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FREEZING REGIMEN AFFECTS PRESERVATION OF CANINE ERYTHROCYTES WHEN USING DIMETHYL SULFOXIDE

This study examined how dimethyl sulfoxide (DMSO) concentration and different freezing regimens influence the preservation of canine erythrocytes during cryopreservation. The degree of erythrocyte hemolysis was assessed after thawing and at each stage of cryoprotectant removal. Increasing the DMSO concentration from 7.5 to 10% reduced hemolysis immediately after freeze–thawing by more than twofold. During cryoprotectant removal, the extent of erythrocyte damage did not differ significantly between samples frozen with 7.5 and 10% DMSO. Overall hemolysis after freeze–thawing varied markedly depending on the cooling protocol. The highest level of cell damage (73%) occurred when samples were frozen in nitrogen vapor followed by immersion in liquid nitrogen. Direct immersion of the samples into liquid nitrogen reduced hemolysis to 37%. The most effective protocol involved briefly immersing the bottom of the cryotube in liquid nitrogen prior to complete freezing, resulting in only 31% hemolysis. This approach initiates crystallization at the lower part of the cryotube, substantially reducing the risk of mechanical cell damage during ice structure formation.

Key words: *cryopreservation, cryoprotectant, canine erythrocytes, dimethyl sulfoxide, freezing regimens.*

Cryopreservation of animal erythrocytes plays a key role in the development of veterinary transfusion medicine, particularly under conditions of acute donor blood shortage [18]. The demand for high-quality cryopreserved blood components continues to grow due to the increasing number of surgical interventions, traumatic injuries, and cases of severe anemia in animals [6, 18]. Despite significant progress in the cryopreservation of human blood components [15], the preservation of animal erythrocytes for veterinary use remains insufficiently studied [2, 6, 7, 18], and existing methods still have notable limitations and require substantial improvement.

The development of effective cryopreservation techniques for animal erythrocytes is one of the priority directions in modern veterinary medicine, aimed at improving transfusion support and reducing mortality associated with severe trauma and acute blood loss [5]. During wartime, this issue becomes especially urgent in the context of maintaining the viability of service dogs involved in high-risk operations, including demining, search-and-rescue missions, and patrol activities.

The effectiveness of cell cryopreservation depends on the choice of cryoprotective agent and the freezing protocol used [19]. Several studies have attempted to develop methods for preserving canine erythrocytes in the frozen state using two types of cryoprotectants: permeating agents, which can cross the cell membrane and protect intracellular structures from freezing-induced damage,

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and non-permeating agents, which act primarily in the extracellular environment [1, 2, 8—10, 12, 17, 18].

The use of glycerol — highly effective for cryopreserving human erythrocytes [14] — proved to be of limited value in dogs: hemolysis levels reached 75—99 % [4, 12], likely due to the low permeability of canine erythrocyte membranes to this cryoprotectant [11]. This indicates that glycerol is unsuitable for the cryopreservation of canine erythrocytes at −196 °C.

Dimethyl sulfoxide (DMSO) is considered the most promising permeating cryoprotectant for the low-temperature preservation of animal erythrocytes, including those of dogs [6], due to its high membrane permeability [11]. However, existing data on the effectiveness of this cryoprotectant remain inconsistent. For example, in the study by D. Pogozhykh *et al*. [12], the use of 10% DMSO (with a 20-minute pre-incubation) resulted in a hemolysis level of (42.41 ± 1.06) %, which is insufficient for practical application. Other authors have reported that the hemolysis level of erythrocytes from various mammalian species (cattle, rabbit, horse, dog) frozen in a medium containing 10% DMSO ranged from 15 to 23% but increased to 44—47% after cryoprotectant removal [13, 17].

