 13(3), pp. 232–241. doi: 10.1002/j.1939-4640.1992.tb00306.x.

Baskurt, O. K. and Meiselman, H. J. (2013) 'Red blood cell mechanical stability test', *Clinical Hemorheology and Microcirculation*, 55(1), pp. 55–62. doi: 10.3233/CH-131689.

CE (The Council of Europe). (1986) *European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes*. (European Treaty Series, No. 123). Strasbourg: The Council of Europe. Available at: https://conventions.coe.int/treaty/en/treaties/html/123.htm.

CEC (The Council of the European Communities) (2010) 'Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes', *The Official Journal of the European Communities*, L 276, pp. 33–79. Available at: http://data.europa.eu/eli/dir/2010/63/oj.

Denysova, O. and Zhegunov, G. (2021) 'Cryopreservation of canine erythrocytes using dimethyl sulfoxide, polyethylene glycol and sucrose', *Problems of Cryobiology and Cryomedicine*, 31(1), pp. 38–50. doi: 10.15407/cryo31.01.038.

Elliott, G. D., Wang, S. and Fuller, B. J. (2017) 'Cryoprotectants: A review of the actions and applications of cryoprotective solutes that modulate cell recovery from ultra-low temperatures', *Cryobiology*, 76, pp. 74–91. doi: 10.1016/j.cryobiol.2017.04.004.

Gao, D. and Critser, J. K. (2000) 'Mechanisms of cryoinjury in living cells', *ILAR Journal*, 41(4), pp. 187–196. doi: 10.1093/ilar.41.4.187.

Ishiguro, H. and Rubinsky, B. (1994) 'Mechanical interactions between ice crystals and red blood cells during directional solidification', *Cryobiology*, 31(5), pp. 483–500. doi: 10.1006/cryo.1994.1059.

Kameneva, M. V., Repko, B. M., Krasik, E. F., Perricelli, B. C. and Borovetz, H. S. (2003) 'Polyethylene glycol additives reduce hemolysis in red blood cell suspensions exposed to mechanical stress', *ASAIO Journal*, 49(5), pp. 537–542. doi: 10.1097/01.MAT.0000084176.30221.CF.

Saragusty, J., Gacitua, H., Rozenboim, I. and Arav, A. (2009) 'Do physical forces contribute to cryodamage?', *Biotechnology and Bioengineering*, 104(4), pp. 719–728. doi: 10.1002/bit.22435.

Shpakova, N. M., Orlova, N. V. and Alexandrova, D. I. (2010) *Method for Destruction of Erythrocytes [Sposib destruktsii erytrotsytiv]*. Patent no. UA 52701. Available at: https://sis.nipo.gov.ua/uk/search/detail/257082. [in Ukrainian].

Shpakova, N. M., Orlova, N. V., Nipot, E. E. and Aleksandrova, D. I. (2015) 'Comparative study of mechanical stress effect on human and animal erythrocytes' [Porivnialne vyvchennia dii mekhanichnoho stresu na erytrotsyty liudyny i tvaryn], *Fiziologichnyi Zhurnal*, 61(3), pp. 75–80. doi: 10.15407/fz61.03.075. [in Ukrainian].

Simmonds, R. C. (2017) 'Chapter 4. Bioethics and animal use in programs of research, teaching, and testing', in Weichbrod, R. H., Thompson, G. A. and Norton, J. N. (eds.) *Management of Animal Care and Use Programs in Research, Education, and Testing*. 2nd ed. Boca Raton: CRC Press, pp. 35–62. doi: 10.1201/9781315152189-4.

Tarasev, M., Chakraborty, S. and Alfano, K. (2015) 'RBC mechanical fragility as a direct blood quality metric to supplement storage time', *Military Medicine*, 180(3S), pp. 150–157. doi: 10.7205/MILMED-D-14-00404.

Ugurel, E., Kucuksumer, Z., Eglenen, B. and Yalcin, O. (2017) 'Blood storage alters mechanical stress responses of erythrocytes', *Clinical Hemorheology and Microcirculation*, 66(2), pp. 143–155. doi: 10.3233/CH-160219.

VRU (Verkhovna Rada Ukrainy) (2006) 'Law of Ukraine No. 3447-IV of 21.02.2006 'About protection of animals from cruel treatment' [Zakon Ukrainy № 3447-IV vid 21.02.2006 'Pro zakhyst tvaryn vid zhorstokoho povodzhennia'], *News of the Verkhovna Rada of Ukraine [Vidomosti Verkhovnoi Rady Ukrainy]*, 27, art. 230. Available at: https://zakon.rada.gov.ua/laws/3447-15. [in Ukrainian].

Zemlianskykh, N. G. (2018) 'The effects of cryoprotective substances on the mechanical stability and geometric parameters of human erythrocytes', *Biophysics*, 63(1), pp. 66–76. doi: 10.1134/S0006350918010219.

Zemlyanskikh, N. G. and Denisova, O. N. (2009) 'Changes in the erythrocyte membrane-cytoskeleton complex induced by dimethyl sulfoxide, polyethylene glycol, and low temperature', *Biophysics*, 54(4), pp. 490–496. doi: 10.1134/S0006350909040162.

Zhegunov, G. F. and Denisova, O. N. (2010) 'Permeability of mammalian erythrocytes for the molecules of glycerol and DMSO and the level of cellular viability after freezing-thawing' [Pronitsaemost' eritrotsitov mlekopitayushchikh dlya molekul glitserina i DMSO i stepen' sokhrannosti kletok posle zamorazhivaniya-ottaivaniya], *Reports of the National Academy of Sciences of Ukraine [Dopovidi Natsionalnoi akademii nauk Ukrainy]*, 12, pp. 139–143. Available at: http://dspace.nbuv.gov.ua/handle/123456789/31118. [in Russian].

Zhegunov, G., Denysova, O. and Zhegunova, G. (2022) 'Blood hypothermic storage and erythrocyte cryopreservation in dogs', *Problems of Cryobiology and Cryomedicine*, 32(4), pp. 245–255. doi: 10.15407/cryo32.04.245.