In all these studies, the duration of cell incubation with the cryoprotectant prior to cryopreservation did not exceed 15—20 minutes, which corresponds to the physicochemical properties of DMSO and its high rate of membrane penetration. Extending the incubation time of erythrocytes with DMSO to 30 minutes, as described by D. Pogozhykh *et al*. [12], lacks sufficient theoretical justification. Their reported results—particularly the hemolysis level of canine erythrocytes (26.3 ± 1.12) % after freeze–thawing — appear atypical compared with existing literature and require additional confirmation or a more detailed explanation of the mechanisms that could account for the observed effects.

Among non-permeating cryoprotectants, polyethylene oxide with a molecular weight of 1500 (PEO-1500) has demonstrated high effectiveness in preserving the integrity of canine erythrocytes after thawing. However, its use is limited by substantial cell damage occurring during the washing stage of cryoprotectant removal [4]. Attempts to combine permeating (DMSO) and non-permeating (hydroxyethyl starch, PEO-1500) agents have not yielded consistently positive results [6, 12]. At the same time, combining DMSO with sucrose has shown a clear tendency toward enhanced cryoprotective action compared with the use of DMSO alone [6]. However, these studies were fragmentary and lacked systematic investigation.

Thus, the problem of cryopreserving canine erythrocytes remains unresolved, as no existing method provides sufficient cell preservation for clinical use in veterinary transfusion practice. Addressing this challenge requires the identification of effective and safe cryoprotectants, as well as the optimization of freezing and thawing protocols to preserve cell viability and functional properties. These efforts will create the prerequisites for long-term storage of blood components, the establishment of animal blood banks, and the development of veterinary donor systems — an especially important goal in situations where access to fresh donor blood is limited and emergency transfusions are required.

The aim of this study was to investigate the effect of dimethyl sulfoxide concentration and different freezing regimens on the preservation of canine erythrocytes during cryopreservation.

MATERIALS AND METHODS

Erythrocytes were obtained from blood collected from clinically healthy, sexually mature male dogs (*Canis lupus familiaris*) using a glucose–citrate anticoagulant. Blood collection and all experimental procedures were carried out in accordance with national and international bioethical standards, including the General Principles of Animal Experiments (Ist National Congress on Bioethics, Kyiv, 2001) and the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (Strasbourg, 1986).

Erythrocytes were isolated using a standard procedure. After plasma removal, the erythrocyte mass was washed three times by 3-minute centrifugation at 1500 g in a tenfold volume of physiological saline (0.15 mol/L NaCl, 0.01 mol/L phosphate buffer, pH 7.4). The leukocyte layer and supernatant were removed by aspiration. All solutions were prepared using 0.01 mol/L phosphate buffer (pH 7.4).

DMSO was added dropwise to the erythrocytes at a 1 : 1 ratio, followed by incubation at room temperature for 20 min. The final cryoprotectant concentration was 7.5 or 10%. Cryopreservation was performed using 2 mL or 10 mL cryovials. Samples in 2 mL cryovials were frozen by direct immersion in liquid nitrogen (−196 °C). Samples

in 10 mL cryovials were frozen using three different regimens:

Regimen 1: freezing in liquid nitrogen vapor for 15 min followed by immersion in liquid nitrogen;

Regimen 2: direct immersion in liquid nitrogen (–196 °C);

Regimen 3: pre-immersion of the lower part of the cryovial (approximately 2 mm) in liquid nitrogen for 20 s, followed by complete immersion.

Cells were thawed in a water bath at 38—40 °C until the complete disappearance of the solid phase. The cryoprotectant was removed by sequential centrifugation. At the first stage, an equal volume (1 : 1) of a solution containing 0.6 mol/L NaCl was added to the erythrocytes. The cells were then washed twice with physiological saline.

The condition of the erythrocytes after cryopreservation was assessed by measuring the level of hemolysis. Hemoglobin content was determined spectrophotometrically at 543 nm.

Hemolysis was evaluated immediately after thawing and at each of the three stages of cryoprotectant removal. For each sample, an individual 100% hemolysis value was established, corresponding to the optical density measured after adding 0.1% Triton X-100. The hemolysis level at each stage was expressed as a percentage of the total lysis of erythrocytes subjected to the freeze–thaw cycle, considering the hemolysis that occurred at the preceding stages. The integral hemolysis index reflected the cumulative cell loss during thawing and washing. Adjusted hemolysis values at each stage, accounting for prior hemolysis, as well as the integral hemolysis level, are presented in Tables 1 and 2.

Statistical analysis was performed using analysis of variance (ANOVA) and the Mann–Whitney

Table 1. **Hemolysis level of canine erythrocytes after freezing and washing using 7.5 and 10% DMSO (sample volume 2 mL); Me, Q1—Q3, *n* = 6**

Hemolysis of erythrocytes after the corresponding stage, %	Concentration of DMSO, %	
	7.5	10
Hemolysis of erythrocytes after freeze-thawing	32 (28—38)	12 (9—14) *
Hemolysis of erythrocytes after the first stage of washing	7 (5—8)	4 (4—6)
Hemolysis of erythrocytes after the second stage of washing	10 (8—11)	9 (7—10)
Hemolysis of erythrocytes after the third stage of washing	5 (4—6)	4 (2—4)
Total hemolysis of erythrocytes	54 (47—61)	28 (23—29) *

* Significant difference relative to 7.5% DMSO, $p < 0.05$.

Table 2. **Hemolysis level of canine erythrocytes after freezing and washing using different freezing regimens (sample volume 10 mL); Me, Q1—Q3, *n* = 6**

Hemolysis of erythrocytes after the corresponding stage, %	Freezing regimens		
	1	2	3
	In nitrogen vapor followed by immersion in liquid nitrogen	Immersion in liquid nitrogen	Pre-immersion of the lower part of the cryovial in liquid nitrogen, followed by complete immersion
Hemolysis of erythrocytes after freezing-thawing	60 (47—61)	22 (20—24) *	17 (16—17) #
Hemolysis of erythrocytes after the first stage of washing	5 (4—6)	5 (3—6)	4 (3—4)
Hemolysis of erythrocytes after the second stage of washing	6 (5—7)	7 (6—8)	6 (5—8)
Hemolysis of erythrocytes after the third stage of washing	3 (2—3)	4 (3—5)	4 (3—4)
Total hemolysis of erythrocytes	73 (61—73)	37 (35—42) *	31 (30—32) #

* Significant difference relative to regimen 1, $p < 0.05$; # significant difference relative to regimens 1 and 2, $p < 0.05$.

U test. Differences between groups were considered significant at $p < 0.05$.

RESULTS AND DISCUSSION

Cryoprotective agents exert different effects on cells, and due to the limited understanding of their mechanisms of action, the optimal choice of cryoprotectant is, in most cases, determined empirically. It is important not only to select an effective cryoprotectant but also to determine its appropriate concentration: excessive amounts may be toxic or induce osmotic stress. The addition of a permeating cryoprotectant causes rapid water efflux and a decrease in cell volume, which is subsequently restored once the cryoprotectant enters the cell. Water moves across membranes much faster than cryoprotectant molecules, which explains this dynamic behaviour.

The permeability coefficients of water and cryoprotectants determine the optimal exposure time of cells in cryoprotective media and form the basis for selecting appropriate cooling and warming rates during freezing and thawing. The permeability of human and canine erythrocyte membranes to water does not differ significantly [3], whereas substantial differences have been reported for cryoprotectants: canine erythrocytes exhibit lower permeability to glycerol [16]. G.F. Zhegunov *et al.* demonstrated [16] that the permeability coefficient of DMSO across mammalian erythrocyte membranes is an order of magnitude higher than that of glycerol, which accounts for the improved preservation of cells cryopreserved with DMSO. Considering this, DMSO was used in the present study, as several authors have reported its effectiveness for cryopreserving erythrocytes of various animal species [6, 13, 17]. It was applied at concentrations of 10 and 7.5%, and the cells were frozen to −196 °C by immersion in liquid nitrogen.

The level of erythrocyte hemolysis was assessed after thawing and at each stage of cryoprotectant removal. This approach makes it possible to evaluate the contribution of each stage to the overall damage to erythrocytes during cryopreservation.

As shown in Table 1, the hemolysis level of erythrocytes immediately after the freeze–thaw cycle differed markedly between the two DMSO concentrations: 32 at 7.5% DMSO and 12 at 10% DMSO. This indicates more than a twofold reduction in cell damage at this stage when the cryoprotectant concentration is increased.

The subsequent step — returning erythrocytes to a physiological medium after freeze–thawing in the presence of a permeating cryoprotectant — is critically important. The rapid influx of water into thawed cells causes a sharp increase in cell volume, and when the critical hemolytic threshold is exceeded, membrane damage occurs, leading to erythrocyte hemolysis. To prevent this effect, the intracellular concentration of the cryoprotectant must be reduced gradually by sequential washing in saline solutions (see *Materials and Methods*). As shown in Table 1, during cryoprotectant removal the extent of damage did not differ significantly between erythrocytes frozen with 7.5 and 10% DMSO: 4—7% at the first washing step, approximately 10% at the second, and about 5% at the third.

The total erythrocyte hemolysis level — which accounts for cell damage after thawing and during subsequent cryoprotectant removal — was 54% when erythrocytes were cryopreserved with 7.5% DMSO. Increasing the cryoprotectant concentration to 10% reduced total cell damage to 28%. Thus, the results demonstrate that using 10% DMSO for the cryopreservation of canine erythrocytes is the most appropriate approach, as it ensures minimal hemolysis after the freeze–thaw cycle and maintains stability of this parameter during cryoprotectant removal.

Based on these results, 10% DMSO was selected for subsequent erythrocyte cryopreservation experiments because it provided the best cell preservation.

The next stage of the study was to examine the effect of different cooling regimens on the preservation of canine erythrocytes. For this experiment, a DMSO concentration of 10% was selected, and three freezing protocols were applied. After incubation with the cryoprotectant, samples in 10 mL plastic cryovials were frozen using the three regimens described above. The results are presented in Table 2.

It was found that the overall hemolysis level after freeze–thawing varied substantially depending on the cooling regimen used. The greatest cell damage occurred when samples were frozen in nitrogen vapor followed by immersion in liquid nitrogen, with hemolysis reaching 73%. Freezing by direct immersion of the sample into liquid nitrogen significantly reduced cell damage, lowering hemolysis to 37%. The higher hemolysis level compared with Table 1 is attributable to changes in cooling rate resulting

from the increased sample volume. The most effective protocol was the freezing regimen involving pre-immersion of the lower part of the cryovial (approximately 2 mm) in liquid nitrogen for 20 s prior to complete immersion. This approach likely promoted crystallization in the lower part of the cryovial, substantially reducing the risk of mechanical cell damage during ice crystal formation. Using this regimen reduced overall erythrocyte hemolysis to 31%.

The greatest differences in erythrocyte hemolysis were observed after the freeze–thaw stage. When samples were frozen in nitrogen vapor followed by immersion in liquid nitrogen, hemolysis reached 60%. Direct immersion in liquid nitrogen reduced hemolysis to 22%, while freezing with crystallization initiation followed by immersion resulted in only 17% hemolysis. After the washing procedure, which included three steps of cryoprotectant removal, hemolysis levels ranged from 11 to 16%, regardless of the freezing method used. This indicates that the washing procedure itself did not substantially contribute to overall cell damage.

Thus, the extent of canine erythrocyte injury during cryopreservation depends strongly on the rate and pattern of cooling. The low cooling rate characteristic of freezing in nitrogen vapor leads to significant cell damage. Increasing the cooling rate by direct immersion in liquid nitrogen improves erythrocyte survival preservation; however, this method still does not provide a sufficiently high level of cell viability for practical veterinary transfusion purposes.

The major sources of damage to biological systems during cryopreservation are associated with the water–ice phase transition and subsequent rearrangements of the ice matrix. According to Mazur's theory, which explains the mechanisms of such injuries and their dependence on cooling rate, two key factors are involved: osmotic and mechanical ones [19].

During slow cooling, osmotic stress is the dominant factor causing cellular injury. Ice formation occurs primarily in the extracellular medium, leading to a sharp increase in solute concentration in the remaining liquid phase. This creates an osmotic gradient that drives water out of the cell, resulting in progressive dehydration. The cell shrinks, the cytoplasm becomes dense and viscous, and the membranes undergo substantial deformation. In contrast, during rapid cooling, water does not have enough time to leave the cell, and ice formation may occur simultaneously in both the extra- and intracellular compartments. Intracellular ice crystals exert mechanical stress on cellular structures, disrupting membrane integrity and causing physical destruction of the cell. Under these conditions, mechanical injury becomes the predominant damaging factor. Permeating cryoprotectants can reduce osmotic stress and prevent intracellular ice formation, thereby markedly improving the post-thaw survival of biological systems.

Improvement of the cooling protocol in this study was achieved by freezing canine erythrocytes with prior initiation of ice nucleation in the lower part of the cryovial. This approach ensures controlled formation of the primary crystallization center, promoting directed ice growth and substantially reducing mechanical damage to cell membranes during sample cooling and initial ice matrix formation. The use of this regimen resulted in a low level of erythrocyte hemolysis, indicating its high effectiveness through minimization of cryoinjury.

A priority direction for further research is a comprehensive assessment of the functional state of thawed erythrocytes, including their oxygen-carrying capacity, metabolic activity, and preservation of structural and functional membrane integrity. Such an evaluation will not only confirm the morphological preservation of cells after cryopreservation but also provide an objective measure of the extent to which their physiological properties are restored and their suitability for clinical use.

CONCLUSIONS

The combination of 10% DMSO with controlled initiation of primary ice-nucleation centers to direct ice-crystal growth ensures a significant increase in the preservation of canine erythrocytes and a reduction in hemolysis after cryopreservation. The effectiveness of this approach is attributed to the optimization of ice-formation dynamics, which is a key factor in preventing mechanical cryoinjury. The proposed method may be used to improve erythrocyte cryopreservation protocols in veterinary practice.

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ВПЛИВ РЕЖИМУ ЗАМОРОЖУВАННЯ НА ЗБЕРЕЖЕННЯ ЕРИТРОЦИТІВ СОБАКИ ЗА ВИКОРИСТАННЯ ДИМЕТИЛСУЛЬФОКСИДУ

У роботі досліджено вплив концентрації диметилсульфоксиду (ДМСО) та різних режимів заморожування на збереження еритроцитів собаки в умовах кріоконсервування. Рівень гемолізу еритроцитів визначали після їх відтавання та на кожному етапі відмивання від кріопротектора. Показано, що збільшення концентрації ДМСО з 7,5 до 10 % дозволяє знизити рівень гемолізу еритроцитів собаки безпосередньо після заморожування-відтавання більш ніж у два рази. Під час відмивання від кріопротектора рівень пошкодження еритроцитів, заморожених із концентрацією ДМСО 7,5 та 10 %, значуще не відрізнявся. Встановлено, що рівень загального гемолізу еритроцитів після заморожування-відтавання суттєво варіює залежно від обраного режиму охолодження. Найбільше ушкодження клітин спостерігається за умов заморожування в парах азоту з подальшим зануренням у рідкий азот (73 %). Заморожування шляхом безпосереднього занурення зразка у рідкий азот дозволяє знизити гемоліз до 37 %. Найбільш ефективним виявився режим заморожування з попереднім зануренням нижньої частини кріопробірки у рідкий азот перед її повним заморожуванням (31 %). Такий підхід забезпечує ініціацію кристалоутворення в нижній частині кріопробірки, що значно знижує ризик механічного пошкодження клітин у процесі формування кристалічної структури льоду.

***Ключові слова*:** кріоконсервування, кріопротектор, еритроцити собаки, диметилсульфоксид, режими заморожування.